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Exploring the agronomic and environmental effects of herbal leys on lowland sheep production

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Exploring the agronomic and environmental effects of herbal leys on lowland sheep production

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Philosophiae Doctor (PhD)

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Declaration

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Thesis Abstract

Rapid agricultural intensification has led to the decline of grassland species richness and the disruption of biogeochemical cycles, ultimately contributing to the loss of grassland ecosystem services and multifunctionality. Restoring grassland biodiversity through the reintroduction of herb and legume species into grass-dominated pastures can offer a practical, low-cost grassland degradation mitigation strategy by improving the soil physiochemical characteristics that underpin ecosystem service delivery. In the UK, herbal leys (multispecies swards) are rapidly gaining in popularity due to their potential to deliver greater agronomic and environmental benefits than conventional grass-clover pastures. However, despite their promotion in agri-environment schemes, little is known about the effect of high-diversity (e.g., 9-18 species) commercial herbal leys on lowland sheep production, soil N cycling, and soil quality. The overall aims of this thesis were therefore to: i) determine if herbal leys can improve sward nutritional quality and productivity while enhancing livestock productivity and health (Chapter 3); ii) investigate if a commercial herbal ley can reduce urine N excretion and soil N losses (e.g., NO_3^-, NH_3, N_2O) associated with lowland lamb grazing (Chapter 4); and iii) evaluate if a commercial herbal ley can improve short-term soil physiochemical characteristics responsible for below-ground ecosystem service delivery in a sheep grazed grassland (Chapter 5). To explore this, a 2-ha split-field experiment using either a herbal or grass-clover ley (0.33 ha paddock^{-1}, $n = 3$ per sward) was established at the Henfaes Research Centre (North Wales, UK) in July 2020 and rotationally grazed by Welsh mountain lambs (ca. $n = 40$ per sward) over two experimental seasons: autumn 2020 (males) and spring 2021 (females). Chapter 3 revealed that sward nutritional quality (e.g., crude protein) did not differ between the herbal and grass-clover ley in either experimental season, however, the herbal ley contained higher concentrations of macro- and micronutrients in both seasons. Subsequently, spring liveweight gain was greater in lambs grazing the herbal (172 ± 7 g d^{-1}) *vs*. grass-clover ley (144 ± 7 g d^{-1}), while autumn liveweight gain showed no difference, driven by the high gastrointestinal parasite burden in both treatments. Spring lambs grazing the herbal ley compared to the grass-clover had elevated plasma cobalt (2.0 ± 0.1 nmol l^{-1} *vs*. 1.6 ± 0.1 nmol l^{-1}) and selenium (0.7 ± 0.04 µmol l^{-1} *vs*. 0.5 ± 0.01 µmol l^{-1}), with lower blood urea (7.7 ± 0.3 mmol l^{-1} *vs*. 10.4 ± 0.4 mmol l^{-1}). Chapter 4 found that while there was no difference in lamb dung and urine N concentration between swards, seasonal variations in urination volume increased the urine N loading rate in the herbal ley in autumn (1020 *vs*. 555 kg N ha^{-1}), but not spring. A higher sward sodium content drove greater urinary sodium excretion in herbal ley grazed lambs in autumn (439 *vs*. 71 mg Na l^{-1}) and spring (389 *vs*. 47 mg Na l^{-1}). Surprisingly, sward type did not affect soil N cycling or gaseous N losses (e.g., N_2O) associated with lamb grazing in either season. Chapter 5 examined soil characteristics 2-years after ley establishment and discovered no overall difference in soil physical (e.g., aggregate stability), chemical (e.g., soil organic carbon) and biological (e.g., earthworm abundance) properties between sward types. Despite clear differences in root architecture, X-ray µCT analysis revealed greater pore connectivity in grass-clover ley intact soil cores (0-10 cm depth, 7.5 cm diameter) than herbal ley cores dominated by *Plantago lanceolata*. The findings of this thesis have implications for the design and promotion of agri-environment schemes aimed at promoting soil quality and sustainability, with further research needed to optimise commercial herbal ley mixtures to deliver greater agronomic and environmental benefits and help the UK agri-food system achieve sustainable agricultural intensification.

Acknowledgements

Firstly, I would like to thank my supervisors, Prof Davey Jones and Prof Dave Chadwick, as this thesis would not have been possible without their unwavering support, mentorship, sense of humour, and most of all, their confidence in me. Without their guidance, I would not have overcome many of the hurdles experienced during the making of this thesis. Thank you for allowing me the freedom to take charge and run with my ideas, be it by starting (and sometimes failing) with experiments, seeking additional funding, and establishing new external collaborations. Davey – thank you for involving me in the many side-projects (e.g., Australian soils, Canadian AMR genes) that have provided numerous research opportunities and kept me entertained, these were a welcome break from the PhD! Your mentorship has challenged me to grow and become a better researcher than I thought possible.

Special thanks to the team at Henfaes Research Centre, Mark Hughes, Bleddyn Jones, Llinos Hughes, and Ian Harris for their help with establishing and maintaining the field experiment, assistance with sheep handling and husbandry, providing their agronomic expertise, and for the much-needed chats and words of encouragement over a cup of tea.

Thank you to Joe Cotton for your support with sampling and analyses, and for making every trip to Henfaes (and beyond!) exceptionally fun. I am also extremely grateful for colleagues, past and present, at Environment Centre Wales (ECW) for their support with analyses, statistics, R, and general PhD life. It has taken a small army of people to get this PhD across the line.

Finally, thank you to my family for your unconditional love, patience, understanding, and support throughout the highs and lows of this PhD. Your endless encouragement has kept me going.

Funding and organisational acknowledgements

Many thanks also to the following funding bodies and collaborating colleagues at various organisations for their support throughout the completion of this research project.

1884
PRIFYSGOL
BANGOR
UNIVERSITY

University of Sheffield

UK Centre for Ecology & Hydrology

NIAB InnovationFarm

ROTHAMSTED RESEARCH

UNIVERSITY OF BIRMINGHAM

HERIOT WATT UNIVERSITY

COTSWOLD Grass Seeds DIRECT

NSA National Sheep Association

YorkshireWater

cawood scientific

Game & Wildlife CONSERVATION TRUST

University of Nottingham
UK | CHINA | MALAYSIA

NUVetNA

MU Murdoch University

UKRI Biotechnology and Biological Sciences Research Council

BRITISH SOCIETY OF SOIL SCIENCE

SARIC • SUSTAINABLE AGRICULTURE RESEARCH & INNOVATION CLUB

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Abbreviations

% - Percentage

°C – Degrees Celsius

µCT – Micro Computed Tomography

µg – Microgram(s)

µl – Microlitre(s)

µm – Micrometre(s)

µmol – Micromole(s)

µS – Microsiemen(s)

2D – Two dimensional

3D – Three dimensional

a.s.l. – Above sea level

AMF – Arbuscular mycorrhizal fungi

ANOVA – Analysis of Variance

ASPA – Animals Scientific Procedures Act

BCS – Body condition score

BNI – Biological nitrification inhibitor

C – Carbon

C:N – Carbon to nitrogen ratio

ca. – Circa

CH_3COOH – Acetic acid

cm – Centimetre(s)

CO_2 – Carbon dioxide

d – Day(s)

DCD – dicyandiamide

DI H_2O – Distilled water

DM – Dry matter

DMI – Dry matter intake

DMPP – 3,4-dimethylpyrazole phosphate

DNA – Deoxyribonucleic acid

DW – Dry weight

EC – Electrical conductivity

ECD – Electron capture detector

EF – Emission factor

epg – Eggs per gram

EU – European Union

FEC – Faecal egg count

FID – Flame ionisation detector

g – Gram(s)

GC – Gas chromatograph

GHG – Greenhouse gas

h – Hour(s)

H' – Shannon diversity index

H_3PO_4 – Orthophosphoric acid

ha – Hectare(s)

Hb – Haemoglobin

ICP-MS – Inductively coupled plasma mass spectroscopy

ICP-OES – Inductively coupled plasma optical emission spectroscopy

K_2SO_4 – Potassium sulphate

KCl – Potassium chloride

kg – Kilogram(s)

kV – Kilovolt(s)

l – Litre(s)

LU – Livestock unit

LWG – Liveweight gain

m – Meter(s)

mA – Milliamp(s)

MAOM – Mineral-associated organic matter

ME – Metabolisable energy

Mg – Megagram (tonne)

mg – Milligram(s)

Mha – Megahectare(s)

min – Minute(s)

MJ – Megajoule(s)

ml – Millilitre(s)

mm – Millimetre(s)

mmol – Millimole(s)

N – Nitrogen

n – Sample size

N_2O – Nitrous oxide

NaOH – Sodium hydroxide

NDF – Neutral detergent fibre

NH_3 – Ammonia

NH_3-N – Ammonia-N

NH_4^+ – Ammonium

nmol – Nanomole(s)

NO – Nitric oxide

NO_3^- – Nitrate

P – Phosphorus

PCV – Packed cell volume

PERMANOVA – Permutational multivariate ANOVA

Pg – Petagram(s)

pmol – Picomole(s)

POM – Particulate organic matter

PRP – Pasture, range, and paddock

PSM – Plant secondary metabolite

ROI – Region of interest

SD – Standard deviation

SEM – Standard error of the mean

SOC – Soil organic carbon

SOM – Soil organic matter

t – Tonne(s)

TN – Total nitrogen

TOC – Total organic carbon

UK – United Kingdom

US – United States

VESS – Visual evaluation of soil structure

VSA – Visual scoring assessment

w/v – Weight per volume

WFPS – Water-filled pore space

Y.B.P. – Years before present

yr – Year(s)

Foreword

Thesis Caveat

This PhD thesis was produced while employed as a part-time Research Support Technician on the BBSRC-SARIC funded project *Restoring soil quality through the reintegration of leys and sheep in arable rotations* (see website for more information: http://restoringsoilquality.bangor.ac.uk/) (2019-2023) (BB/R021716/1). The original aim of this thesis was to utilise pre-existing arable-ley experiments established for the BBSRC-SARIC project on commercial farms across eastern England to assess how herbal leys affect soil quality and ecosystem service delivery in degraded arable soils. However, this was not possible, as in March 2020, the COVID-19 pandemic in the UK prohibited travel to field sites in eastern England from North Wales. This consequently resulted in a limited ability to travel to study sites during a crucial sampling period, reducing the control and management of those sites, and ultimately contributing to a communication breakdown between researchers and project farmers.

Instead, in July 2020, a new replicated field-scale experiment was established at Bangor University's Henfaes Research Centre that formed the basis of this thesis. The new experiment at Henfaes required a change in thesis aims and design but provided improved experimental management and greater research freedom to explore the agronomic and environmental benefits of herbal leys in grazed grasslands. The Henfaes experiment facilitated the timely collection of measurements that were able to capture livestock (e.g., blood characteristics) and soil physical (e.g., porosity), chemical (e.g., nitrous oxide emissions) and biological (e.g., microbial community composition) data that was outside of the scope of the original BBSRC-SARIC project.

Funding

This work was primarily funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC) under the Sustainable Agriculture Research and Innovation Club (SARIC) programme (BB/R021716/1). The BBSRC-SARIC funding was shared between a range of collaborating organisations, namely the University of Sheffield, National Institute of Agricultural Botany (NIAB), Bangor University, University of Birmingham, UK Centre for Ecology and Hydrology (UK-CEH), Rothamsted Research, and Heriot-Watt University. Towards the end of the project, budget overspends associated with some of the external project partners resulted in funding shortfalls that limited potential additional experimentation and research mobility.

To overcome this, external funding was sought to continue experiments independently at Bangor University to support the completion of this thesis. Unfortunately, due to the nature of a part-time Research Technician employment, no studentship or doctoral training program support was available. However, a successful entry to the University of Nottingham's X-ray Computed Tomography (CT) access competition enabled the initial X-ray µCT scanning of intact vegetated soil cores and soil aggregates detailed in Chapter 5. Additional funding for further scans was then provided by the British Society of Soil Science (BSSS) Brian Chambers Soils Fund to enable travel and subsistence to the University of Nottingham's Hounsfield Facility to facilitate further research training and the completion of the X-ray µCT dataset. Funding provided by collaborators at Murdoch University (Australia) is also gratefully acknowledged for enabling the analysis of the microbial community composition dataset in Chapter 5.

Publications

In addition to this thesis, the following publications were produced during the PhD:

1. Jones, D.L., **Cooledge, E.C.,** Hoyle, F.C., Griffiths, R.I., Murphy, D.V. (2019). *pH and exchangeable aluminum are major regulators of microbial energy flow and carbon use efficiency in soil microbial communities*. Soil Biology and Biochemistry. DOI: 10.1016/j.soilbio.2019.107584.
2. Schut, A.G.T., **Cooledge, E.C.,** Moraine, M., van de Ven, G.W.J., Jones, D.L., Chadwick, D.R. (2021). *Reintegration Of Crop-Livestock Systems In Europe: An Overview.* Frontiers of Agricultural Science and Engineering. DOI: 10.15302/J-FASE-2020373.
3. Horn, E.L., **Cooledge, E.C.,** Jones, D.L., Hoyle, F.C., Brailsford, F.L., Murphy, D.V. (2021). *Addition of base cations increases microbial carbon use efficiency and biomass in acidic soils.* Soil Biology and Biochemistry. DOI: 10.1016/j.soilbio.2021.108392
4. **Cooledge, E.C.,** Chadwick, D.R., Smith, L.M.J., Leake, J.R., Jones, D.L. (2022). *Agronomic and Environmental Benefits of Reintroducing Herb- and Legume-rich Multispecies Leys into Arable Rotations: A Review*. Frontiers of Agricultural Science and Engineering. DOI: 10.15302/J-FASE-2021439
5. Buckingham, S., Topp, C.F.E., Smith, P., Eory, V., Chadwick, D.R., Baxter, C.K., Cloy, J.M., Connolly, S., **Cooledge, E.C.,** Cowan, N.J., Drewer, J., Duffy, C., Fox, N.J., Jebari, A., Jenkins, B., Krol, D.J., Marsden, K.A., McAuliffe, G.A., Morrison, S.J., O'Flaherty, V., Ramsey, R., Richards, K.G., Roehe, R., Smith, J., Smith, K., Takahashi, T., Thorman, R.E., Williams, J., Wiltshire, J., Rees, R.M. (2023). *Greenhouse Gas and Ammonia Emission Mitigation Priorities for UK Policy Targets.* Frontiers of Agricultural Science and Engineering. DOI: 10.15302/J-FASE-2023495

Academic contributions were also made to the social science and forage quality work-packages of the BBSRC-SARIC research project; however, at the time of writing, no publications have been produced for this as these datasets are gatekept by the work-package leads and thus are still awaiting development.

Outreach

Due to the industry-led nature of this research, stakeholder engagement was at the forefront of this thesis. Results were shared via social media and various academic conferences (e.g., World Congress of Soil Science 2022 conference and the Association of Applied Biologists conference), industry dissemination events (e.g., SARIC meetings, Cereals Live 2020, NIAB and Agri-tech East conference), and on-farm outreach events (e.g., the British Grassland Society and Anglesey Grassland Society visits to Henfaes Research Centre).

Chapter 1 - Introduction

1.1. Background and Rationale

Since the mid-20th century, rapid agricultural intensification has contributed to the widespread increase in soil degradation and the loss of provisioning (e.g., food production), regulating (e.g., carbon storage), and cultural (e.g., heritage) ecosystem services (Adhikari and Hartemink, 2016; Kopittke et al., 2019; Pretty, 2018), with approximately 53 million km^2 of global agricultural land classified as degraded (Hossain et al., 2020). This decline in soil quality undermines the ability of agricultural soils to maintain global food security and reduces their potential to deliver intergovernmental climate change policies (e.g., the Paris Agreement). It will also make it difficult to achieve targets set by the United Nation's Sustainable Development Goals (Arora and Mishra, 2019; Bonfante et al., 2020; Lal, 2007), placing the resilience of agrifood systems at risk (Kopittke et al., 2019; Tilman et al., 2011). Instead, there is an urgent need to identify economically viable sustainable agricultural intensification practices that can increase productivity with minimal ecosystem trade-offs (Clark and Tilman, 2017; Holt et al., 2016; Power, 2010). This is a challenge to achieve in the era of climate change, where extreme weather events (e.g., droughts) affect productivity and the global population is expected to reach 9.4 billion by 2050 (United Nations, 2017). This will require a 70 % increase in food production to maintain global food security, equivalent to a 200 million tonne and 3 billion tonne increase in meat and cereal production, respectively (Hossain et al., 2020).

In grazed grasslands, agricultural expansion (i.e., land conversion), intensification, and poor grazing management practices (e.g., overgrazing) has led to the decline of plant and animal biodiversity (Cardinale et al., 2012), the loss of soil organic matter (Abdalla et al., 2018; Liu et al., 2023), and the disruption of biogeochemical cycles at various temporal and spatial scales (Lal, 2007), ultimately contributing to the loss of grassland ecosystem services (Brynes, et al., 2018) and multifunctionality (Schils et al., 2022). As such, approximately 49 % of global grasslands are classified as degraded (Bardgett et al., 2021), with the global economic cost of the loss of meat and milk production arising from degradation valued at ca. 7.7 billion US dollars in 2007 (Nkonya et al., 2015).

In temperate regions, the prevalence of perennial ryegrass (*Lolium perenne*) monocultures and grass-clover swards, due to their high sugar content and persistence under grazing (Fraser et al., 2022; Waghorn and Clark, 2004), has inadvertently increased reliance on agrochemicals (e.g., synthetic N inputs) to maintain productivity (Allan et al., 2015). This can have serious economic and environmental implications, such as watercourse eutrophication (Clark and Tilman, 2017), soil acidification (Goulding, 2016), ammonia (NH_3) volatilisation and nitrate (NO_3^-) leaching (Di and Cameron, 2002) leading to direct and indirect nitrous oxide (N_2O) emissions (Bell et al., 2015). As N_2O emissions are a significant driver of anthropogenic climate change with a global warming potential 265-298 times greater than carbon dioxide (CO_2) (IPCC, 2021), mitigation strategies in grasslands often target emissions either directly at the pasture scale (e.g., by introducing bioactive plant species) (Luo et al., 2018) or in the ruminant (e.g., by reducing dietary protein intake) (de Klein et al., 2020), as alternative mitigation practices (e.g., afforestation) are often not cost-effective and can further reduce productivity and ecosystem service delivery (Bardgett et al., 2021). Instead, restoring grassland biodiversity through the reintroduction of herb and legumes species into grass dominated pastures can offer a practical, low-cost grassland degradation mitigation strategy by altering the underlying soil physiochemical characteristics (e.g., soil porosity) that underpin ecosystem service delivery without compromising food production (Gould et al., 2016; Schils et al., 2022).

In recent years, UK agri-environment schemes have promoted the use of herbal leys (multispecies swards) for their potential to provide greater agronomic and environmental benefits than conventional grass or grass-clover pastures (Jordon et al., 2022). Herbal leys consist of a highly diverse mixture of grass, legume, and herb species and are typically established in grasslands for 1-4 years to improve soil quality (Cooledge et al., 2022). Although their exact composition varies with commercial vendor and geographical location, key ‘bioactive’ herb and legume species established in herbal leys such as such as ribwort plantain (*Plantago lanceolata*), chicory (*Cichorium intybus*), and lucerne (*Medicago sativa*) contain high levels of plant secondary metabolites that can improve livestock productivity (Golding et al., 2011; Grace et al., 2019) once consumed by reducing the gastrointestinal parasite burden (Marley et al., 2003; Peña-Espinoza et al., 2018) and rumen protein degradation (Minneé et al., 2017). This can subsequently alter the ratio of nitrogen (N) excretion in urine and dung (Bryant et al., 2018; Cheng et al., 2017; Totty et al., 2013; Wilson et al., 2020), reducing potential NO_3^- leaching and N_2O emissions through the urinary excretion of biological nitrification inhibition compounds (Gardiner et al., 2018; Simon et al., 2019). The complementarity nature of combining plant species also enables greater sward productivity (Finn et al., 2013) and improvements in soil structure (Uteau et al., 2013), with deep-rooting taproot species (e.g., *Cichorium intybus*) accessing subsoil nutrient reserves to increase sward drought tolerance (Grange et al., 2021; Hofer et al., 2016) and accumulate greater macro- and micronutrients than other plant species (Barry, 1998; Darch et al., 2020; Kao et al., 2020; Watson et al., 2012), improving the overall sward quality for grazing (Grace et al., 2018; Jing et al., 2017). These benefits are summarised in Figure 1.1.

However, many of these studies have only examined the effect of low-diversity (e.g., 3-9 species) experimental herbal ley mixtures on ecosystem services, often under cattle grazing, with no studies to-date investigating the effect of a high diversity (e.g., 9-18 species) commercial herbal ley mixture on lowland sheep production, soil quality, and N_2O emissions. As such, this presents a significant research gap that forms the focus of this thesis.

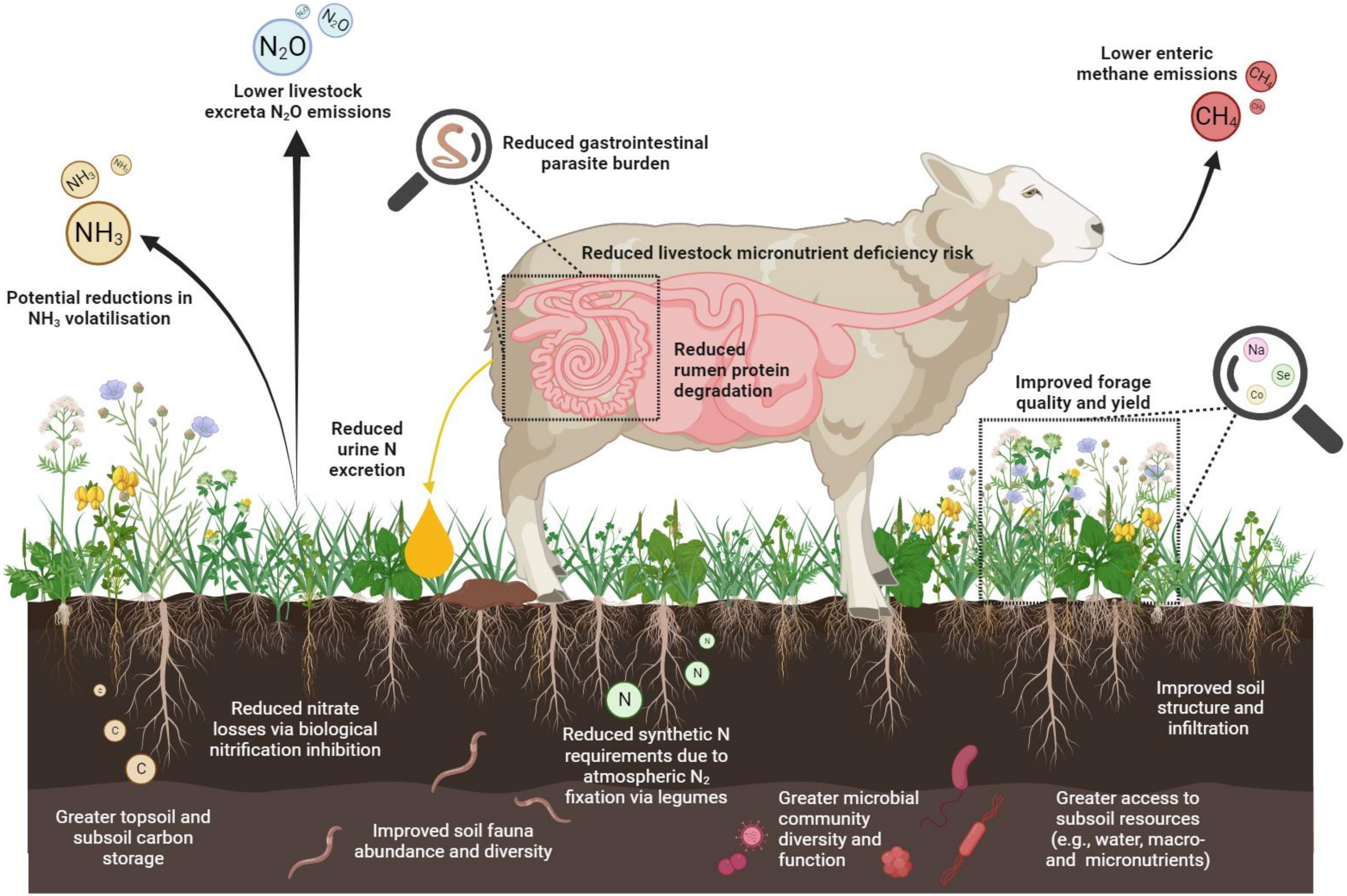


***Figure 1.1**. Schematic diagram illustrating the key potential ecosystem services delivered by herbal leys.*

1.2. Thesis Aims and Objectives

This thesis utilises a multidisciplinary approach to evaluate the agronomic and environmental benefits of a current commercial herbal ley mixture, with the overarching aim to investigate the effect of herbal leys on lowland sheep production, soil quality, NH_3 volatilisation and N_2O emissions compared to a conventional grass-clover ley. To address this, the following core objectives were investigated:

I. **Objective 1:**
   a. To determine if greater plant species richness established in a commercial herbal ley can improve sward nutritional quality and productivity while enhancing livestock productivity and health compared to a conventional (grass-clover) pasture (Chapter 3).

II. **Objective 2:**
   a. To investigate if a commercial herbal ley mixture can reduce urine N excretion, soil N losses, NH_3 volatilisation and N_2O emissions associated with lowland lamb grazing through the consumption of key herb and legume species containing high levels of plant secondary metabolites, potentially enabling biological nitrification inhibition in the soil compared to a conventional (grass-clover) pasture (Chapter 4).

III. **Objective 3:**
   a. To evaluate if a commercial herbal ley can improve short-term below-ground ecosystem service delivery in a grazed grassland through enhancing soil physical, chemical, and biological characteristics, compared to a conventional (grass-clover) pasture (Chapter 5).

1.3. Thesis Outline

This thesis is formed of 6 chapters, with Chapter 2 critically reviewing previous research on herbal leys, and Chapters 3-5 detailing experimental research undertaken to address the hypotheses and aims previously discussed. Chapters 3-5 are written as journal articles for submission, with Chapters 3 and 4 currently under review in Agriculture, Ecosystems and Environment at the time of writing. An overview of key research themes and the relationship between each experimental chapter is shown in Figure 1.2, with a timeline of key sampling events that occurred during the Henfaes field experiment detailed in Figure 1.3. Detailed hypotheses are described within individual chapters.

A brief description of each chapter is as follows:

***Chapter 2**: Agronomic and Environmental Benefits of Reintroducing Herb- and Legume-rich Multispecies Leys into Arable Rotations: A Review*

This chapter introduces the issue of soil degradation arising from conventional agricultural practices and critically reviews how the introduction of temporary grasslands (leys) and livestock in arable rotations may be used to alleviate this. The literature review introduces herbal leys and assesses their potential to improve key ecosystem services (e.g., yield), outlining the impact on soil carbon stocks, microbial community composition, carbon and nitrogen cycling, greenhouse gas emissions, and livestock health and productivity. Key research gaps are identified, with recommendations for including herbal leys in future UK agri-environment schemes and policy made.

This literature review has since been published in Frontiers of Agricultural Science and Engineering (Cooledge et al., 2022).

Chapter 3: *Herbal leys increase forage macro- and micronutrient content, spring lamb nutrition, liveweight gain, and reduce gastrointestinal parasites compared to a grass-clover ley*

The aim of this chapter was to assess the impact of herbal leys on lowland sheep production, covering aspects of forage quality (e.g., crude protein content), lamb productivity (e.g., liveweight gain), and health (e.g., gastrointestinal parasite burden, plasma macro- and micronutrient content). Following the establishment of the 2-ha field experiment at Henfaes Research Centre in July 2020, weaned Welsh mountain lambs (*Ovis aries*) were grazed on either a commercial herbal ley or a conventional grass-clover ley (ca. $n = 40$ per sward, 3.2 LU ha^{-1}) over two grazing seasons (autumn 2020 *vs*. spring 2021). Forage, liveweight gain, and faecal egg count measurements were made at regular intervals within each grazing season, with one blood sample of $n = 10$ lambs per sward occurring at the end of the spring 2021 grazing season to investigate the effect of sward type on lamb mineral status.

This chapter has since been submitted and is under review at Agriculture, Ecosystems and Environment.

Chapter 4: *Grazing lambs on herbal leys increases urinary sodium excretion but does not affect excreta N concentration or nitrous oxide emissions compared to a grass-clover ley*

This chapter follows on from the grazing experiment described in Chapter 3 and explores the role of sward type (herbal *vs*. grass-clover ley) on livestock excreta composition and nutrient cycling in a grazed grassland. Sward-specific lamb urine and dung was collected during the autumn 2020 and spring 2021 grazing experiments and reapplied to the soil of each respective pasture. Regular soil (0-10 cm depth) and N_2O measurements were then taken from each treatment (urine, dung, 50 kg N ha^{-1} ammonium nitrate, control) over 103 and 78 days in autumn and spring, respectively. Seasonal N_2O measurements were used to develop a sward-specific urine emission factor (EF_{3PRP}). A bench-scale ammonia volatilisation system was used to assess NH_3 loss from urine addition in intact vegetated soil cores (0-12 cm depth, 9 cm diameter) over 12 days.

This chapter has since been submitted and is under review at Agriculture, Ecosystems and Environment.

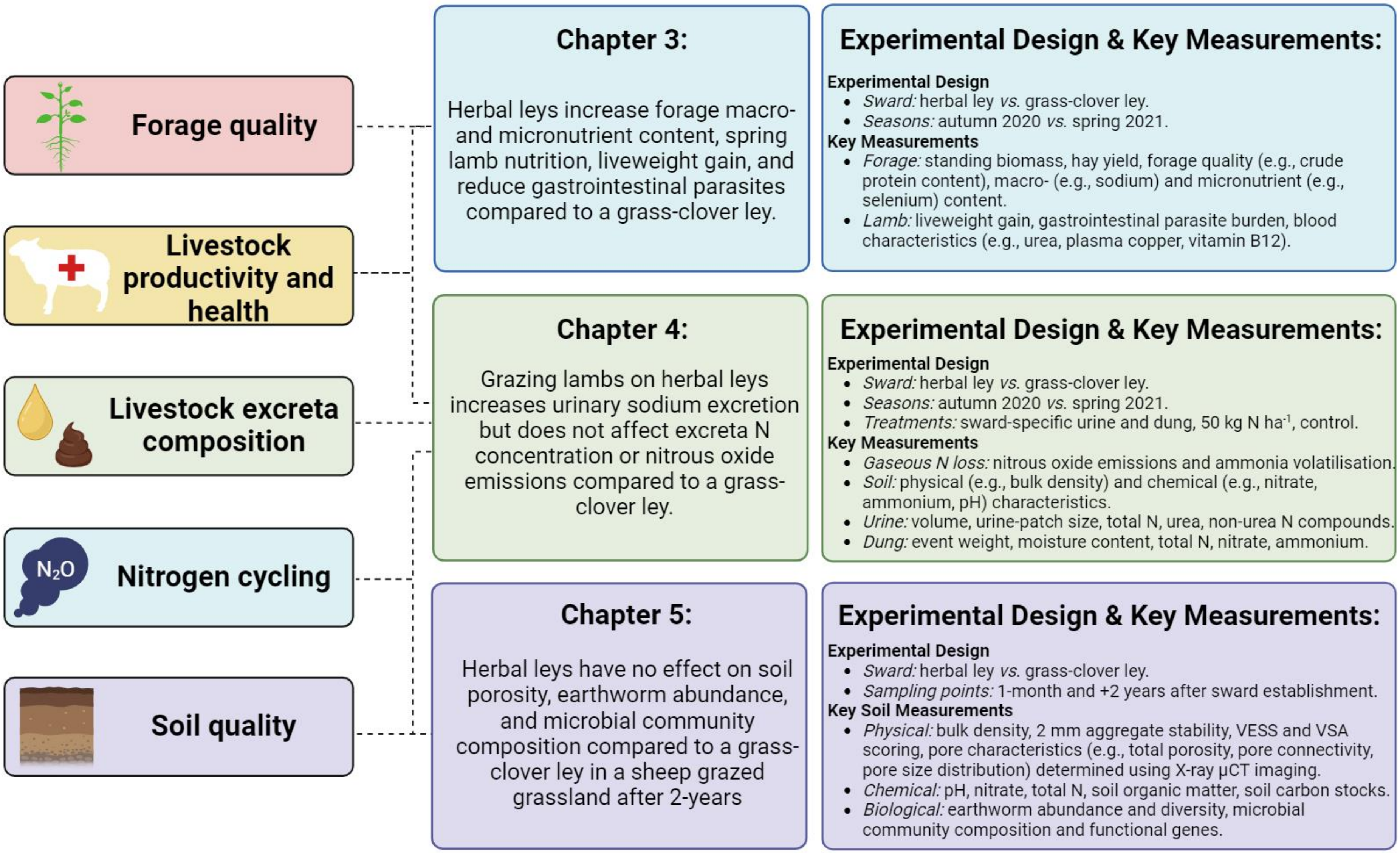
Chapter 5: *Herbal leys have no effect on soil porosity, earthworm abundance, and microbial community composition compared to a grass-clover ley in a sheep grazed grassland after 2-years*

This final experimental chapter assesses below-ground ecosystem services and soil quality beneath the herbal and grass-clover ley. Soil physical (e.g., porosity), chemical (e.g., carbon stocks) and biological (e.g., microbial community composition) characteristics were assessed in the topsoil (0-10 cm) 2-years after sward establishment, using techniques such as X-ray micro-computed tomography (µCT) and shallow shotgun sequencing. Intact vegetated soil cores (0-10 cm depth, 7.5 cm diameter) used for X-ray µCT imaging were collected from the grass-clover and herbal ley (n = 9 per sward) after two grazing seasons, with key plant species within the herbal ley such as *Cichorium intybus* and *Plantago lanceolata* targeted to assess the effect of taproot herbs on soil structure. Once imaged, these cores were then used to assess aggregate stability and pore characteristics across two soil depths (0-5 cm and 5-10 cm).

This chapter has been published in Agriculture, Ecosystems and Environment.

Chapter 6: *Discussion and Conclusion*

This chapter evaluates the findings of this study and explores the limitations and implications of these results. Recommendations for government policies and priorities for future research are also provided.



Chapter 3:

Herbal leys increase forage macro- and micronutrient content, spring lamb nutrition, liveweight gain, and reduce gastrointestinal parasites compared to a grass-clover ley.

Experimental Design & Key Measurements:

Experimental Design

- *Sward:* herbal ley *vs.* grass-clover ley.
- *Seasons:* autumn 2020 *vs.* spring 2021.

Key Measurements

- *Forage:* standing biomass, hay yield, forage quality (e.g., crude protein content), macro- (e.g., sodium) and micronutrient (e.g., selenium) content.
- *Lamb:* liveweight gain, gastrointestinal parasite burden, blood characteristics (e.g., urea, plasma copper, vitamin B12).

Chapter 4:

Grazing lambs on herbal leys increases urinary sodium excretion but does not affect excreta N concentration or nitrous oxide emissions compared to a grass-clover ley.

Experimental Design & Key Measurements:

Experimental Design

- *Sward:* herbal ley *vs.* grass-clover ley.
- *Seasons:* autumn 2020 *vs.* spring 2021.
- *Treatments:* sward-specific urine and dung, 50 kg N ha^{-1}, control.

Key Measurements

- *Gaseous N loss:* nitrous oxide emissions and ammonia volatilisation.
- *Soil:* physical (e.g., bulk density) and chemical (e.g., nitrate, ammonium, pH) characteristics.
- *Urine:* volume, urine-patch size, total N, urea, non-urea N compounds.
- *Dung:* event weight, moisture content, total N, nitrate, ammonium.

Chapter 5:

Herbal leys have no effect on soil porosity, earthworm abundance, and microbial community composition compared to a grass-clover ley in a sheep grazed grassland after 2-years

Experimental Design & Key Measurements:

Experimental Design

- *Sward:* herbal ley *vs.* grass-clover ley.
- *Sampling points:* 1-month and +2 years after sward establishment.

Key Soil Measurements

- *Physical:* bulk density, 2 mm aggregate stability, VESS and VSA scoring, pore characteristics (e.g., total porosity, pore connectivity, pore size distribution) determined using X-ray µCT imaging.
- *Chemical:* pH, nitrate, total N, soil organic matter, soil carbon stocks.
- *Biological:* earthworm abundance and diversity, microbial community composition and functional genes.

***Figure 1.2**. Schematic diagram showing key research themes and how they interlink with thesis chapters, treatments, and key measurements.*

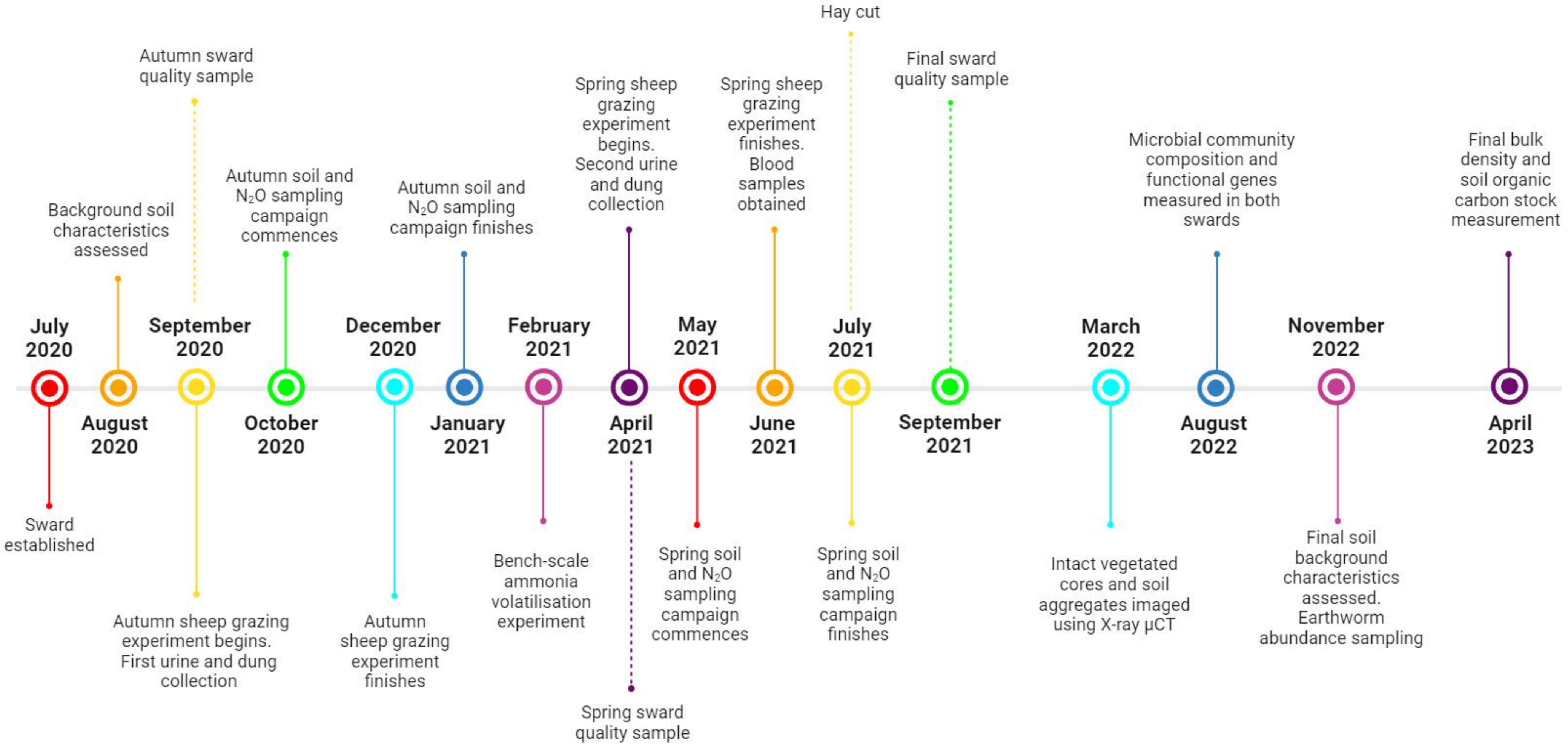


Figure 1.3. *Timeline displaying key sampling points of the Henfaes Research Centre field experiment from July 2020 to April 2023.*

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Chapter 2

Agronomic and Environmental Benefits of Reintroducing Herb- And Legume-Rich Multispecies Leys into Arable Rotations: A Review

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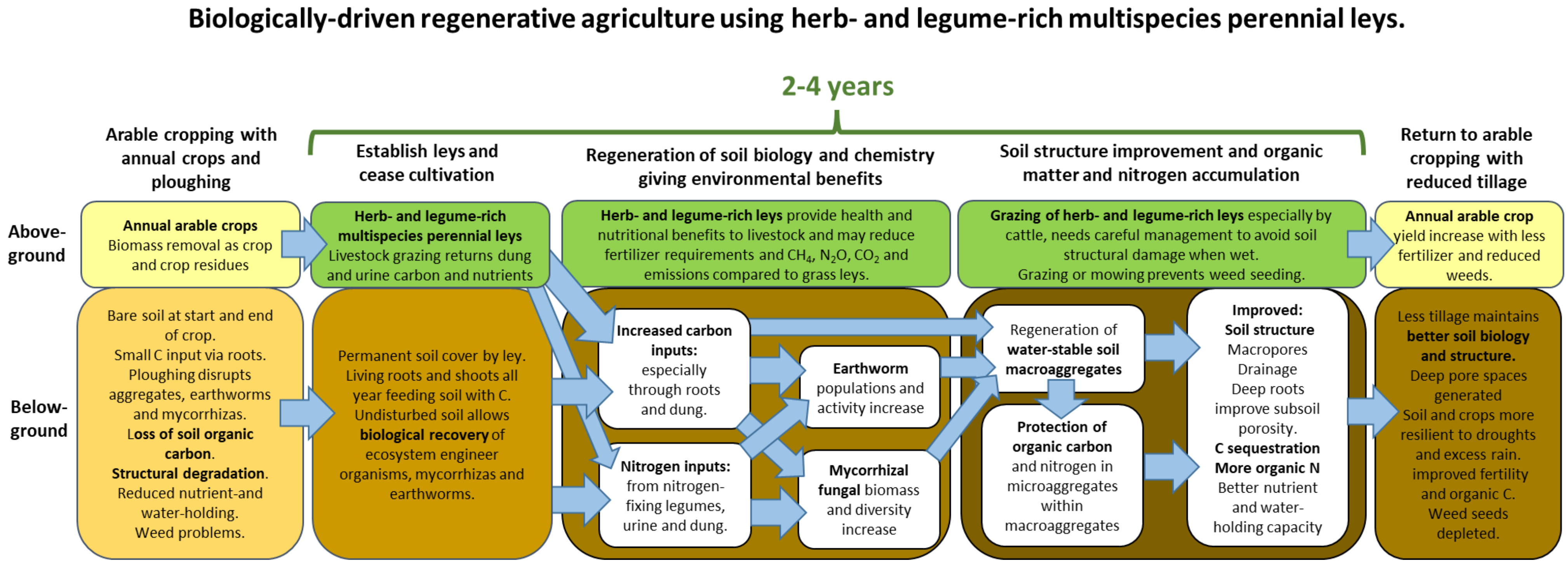

Biologically-driven regenerative agriculture using herb- and legume-rich multispecies perennial leys.

2-4 years

Arable cropping with annual crops and ploughing

Establish leys and cease cultivation

Regeneration of soil biology and chemistry giving environmental benefits

Soil structure improvement and organic matter and nitrogen accumulation

Return to arable cropping with reduced tillage

Above-ground

Annual arable crops Biomass removal as crop and crop residues

Herb- and legume-rich multispecies perennial leys Livestock grazing returns dung and urine carbon and nutrients

Herb- and legume-rich leys provide health and nutritional benefits to livestock and may reduce fertilizer requirements and CH$_4$, N$_2$O, CO$_2$ and emissions compared to grass leys.

Grazing of herb- and legume-rich leys especially by cattle, needs careful management to avoid soil structural damage when wet. Grazing or mowing prevents weed seeding.

Annual arable crop yield increase with less fertilizer and reduced weeds.

Below-ground

Bare soil at start and end of crop. Small C input via roots. Ploughing disrupts aggregates, earthworms and mycorrhizas. **Loss of soil organic carbon**. **Structural degradation**. Reduced nutrient-and water-holding. Weed problems.

Permanent soil cover by ley. Living roots and shoots all year feeding soil with C. Undisturbed soil allows **biological recovery** of ecosystem engineer organisms, mycorrhizas and earthworms.

Increased carbon inputs: especially through roots and dung.

Nitrogen inputs: from nitrogen-fixing legumes, urine and dung.

Earthworm populations and activity increase

Mycorrhizal fungal biomass and diversity increase

Regeneration of **water-stable soil macroaggregates**

Protection of organic carbon and nitrogen in microaggregates within macroaggregates

Improved: **Soil structure** Macropores Drainage Deep roots improve subsoil porosity. **C sequestration** **More organic N** Better nutrient and water-holding capacity

Less tillage maintains **better soil biology and structure.** Deep pore spaces generated Soil and crops more resilient to droughts and excess rain. improved fertility and organic C. Weed seeds depleted.

Figure 2.0. *Graphical abstract for Chapter 2.*

Highlights

- Arable-ley rotations can alleviate soil degradation and erosion.
- Multispecies (herbal) leys can improve livestock health and reduce greenhouse gas emissions.
- Ley botanical composition is crucial for determining benefits.
- Lack of livestock infrastructure in arable areas may prevent arable-ley uptake.
- Long-term (10-25 years) research is needed to facilitate evidence-based decisions.

Keywords bioactive forages, integrated crop-livestock systems, nitrogen cycling, plant secondary metabolites, soil carbon, soil quality

Abstract

Agricultural intensification and the subsequent decline of mixed farming systems has led to an increase in continuous cropping with only a few fallow or break years, undermining global soil health. Arable-ley rotations incorporating temporary pastures (leys) lasting 1-4 years may alleviate soil degradation by building soil fertility and improving soil structure. However, the majority of previous research on arable-ley rotations has utilized either grass or grass-clover leys within ungrazed systems. Multispecies (herbal) leys, containing a mix of grasses, legumes, and herbs, are rapidly gaining popularity due to their promotion in agri-environment schemes and potential to deliver greater ecosystem services than conventional grass or grass-clover leys. Livestock grazing in arable-ley rotations may increase the economic resilience of these systems, despite limited research of the effects of herbal leys on ruminant health and greenhouse gas emissions. This review aims to evaluate previous research on herbal leys, highlighting areas for future research and the potential benefits and disbenefits on soil quality and livestock productivity. The botanical composition of herbal leys is crucial, as legumes, deep rooted perennial plants (e.g., *Onobrychis viciifolia* and *Cichorium intybus*) and herbs like *Plantago lanceolata* can increase soil carbon, improve soil structure, reduce nitrogen fertilizer requirements, and promote the recovery of soil fauna (e.g., earthworms) in degraded arable soils while delivering additional environmental benefits (e.g., biological nitrification inhibition and enteric methane reduction). Herbal leys have the potential to deliver biologically driven regenerative agriculture, but more long-term research is needed to underpin evidence-based policy and farmer guidance.

2.1. Introduction

With the global population expected to reach 10.9 billion by 2100, sustainable agricultural intensification is required to increase food production by 48.6 % to meet the projected demand (FAO, 2017; United Nations, 2019). However, current agricultural practices are unsustainable and have contributed to a global decline in soil quality, risking future crop and livestock production, especially with intensifying climate change (Committee on Climate Change, 2018). Reliance on agrochemicals and mineral fertilizers to increase productivity has led to increases in greenhouse gas (GHG) emissions (Bell et al., 2015; Bouwman et al., 2002), pest and herbicide resistance (Hicks et al., 2018; Ramsden et al., 2017), loss of biodiversity (Donald et al., 2001), and soil degradation (Graves et al., 2015; Gregory et al., 2015).

Soil degradation is widely recognized as a key threat to soils and the ecosystem services they provide (Panagos et al., 2018). Due to their slow rate of formation, ca. 0.3-1.4 t ha^{-1} yr^{-1} for Europe, soils are regarded as a non-renewable resource that need careful protection and management to preserve them for future use (Verheijen et al., 2009). Soils can provide multiple ecosystem services, such as carbon sequestration, food and fiber production, disease control, water quality management, and flood and climate regulation (Kibblewhite et al., 2008; Panagos et al., 2018). Arable land management and cultivation practices, for example, deep tillage and removal of crop residue, can contribute to progressive soil degradation through damage to soil structure, loss of soil organic matter (SOM), increased compaction and an increased risk of erosion, especially in hilly regions. Poor soil structure also leads to more diffuse pollution; especially excessive nitrogen and increasingly phosphate leaching (Graves et al., 2015). Although it is difficult to accurately quantify the full economic impact of the loss of ecosystem services, soil degradation has a significant impact on the global economy. The 2006 Thematic Soil Strategy for the EU estimated the annual cost of soil degradation to be 38 billion EUR for the EU25 member states, with soil erosion and loss of soil organic matter costing 0.7-14.0 and

3.4-5.6 billion EUR yr^{-1}, respectively (European Commission, 2006). In a wider perspective the global economic cost of land degradation has been estimated at 231 billion USD yr^{-1}, equivalent to ~0.41 % of the global GDP (Nkonya et al., 2016).

Technological advancements in farm machinery, introduction of high-yielding crop varieties, increased consumer demand and affluence, and government policies, subsidies and grants have improved farm efficiency and productivity per unit of labor but inadvertently accelerated the degradation of agricultural soils in many European nations (Robinson and Sutherland, 2002). This has encouraged agricultural intensification and specialization, leading to the decline of mixed farming and the infrastructure to support this. Consequently, there has been an increase in monoculture farming and continuous cropping with no fallow or break years, which formerly involved rotations with grazed grass-clover leys, and subsequently decreasing the heterogeneity of the landscape and creating regional areas of soil degradation and environmental pollution (Knox et al., 2011; Posthumus et al., 2015). In the UK, for example, increases in specialization were initiated in 1947 by the introduction of the Agriculture Act following World War II to improve self-sufficiency (Knox et al., 2011). This resulted in a decline in mixed farming and a progressive geographical separation of land use with livestock-grazed grasslands becoming dominant in the wetter west of the country and arable farming becoming dominant in the drier east of the country (DEFRA, 2019; Robinson and Sutherland, 2002).

In many regions of the world, integrated crop-livestock systems, also referred to as mixed farming systems, have been reintroduced to promote more climate-resilient, sustainable and economically viable agricultural systems, compared to specialized and intensive systems (Sekaran et al., 2021). However, coupled crop-livestock farming systems maintain on-farm specialization but utilize neighboring farms to manage system inputs effectively (e.g., muck-for-straw deals), integrated crop-livestock systems utilize systems more efficiently and can produce higher economic returns than coupled crop-livestock farms (Schut et al., 2021). Integrated crop-livestock systems employ arable-ley rotations to alleviate soil degradation, improve soil quality for future use, build soil fertility via symbiotic nitrogen fixation in legumes, and increase resilience by diversifying the farm enterprise (Johnston et al., 2017; Lemaire et al., 2014). Incorporating leys, temporary grasslands lasting up to 5 years, and integrating livestock into arable rotations can help to better manage arable weeds, pests, improve soil structure, enhance nitrogen fixation and recycle nutrients from livestock excreta back into the soil (Kumar et al., 2019). However, the effect of integrated crop-livestock systems on soil quality and productivity can vary significantly depending on grazing management regime, soil type and ley species sown, for example, perennial ryegrass, grass-legume, or a herbal ley mixture containing grasses, herbs and legumes (Cong and Eriksen, 2018; Jing et al., 2017; Moloney et al., 2020). Ryegrass-based leys are often used due to their wide tolerance of different conditions, versatility of use for silage, hay, haylage and grazing, and high digestibility for livestock (Kingston-Smith et al., 2013), however, ryegrass monocultures provide limited ecosystem service benefits (Pembleton et al., 2016, 2015). By comparison, grass-legume leys have the benefit of reducing the need for mineral N fertilizers in the subsequent crop due to their N fixing abilities (Ten Berge et al., 2016). It should be noted, however, that plowing in leys in preparation for the following crop can lead to increased N leaching into watercourses and thus indirect GHG emissions (Hansen and Eriksen, 2016). Based on the study of natural ecosystems, however, it is clear that resilience and ecosystem delivery increases with plant diversity (Quijas et al., 2010). Pasture management for increased plant species diversity, however, is not simply a case of mixing and planting as many forage species as possible. It is clear that the kinds and amounts of different forage species along with their arrangement within and among pastures at the farm scale are critical features that must be considered (Sanderson et al., 2007).

Although research often focuses on the effect of herbal leys (also known as multispecies swards) on livestock health and productivity (Grace et al., 2019), grass yield (Moloney et al., 2020) and GHG emissions (Bracken et al., 2020), there is currently limited information available on the combined effects of these leys on restoring soil quality in degraded arable soils over and above simple mixtures of two to three species. This review focusses on the potential benefits and implications of reintroducing herbal leys and livestock grazing in arable rotations for (1) ecosystem services, (2) soil structure, (3) soil carbon and nitrogen cycling, (4) livestock GHG emissions, (5) livestock productivity, and (6) sustainable agriculture. Finally, we highlight areas that require further research.

2.2. Reintroducing leys into arable rotations

2.2.1. Ecosystem services delivered by leys

Policy, land use and management regime heavily influence the ecosystem services that modern agriculture can provide. The decline in mixed farming, and subsequently the intensification of arable agriculture, has contributed to the generation of an unbalanced agroecosystem and the poor delivery of some ecosystem services. Such services are split into four core categories; provisioning (e.g., food and fiber production), regulating (e.g., climate and flood regulation), cultural (e.g., heritage and recreation), and supporting (e.g., nutrient cycling and biodiversity) (Millennium Ecosystem Assessment, 2005). The drive to increase provisioning services to meet consumer demand often comes at the cost of long-term regulating, supporting and cultural services (Foley et al., 2005). Disservices, for example, eutrophication from excessive nutrient use or species loss from agrochemical use, caused by intensive agriculture are not always experienced just at the local scale but can apply at a range of spatial scales impacting the wider ecosystem (Power, 2010). These disservices are often described as trade-offs, where certain regulating or supporting services are reduced as a result of maintaining or increasing current food, fiber and bioenergy production (Power, 2010).

Although some agri-environment schemes (AES) encourage the use of amelioration measures to reduce disservices from agriculture, for example, promoting extensification or introduction of buffer strips, they have attracted criticism for increasing production pressure elsewhere to account for a reduction in provisioning services (Ekroos et al., 2014; Horrocks et al., 2014). In the UK, for example, the Higher Level Stewardship scheme in England, and the Tir Gofal and Glastir agri-environment schemes in Wales, promoted conversion of arable land to species-rich permanent pastures as an extensification measure (UK Government, 2020a; Welsh Audit Office, 2007). However, it is important to discriminate between the aims of restoring and establishing species-rich permanent pastures compared to the desired ecosystem services of establishing herbal leys. Species-rich permanent pastures, such as described in the GS6 and GS7 scheme in England, aim to restore, maintain and protect important habitats such as lowland meadows and rush pastures (UK Government, 2021, 2020b). In contrast, schemes promoting the introduction of herbal leys in arable rotations (e.g., GS4 scheme in England) aim to restore soil quality and provide new habitats (e.g., for pollinators and soil invertebrates) (UK Government, 2020a). However, as with other AES, farmer willingness and the voluntary nature of schemes is recognized as a key limitation to uptake (Arnott et al., 2019).

Further, assessment of the benefits of these schemes indicated that they provided little tangible improvement in key indicators such as biodiversity, carbon storage, greenhouse gas reduction, and water quality (Arnott et al., 2019; Jones et al., 2017). Alternative strategies are therefore needed to promote current and future AES, particularly those that can be adopted at the landscape scale. In Ireland, an ongoing Results-Based Environment Agri Pilot Programme (REAP) is testing a results-based payment system to reward farmers for maintaining or improving their farm environment (Teagasc, 2021). Payments within this 2-year trial scheme are dependent on the results of an environmental scorecard, assessing ecological integrity (e.g., species richness), field margins (e.g., width) and the field boundary (e.g., hedgerow condition and density) (Teagasc, 2021). Farmers that establish herbal leys within their grassland system can receive payments up to 275 EUR ha^{-1} if achieving the maximum scorecard result (Teagasc, 2021). However, currently REAP does not include tilled fields or herbal leys sown within a crop rotation (Teagasc, 2021). This may be an avenue that future AES explore to encourage uptake of herbal leys to deliver multiple ecosystem services at a landscape level.

Although the establishment of species-rich pastures encourages improvements in biodiversity, it often fails to account for the persistent effects of previous intensive management on soil properties (Horrocks et al., 2016; Millennium Ecosystem Assessment, 2005). This can limit the potential ecosystem services that the conversion of arable land to herbal leys can offer. Incorporating leys and livestock into arable rotations offers the potential to increase provisioning services and ameliorate the disservices created by intensive arable agriculture. These potential services and disservices are illustrated in Figure 2.1.

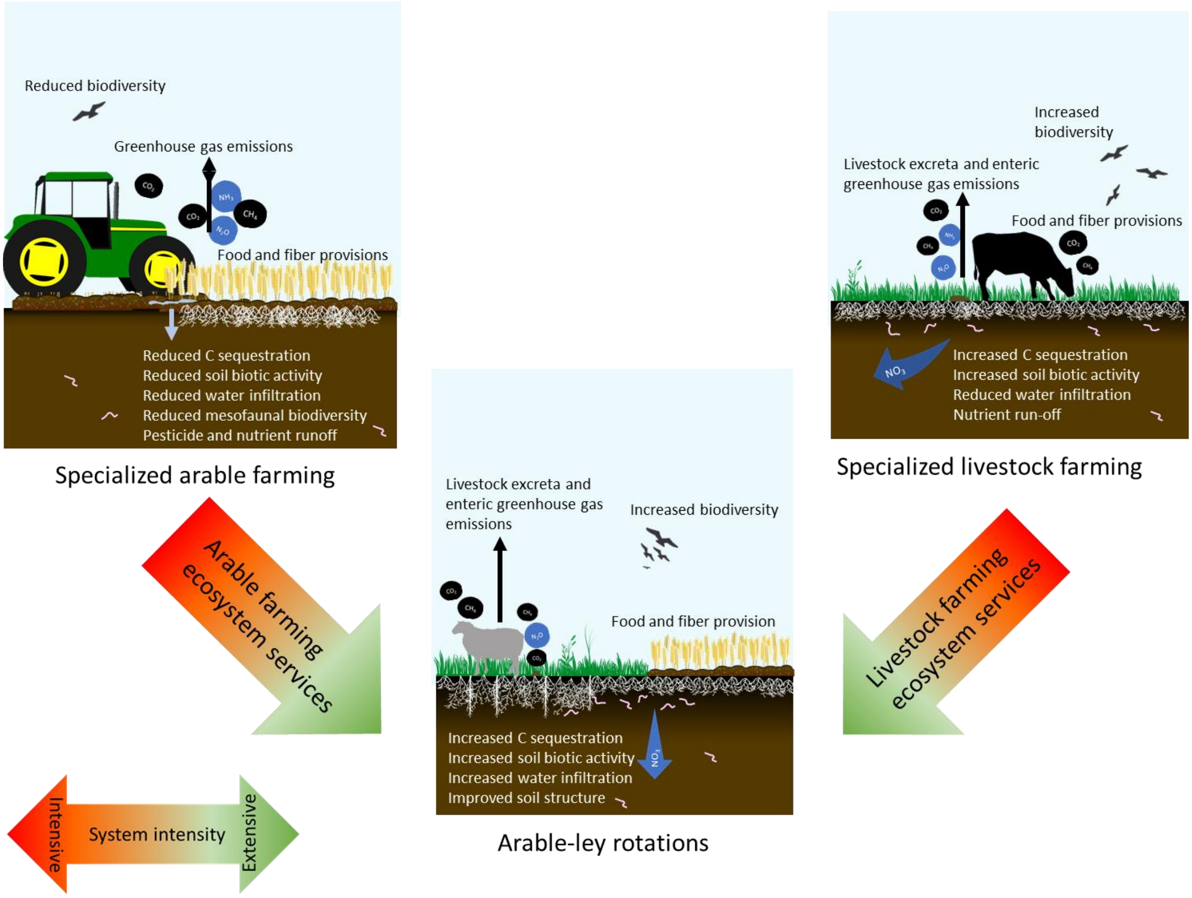

Figure 2.1. *Comparison of the ecosystem services and disservices produced by arable farming, livestock farming and arable-ley rotations.*

In Sweden, a system including a one-year grass ley in rotation did not encounter disservices between provisioning services and supporting or regulating soil services from the agroecosystem, but instead maximized the delivery of soil-focused ecosystem services (Albizua et al., 2015). Under the zero N fertilizer regime, the grass ley system produced a greater yield than the conventional continuous commodity crop system, which produced the least ecosystem services delivery overall (Albizua et al., 2015). If a herbal ley is introduced into cropping systems, it may provide a greater abundance of ecosystem services than a basic grass ley through increased resilience and complementarity of species (Horrocks et al., 2014; Lemaire et al., 2015; Malézieux et al., 2009). This was demonstrated in a 3-year, 31-site study across Europe where a four-species mixture consisting of two grasses and two legumes consistently outperformed the respective monoculture comparison of each plant species (Finn et al., 2013). This was attributed to the synergistic interactions between the plant types delivering transgressive overyielding and a greater resistance to weed invasion in the herbal ley than in the grass monoculture (Finn et al., 2013). During the 3-year study, only 4 % of the total yield was weed biomass in the herbal ley whereas the weed biomass of the monoculture mixtures increased from 15 % to 32 % over 3 years (Finn et al., 2013). The beneficial yield effects were also highlighted in Switzerland, where a temporary 3-year four-species mixture consisting of two grasses and two legumes receiving 50 kg ha^{-1} N delivered multifunctional ecosystem services at the same level as grass or legume monocultures receiving 450 kg N ha^{-1} (Suter et al., 2021). The greater delivery of ecosystem services such as N cycling, forage quality and production in the four-species mixture at a low N rate was mainly attributed to the symbiotic nitrogen fixation in legumes (Suter et al., 2021). Similar findings were reported in Ireland under rainfed conditions, where the annual yield of a 2-year six-species mixture receiving 150 kg N ha^{-1} outperformed the 2-year perennial ryegrass (*Lolium perenne*) monoculture receiving 300 kg N ha^{-1} by 1.3 t ha^{-1} (Grange et al., 2021).

In addition to the inclusion of nitrogen fixation from legumes in herbal leys, attributes such as deep rooting of plant species including cocksfoot (*Dactylis glomerata*), *Festulolium*, chicory (*Cichorium intybus*), lucerne (*Medicago sativa*), sainfoin (*Onobrychis*), and sweet clover (*Melilotus officinalis*) can increase regulating and supporting services such as C storage, nitrogen fixation, soil structure and biodiversity (McNally et al., 2015; Modernel et al., 2018; Nicholls and Altieri, 2013; Wagner, 2011). The deep rooting capabilities of these species enable access to water in deeper soil horizons to increase herbage production during dry periods compared to ryegrass leys, thus improving provisioning services (Albizua et al., 2015; Mueller et al., 2013). The benefits of species diversity under drought conditions were shown in Ireland by Grange et al. (2021), where a 2-year six-species mixture receiving 150 kg ha^{-1} N under drought conditions produced a similar yield (10.7 t ha^{-1}) to a rainfed 2-year perennial ryegrass monoculture receiving 300 kg N ha^{-1} (10.5 t ha^{-1}). The deep rooting capabilities of herbal leys may also recover both macro- and micronutrients from depth which would have otherwise been lost. Although leys can be used for silage, hay or haylage production, grazing livestock on herbal leys may increase farm productivity to address the economic gap that removing land from cultivation can cause. The supporting and regulating services that grazed herbal leys in arable rotations can provide is explored in detail in the following sections. Although there has been extensive research into the delivery of ecosystem services in species-rich permanent grasslands, there is a lack of research into the ecosystem services delivered by temporary herbal leys introduced to degraded arable land. Further research is needed to evaluate the potential benefits and disservices delivered by herbal leys, with an emphasis on how these services can be maintained in the following crop.

2.3. Impact of reintroducing leys on soil quality

2.3.1. Soil quality under cropping systems

Application of agrochemicals for crop protection, use of mineral fertilizers, tillage regime, high temperature and increased rainfall intensity are all factors that contribute to the loss of soil structure and soil fertility (Gregory et al., 2015; Nevens and Reheul, 2002; Pagliai et al., 2004). Tillage is frequently used in arable agriculture to provide an effective seedbed, aid the decomposition of plant residues, and reduce crop pests, pathogens and weeds (Kabir, 2005). However, continuous cultivation and intensive tillage practices such as moldboard plowing and harrowing frequently deplete SOC through enhanced oxidation, and also damage the soil crumb structure; reducing the macroporosity and contributing to greater compaction and erosion, which drive further soil degradation (Graves et al., 2015; Gregory et al., 2015; Panagos et al., 2018). The increased use of heavy farm machinery and the decline of mixed farming systems has increased reliance on mineral fertilizer inputs to maximize yield. The intensification of growing crop species that deliver only small amounts of organic matter that is stabilized in the soil has contributed to long-term depletion of SOM from arable soils, undermining soil stability that cannot be alleviated by normal tillage alone (Yang et al., 2020). Fine-textured arable soils with reduced SOM content are vulnerable to structural collapse when wet, and especially under compaction and prone to losses from water and wind erosion (Gregory et al., 2015). Soils with poor aggregate stability suffer from increased susceptibility to water erosion that can lead to on-site and off-site environmental and economic impacts, such as reduced water holding capacity, loss of valuable nutrients such as N, P and K, reduced water quality, eutrophication, increased flood risk, and erosion leading to the siltation of watercourses and estuaries (Pagliai et al., 2004). Under current practices, it is estimated 112 Mha (12 %) of European land area is under threat from water erosion, with a further 42 Mha affected by wind erosion (European Commission, 2006).

Maintaining a healthy soil structure is crucial in arable agriculture, as soil structure determines seedling establishment and root development, and thus nutrient use efficiency and yield (Pagliai et al., 2004). Tillage disrupts key biological processes responsible for soil structure formation and crop productivity. Arbuscular mycorrhizal fungal (AMF) hyphae, polysaccharides produced by microbial communities and mucilage excreted in earthworm casts act as an adhesive between soil particles and humus, forming micro- and macroaggregates and increasing soil stability and macropores that control infiltration rates (Berdeni et al., 2021; Lehmann et al., 2017). However, tillage can alter the composition and distribution of microbial communities in the soil profile, reduce earthworm populations and disrupt AMF hyphal networks, reducing their symbiotic ability to increase crop P uptake (Anderson et al., 2017; Kabir, 2005; Sun et al., 2018; Yvan et al., 2012). The effects of tillage on earthworms is seen immediately after compaction events, where earthworm populations experience a 70 % decline in total biomass due to animal death from crushing and lateral escape of the remaining population (Yvan et al., 2012). Effects of compaction on soil porosity can be seen for up to 2 years after the initial compaction event (Yvan et al., 2012). This has consequences for the restoration of soil structure and porosity of arable soils, as earthworm burrows aid the mechanical working of the soil and create interconnected macro- transmission pores and channels that allow plant roots rapid access to nutrients at depth and influence water and air infiltration (Langmaack et al., 2002; Shah et al., 2017). Additionally, reduced soil porosity and inadequate drainage from compaction can create anaerobic conditions ideal for denitrifying bacteria, also favoring increased nitrous oxide (N_2O) production (Grave et al., 2018).

Although some farmers attempt to ameliorate compaction and remove the plow pan through subsoiling, also referred to as deep tillage or subsoil ripping, this is fuel and labor intensive and creates a new soil structure inferior to that of uncompacted soil under grassland or reduced tillage management (Schneider et al., 2017). Numerous studies and intergovernmental bodies have recognized the damaging impact excessive tillage has for soil degradation and encourage the adoption of alternative tillage methods, for example, minimum tillage (min-till) and no-tillage (no-till), to alleviate environmental issues (Shukla et al., 2019). However, adoption of no-till methods remain slow in some areas due to concerns about soil compaction, reduction in pathogen inoculum, pest control (e.g., slugs), and perceived losses of crop productivity from herbicide resistant arable weeds, for example, black-grass (*Alopecurus myosuroides*), which would be buried deeper in the soil under an intensive tillage system reducing germination (Arai et al., 2018; Lenssen et al., 2013).

Repeated herbicide and pesticide applications to control arable weeds and pests in no-till systems have contributed to a greater increase in herbicide resistant weeds, and herbicide and pesticide runoff into watercourses than multi-pass tillage systems (Elias et al., 2018; Hull et al., 2014). In the UK, common herbicide resistant weeds such as black-grass, wild oats (*Avena* spp.), Italian ryegrass (*Lolium multiflorum*), common poppy (*Papaver rhoeas*), common chickweed (*Stellaria media*), scentless mayweed (*Tripleurospermum inodorum*) and sterile brome (*Bromus sterilis*) threaten crop yield and farm productivity in arable systems (Davies et al., 2019; Hull et al., 2014). Development of herbicide resistant weeds, and recently the discovery of glyphosate-resistant Italian ryegrass (Collavo and Sattin, 2012) and sterile brome (Davies et al., 2019), has resulted in some no-till farmers returning to more intensive tillage (Alskaf et al., 2020). This has increased pressure to develop alternative pest management regimes for no-till systems. Incorporating leys and livestock, such as sheep and goats, into no-till and min-till systems can provide biocontrol for arable weeds, preventing seed set and thereby depleting seed banks over consecutive years, and pests without sacrificing the ecosystem services and improved soil structure of no-till (Shipitalo and Owens, 2006).

2.3.2. Improving soil quality using arable-ley rotations

Integrated crop-livestock systems have long utilized cover-crops, leys and livestock in arable rotations to ameliorate soil degradation without excessive chemical and mechanical inputs (Schut et al., 2021). Due to their ease of establishment and diversity of use, grass or grass-clover leys are used to improve arable soil structure, increase soil fertility, improve yield, and disrupt pest and pathogen life cycles (Da Silva et al., 2014; Detheridge et al., 2016; Tracy and Davis, 2009). Most commonly, these leys are used for conservation, grazing or forage (e.g., hay or silage) production and are plowed into the topsoil after 1-4 years of use, losing some of the newly developed soil structure and accumulated SOC, and leaving soil and soil nutrients vulnerable to losses (Christensen et al., 2009). Despite requiring increased herbicide inputs to remove competition from unwanted plant species, no-till management can help to preserve the improved soil structure and biological activity post-ley (Franzluebbers and Stuedemann, 2008).

Improvement of arable soil quality under ley is dependent on several key factors: ley duration, botanical composition, soil type, grazing density of livestock and agronomic management (Christensen et al., 2009). It can take between 5 to 10 years for coarse sandy soils under an ungrazed grass ley to return to permanent pasture conditions and to up to 50 years for clay soils (Low, 1955). Perennial ryegrass leys lack deep rooting capabilities, limiting their potential to bioturbate the soil and remove subsoil compaction. Perennial legumes and herbs, for example, chicory and lucerne, with deep primary roots (i.e., taproots) in the ley can influence soil microbial community composition, nutrient cycling, and increase soil porosity and infiltration through the generation of large pores (> 2 mm diameter) in the subsoil (Cong and Eriksen, 2018; Kautz et al., 2014; Mytton et al., 1993). Perennial plant taproots generate large continuous pores from the topsoil to the subsoil through root compression of soil particles and mucilage excretion from the root tip. Once decayed, this produces large pores, encouraging earthworm activity and root growth for the following crop and offering opportunities for subsoil C deposition and storage (Kautz et al., 2014; Kell, 2012; Riley et al., 2008). However, it should be noted that short-term leys (< 3 yr) may be insufficient to realize the synergies between deep rooted crops and deep burrowing earthworms (Kautz et al., 2014).

Inclusion of legumes in the ley composition can have persistent effects on soil, often observed to affect the following crop (Detheridge et al., 2016). Legumes can further encourage additional symbiotic relationships between N fixing bacteria in their root nodules and AMF, which enhances P sequestration in return for nitrogen fixation and plant assimilated C (Hodge, 2000; Püschel et al., 2017). This can increase the abundance of AMF hyphae and improve aggregate stability of arable soils under ley, encouraging an improvement in soil structure (Haynes, 1999). Unlike arable soils where the AMF network is regularly disrupted by frequent tillage, leys allow AMF to establish a new permanent network that can be preserved by no-till management during the establishment of the following crop (Franzluebbers and Stuedemann, 2008; Hodge, 2000). In Argentina, arable soils under a temporary grass-clover ley experienced a rapid restoration of soil properties (i.e., SOC and microbial biomass N) to original values within 3-4 years (Studdert et al., 1997). Similarly, in New Zealand, microaggregates (< 0.25 mm) in arable soil under a temporary grass or grass-clover ley became highly water-stable macroaggregates (> 1 mm) after 5 years, attributed to the enmeshing of soil particles by grass roots and AMF hyphae (Haynes, 1999). Introduction of leys into arable rotations allows the development of a denser root system that encourages increases in microbial biomass, earthworm and mesofauna activity, and subsequently the production of binding agents (e.g., mucilage and exopolysaccharides) which enable soil aggregate stability (Shipitalo and Owens, 2006).

Increases in earthworm populations in soils under leys can accelerate the restoration of degraded arable soils (Hallam et al., 2020). Recovery of earthworm populations is relatively quick and increases with the duration of the ley (Kautz et al., 2014; Prendergast-Miller et al., 2021). Leys encourage the restoration of earthworm populations by no-till management for the duration of the ley, increased C inputs from roots compared to an arable crop, and increased humus and detritus from ley litter, providing food and habitat (Arai et al., 2018; Berdeni et al., 2021). Under herb and grass leys, populations of the anecic earthworm *Lumbricus terrestris* in degraded soils in Germany experienced a rapid increase over 1-2 years, but did not increase further when the duration of ley cropping was extended to 2-3 years (Kautz et al., 2014). Prendergast-Miller et al. (2021) corroborate this; earthworm recovery and abundance in a degraded arable soil in England was found to improve under a 2-year grass-clover ley (732 ± 244 earthworms m^{-2}) and this was four times higher than the arable control (185 ± 132 earthworms m^{-2}) which could potentially exceed the permanent grassland earthworm populations (619 ± 355 earthworms m^{-2} yr^{-1}) in field margin soils. Similarly, within a 6-year arable-ley rotation, earthworm biomass under a 3-year simple grass ley reached 187 g m^{-2} compared to 62 g m^{-2} and 30 g m^{-2} under temporary and permanent arable crop, respectively (van Eekeren et al., 2008). This was estimated as an increase of 40-45 g m^{-2} yr^{-1} under ley, indicating that within 4-5 years the earthworm biomass could reach that of a permanent grassland, although sometimes this recovery can happen sooner (van Eekeren et al., 2008). Earthworms are crucial engineers of soil structure, improving soil porosity through burrows, reducing bulk density, increasing SOM and redistributing AMF spores and mycelium through grazing (Hodge, 2000; Säle et al., 2015). Similarly, within 1 year of establishment of a grass-clover ley, Hallam et al. (2020) reported a decrease in soil bulk density of 6 % and an increase in SOM by 9 % due to increased earthworm populations.

Although the influence of livestock on soil quality has been well explored in pasture systems (e.g., Abdalla et al., 2018; Stavi et al., 2011), there is relatively limited information available for the influence of grazed leys on soil structure and key biological indicators of soil quality when incorporated into arable rotations, for example, microbial and fungal community composition or earthworm activity. This is important as arable soils depleted of SOM may be structurally weak, so could be less resilient to the effects of poaching and trampling by livestock when soils are moist. Studies on arable-ley rotations instead focus on soil C and N cycling of ungrazed or cattle grazed leys, discussed in detail in the next section; with limited attention given to the role of sheep grazed leys on soil structure in comparison to those grazed by cattle. The previously discussed studies in this section detailing the influence of ungrazed leys on earthworms, AMF and aggregate stability fail to consider the potential of compaction and excreta returns from grazing livestock. Inclusion of livestock on leys can stimulate increases in earthworm population, soil macrofauna (e.g., dung beetles), microbial and fungal biomass, and above- and below-ground plant biomass, but risk increased topsoil compaction (Martin et al., 2016; Shah et al., 2017).

Partial improvements in soil physical properties have been observed in cattle-grazed integrated crop-livestock systems within the first annual crop cycle after the overwinter ley or cover crop has been reverted back to arable (Ambus et al., 2018). Integrated crop-livestock systems typically utilize winter cover-crop grazing by cattle in arable rotations to provide green manure to the soil, control arable weeds and stimulate bioturbation of the soil (Da Silva et al., 2014; Nascimento et al., 2019; Schuster et al., 2016). These winter cover-crops are often plowed into the soil in spring to prepare for the following crop. However, cattle-grazed integrated crop-livestock systems often suffer from increased topsoil (0-5 cm) compaction from livestock trampling of the already weakened arable soil structure (Ambus et al., 2018). Static pressure exerted from sheep and cattle hooves averages 66 kPa and 138 kPa, respectively, whereas nominal tire pressures of farm machinery range from 74-81 kPa (Greenwood and McKenzie, 2001). This can collapse macropores in the soil surface, reduce soil porosity and hydraulic connectivity, thus reducing water infiltration and promoting surface runoff and flooding (Shah et al., 2017). Soils with high moisture content are vulnerable to collapse and deformation under livestock trampling, leading to soil poaching and erosion (Greenwood and McKenzie, 2001; Laurenson and Houlbrooke, 2014). Farmers reintroducing livestock into arable rotations need to avoid overstocking and grazing livestock on weakly structured and fine-textured soils, especially when saturated, to preserve soil structure. An increase in compaction and penetration resistance can have persistent effects on the following crop, reducing root growth and thus yield (Shah et al., 2017). It has therefore been suggested that preference when grazing should be given to sheep over cattle due to lower static pressures and smaller hoof sizes. In an integrated crop-livestock system in New Zealand, Laurenson and Houlbrooke (2014) found that the soil bulk density under sheep grazing of overwinter forage crops was 1.26 Mg m^{-3} (0-5 cm) compared to 1.35 Mg m^{-3} (0-5 cm) for cattle grazing.

In arable-ley rotations, sheep grazing of a 3-4 year grass-clover ley resulted in increased bulk density in the 0-35 cm layer, reduced air permeability and macropore continuity compared to ungrazed undersown oats (Ball et al., 2007). However, since grass-clover leys have a particularly dense root system, macroporosity (pores > 100 µm) was greater in the 0-10 cm layer than in the undersown oats (Ball et al., 2007). Conversely, Riley et al. (2008) examined soil physical properties within a 15-year mixed dairy-arable field trial under different grazing intensities and management (e.g., organic *vs.* conventional, and different durations of grass-clover ley) on a loam soil in Norway. They found that soil bulk density increased by 0.14 Mg m^{-3} for the continuous arable, but in the cattle-grazed grass-clover ley undersown with cereals in a 4-year rotation, bulk density decreased by 0.03 Mg m^{-3} for a 2-year ley in a standard dairy production rotation and by 0.02 Mg m^{-3} for an organic rotation with a 3-year ley. This was a relatively small reduction in bulk density for both systems with a ley, but may be attributed to post-ley reversion (e.g., plowing) and duration of ley within the 15-year trial. Similar findings were also found for soil porosity and aggregate stability of cattle grazed leys. Soil porosity within continuous arable rotations decreased by 4.3 %, but increased by 1.4 % for the conventional dairy system with a 2-year ley in rotation and by 0.2 % in the organic dairy system with a 3-year ley in rotation (Riley et al., 2008). Notably, continuous arable soils under reduced tillage management had the same aggregate stability as the rotation with a 3-year ley, highlighting the importance of preserving aggregate stability through reduced tillage and reduced compaction (Ball et al., 2007; Riley et al., 2008).

Currently, there are no reported studies that have compared continuous arable cultivation with mown, cattle or sheep grazed leys in rotation under the same soil type, sown botanical composition or different tillage management regimes. However, maintaining the same botanical composition under different sward management in future research may be difficult due to differences in grazing pressure and selective grazing between sheep and cattle; this may impact the persistence of certain plant species in the mixture and thus affect subsequent ley species composition. The influence of both sheep grazing, and in particular, complex herbal leys, on soil structure and associated biological functions requires further research to critically evaluate the potential benefits and disbenefits sheep grazed arable-ley rotations can provide.

2.4. Carbon and nitrogen cycling in arable-ley rotations

2.4.1. Changes in microbial communities

Arable-ley rotations can alter the microbial community composition, biomass, and activity of agricultural soils and the subsequent cycling of C and N (Six et al., 2006). Soil temperature, soil properties (e.g., pH), climate, botanical composition of the ley, nutrient and cultivation management can influence the soil microbial community and conversely C sequestration of arable soils (Jones et al., 2019; Murphy et al., 2007; Six et al., 2006; van Eekeren et al., 2008). Formation of SOC stocks is regulated by the decomposition of SOM and root exudates by Gram-positive and Gram-negative bacteria and saprotrophic fungi (Mellado-Vázquez et al., 2019). However, microbial communities responsible for the decomposition of SOC in arable soils are sensitive to temperature changes. With global temperature increases projected to exceed 2 °C under different climate scenarios, this could affect the breakdown of SOC stocks and undermine efforts for C sequestration (Collins et al., 2013). Microbial carbon use efficiency (CUE) determines the allocation of C for biomass growth, respiration (CO_2 emissions), and ultimately necromass (Bölscher et al., 2020). In a study by Bölscher et al. (2020), sensitivity of CUE to increases in temperature reduced SOC stocks in Swedish grassland soils by 0.1-0.18 kg C m^{-2}, 4 % of their current stocks. Unlike grassland, forest or ley farming soils, microbial CUE in continuously cropped soils was not sensitive to temperature changes between 5-20 °C (Bölscher et al., 2020).

Although there has been extensive research on the effects of grassland (McAuliffe et al., 2020; Qu et al., 2020) or arable farming (Kautz et al., 2013) on soil microbial communities and functioning, there have been few attempts to measure this in arable-ley rotations (Jarvis et al., 2017). Limited studies, however, have revealed that ley farming can increase the soil microbial biomass (Albizua et al., 2015; Zarea et al., 2009). Long-term research on a sandy loam soil in a temperate climate at the Rothamsted Woburn Arable-Ley field trial in England also found that microbial biomass C and N pool was significantly larger in the arable-ley soil (964 kg C ha^{-1} and 122 kg N ha^{-1}) than the continuous arable control (518 kg C ha^{-1} and 92 kg N ha^{-1}) following an 8-year fertilized grass ley (Murphy et al., 2007). Under different conditions, results from a 25-year arable-ley cropping system experiment in Norway on a silty-sandy loam soil in a humid continental climate indicated that there was no increase in microbial species richness or diversity, despite increasing microbial biomass (Chen et al., 2020). The leys in both these studies used either grass or grass-clover leys, with low species diversity. Inclusion of herbs, such as plantain (*Plantago lanceolata*) or caraway (*Carum carvi*), into grass-clover mixtures were found to increase the ratio of fungi to bacteria but decrease Gram-positive bacteria, indicating a faster growing and more active microbial community (Cong and Eriksen, 2018). However, as far as it known, there have been no studies on microbial community composition, biomass, or diversity within herbal leys under field conditions.

2.4.2. Carbon sequestration using arable leys

The *4-per-mille* initiative aims to increase global SOM content by 0.4 % per year to compensate for increases in atmospheric GHG emissions (Minasny et al., 2017). In particular, the initiative has promoted arable-ley rotations to increase global SOC stocks. In the UK, uptake of arable-ley rotations is projected to increase UK net SOC stocks by 1.6 t C ha^{-1} yr^{-1}, with England in particular accumulating 0.20 t C ha^{-1} annually in the 0-23 cm layer (Minasny et al., 2017; Ostle et al., 2009). However, the *4-per-mille* initiative has attracted criticism as being impractical and uneconomical in practice for land managers (Poulton et al., 2018). This largely depends on the payments system and AES employed on-farm, as under certain schemes, such as the Environmental Land Management scheme in England, this may become economically viable (DEFRA, 2018). The use of arable-ley rotations to increase SOC is highly dependent on soil conditions and management; the limited evidence from long-term replicated trials and measurements of SOC post-ley conversion back to arable requires SOC results to be interpreted with caution. In addition, most studies have focused on a single ley rotation, rather than repeated cycles of herbal leys and arable cropping. Table 2.1 presents changes in SOC content reported from previous global field trials utilizing arable-ley rotations; studies relying solely on modeling changes in SOC were not considered.

Table 2.1. *Changes in soil organic carbon (SOC) content in arable-ley rotations, adapted from Schut et al. 2021.*

Publication	Country	Average annual rainfall (mm)	Soil type	Arable-ley rotation	Ley composition	Ley Management	Soil depth (cm)	Annual C input to soil	Change in SOC	Comments
Börjesson et al. (2018)	Sweden	569 (Lönnstorp), 558 (Lanna)	Loam (Lönnstorp), clay (Lanna)	4-year rotation: 3 years ley, 1-year cereal crop.	Grass-clover (meadow fescue, timothy, and red clover).	Mown with biomass removed. Four N fertilizer treatments: 0, 50, 100, and 150 kg N ha^{-1} yr^{-1}.	0-20	1.7-2.5 t ha^{-1} C in leys. 0.25-1 t C ha^{-1} in cereals.	+0.28 (-N) and +0.35 (+N) t C ha^{-1} at Lönnstorp. +0.07 (-N) and +0.17 (+N) t C ha^{-1} at Lanna.	No significant effect of N fertilizer on SOC stocks in the ley rotations.
Johnston et al. (2017)	UK	640	Sandy loam	Alternating rotation lengths followed by 8-year ley rotation. All leys followed by 2-year arable crop.	Grass or grass-clover (ley composition not given).	Grazed by sheep or mown with biomass removed.	0-25	n.d.	+0.01 t C ha^{-1} for 3-year grass ley. +0.16 t C ha^{-1} for 3-year grass-clover ley. +0.36 t C ha^{-1} for 8-year grass ley. +0.28 t C ha^{-1} for 8-year grass-clover ley.	Long-term field trial started in 1938. Changes in SOC data is measured from 1965-74 to 2000-2009.
Krauss et al. (2017)	Switzerland	1303 (2012), 1112 (2013), 966 (2014)	Calcareous clay	6-year rotation: 2-year ley, 4-year arable crop.	Grass-clover (ley composition not given).	Mown with biomass removed. Cattle slurry applied after each cut.	0-50	n.d.	+8.1 t C ha^{-1} for arable-ley soils under reduced tillage and manure application compared to conventional tillage.	Increased stratification in soils under no-till management with manure applications, with highest SOC content in the surface soil. SOC did not increase in lower layers.

Albizua et al. (2015)	Sweden	655	Sandy loam, sandy clay loam, and coarse-loamy soil	4-year rotation: 1-year ley, 3-year crop.	Grass (ley composition not given).	Mineral N fertilizer addition: 0-150 kg N ha^{-1}. 20-30 t ha^{-1} of manure applied following wheat harvest at the end of the 4th year in rotation.	0-20	n.d.	+0.39 % SOC in 0 kg N ha^{-1} ley system. +0.1 % SOC in 150 kg N ha^{-1} ley system.	Leys in rotation with additional mineral N fertilizer inputs results in positive effect on SOC content.
Chan et al. (2011)	Australia	544	Clay loam	3- or 6-year rotation: see paper for more details.	Grass-legume (reed canary grass, cocksfoot, lucerne, and subterranean clover) or grass-clover (annual ryegrass and subterranean clover).	Grazed by sheep or mown with biomass removed.	0-30	n.d.	+500-700 kg C ha^{-1} yr^{-1}.	Long-term field trials ranging 13-25 years. Increased SOC stocks following ley are quickly depleted by tillage and crop residue management.
Bolinder et al. (2010)	Sweden	567 (Offer), 490 (Ås), and 566 (Röbäcks dalen)	Silty clay loam	6-year rotation: 1-5 years of ley and arable crop.	Grass-clover (meadow fescue, timothy, and red clover).	Ungrazed. Manure applied to ley in autumn (20 t ha^{-1}).	0-25	n.d.	+12 g C m^{-2} yr^{-1} for rotation A (5 years ley, 1-year crop).	60-year field-trial of leys in organic dairy cropping systems.

Chirinda et al. (2010)	Denmark	704	Sandy loam	4-year rotation: 1-year ley, 3-year arable with catch crops undersown in the barley crop.	Grass-clover (white clover and perennial ryegrass or red clover and perennial ryegrass)	Mown with biomass removed. Anaerobically digested pig slurry used as N fertilizer.	0-30	3.95 ± 0.06 t C ha^{-1} between 1997-2007.	-1 g C kg^{-1} between 1996 and 2008.	10-year field trial. Increased C inputs resulted in increased microbial activity but not C storage.
Christensen et al. (2009)	Denmark	862	Sandy loam	6-year rotation: 1-6-year ley in rotation with barley undersown with grass.	Grass (perennial ryegrass, meadow fescue, timothy, smooth meadow grass).	Mown with biomass removed. 225 kg N ha^{-1} yr^{-1} of mineral fertilizer applied. 75 kg N ha^{-1} after the first and 50 kg N ha^{-1} second cut.	0-20	n.d.	+1100 kg C ha^{-1} yr^{-1} in soils under ley.	Lack of residual effect highlights need for legumes in the ley composition.
van Eekeren et al. (2008)	Germany	n.d.	Sandy loam	6-year rotation: 3-year grass-clover ley, 3-years arable crop.	Grass-clover (perennial ryegrass and white clover).	Grazed by dairy heifers from 1966 to 1989. Mineral N fertilizer: either 0 or 354 kg N ha^{-1} yr^{-1}.	0-10	n.d.	+7.5 g SOC per kg dry soil*.	Long-term field trial established in 1966. (* = SOC content of permeant arable minus average SOC of temporary grassland and temporary arable).

Studdert et al. (1997)	Argentina	870	Loam	7-year rotation: 2-year or 4-5-year ley with respective crops in rotation.	Grass-legume (cocksfoot, bulbous canary grass, tall fescue, perennial ryegrass, white clover and alfalfa).	Mown with biomass removed.	0-15	n.d.	Returned to original SOC content 3-4 years under ley (37.2 g SOC per kg).	Long-term field trial established in 1976-1993.
Clement and Williams (1964)	UK	n.d.	n.d.	4-year ley.	Grass-clover (see paper for details).	Grazed and mown for hay, with the post-mown ley grazed.	0-15	n.d.	+0.23 % SOC for grazed ley. +0.17 % SOC for cut and then grazed ley.	Increases in SOC was limited to 2-3 cm of the topsoil.

n.d., not determined; SOC, soil organic carbon; +, increases in SOC; -, decreases in SOC; and +N or -N with or without mineral N fertilizer.

Plant species scientific names: Bulbous canary-grass (*Phalaris aquatica*), cocksfoot (*Dactylis glomerata*), lucerne or alfalfa *(Medicago sativa*), meadow fescue (*Festuca pratensis*), meadow grass (*Poa annua*), red clover (*Trifolium pratense*), reed canary grass (*Phalaris arundinacea*), ryegrass (*Lolium perenne*), subterranean clover (*Trifolium subterraneum*), timothy (*Phleum pratense*), white clover (*Trifolium repens*).

The information summarized in Table 2.1 highlights the lack of homogeneity in reporting changes in SOC in arable-ley rotations in the literature. These studies report SOC content to different depths, different units (e.g., weight *vs*. volume basis), and often without reporting the annual C input from the ley in rotation. Further, the lack of bulk density information prevents changes being expressed on a land area basis. Where livestock are used, such as in Clement and Williams (1964), van Eekeren et al. (2008), Chan et al. (2011) and Johnston et al. (2017), the authors did not estimate nutrient recycling from grazing livestock to allow comparison between studies.

Studies on arable-ley rotations (Table 2.1) have mainly utilized grass or grass-clover leys, with limited attention given to herbal leys with deep rooted plants that can provide other desirable traits (e.g., pollinator potential and biological nitrification inhibitor production). Botanical composition of the leys is crucial, as inclusion of legumes in grass-clover mixtures provide greater SOC increases than grass-only leys (Christensen et al., 2009; Hanley et al., 1973). Diverse herbal leys can sequester more C than simple grass or grass-clover leys due to the greater root mass and rooting depth. In New Zealand, diverse swards including herbs such as chicory, plantain and lucerne increased root mass by 5.32-9.35 t ha^{-1} compared to 3.81-5.70 t ha^{-1} root mass of a ryegrass-clover pasture, thus increasing C inputs into the topsoil (0-30 cm) by 1.20 t C ha^{-1} yr^{-1} (McNally et al., 2015). In comparison, annual soil C inputs from wheat (*Triticum aestivum*) roots were estimated to be only 0.40 t C ha^{-1} yr^{-1} (Sun et al., 2018). Although topsoil SOC is recognized as the most important functionally, the grass only and grass-legume leys listed in Table 2.1 were reported to suffer from C stratification and limited subsoil SOC increases, attributed to the shallower root depths in common grass-clover leys (Clement and Williams, 1964; Krauss et al., 2017).

Management of leys is also an important factor in C sequestration. Unsurprisingly, SOC content in the topsoil increased with the duration of the ley and the proportion of pasture in the crop rotation (Chan et al., 2011). Grazed leys were found to sequester more C than mowing and removal of biomass as livestock return about a quarter of the OM they consume to the soil increasing SOM content (Christensen et al., 2009; Clement and Williams, 1964). Livestock excreta inputs and trampling of OM (e.g., plant litter and fecal matter) into the topsoil can increase SOC content by 0.23 % compared to 0.17 % for mown swards (Clement and Williams, 1964). This was also seen in Johnston et al. (2017), where SOC content within a 28-year sheep grazed ley rotation increased by 0.33 t SOC ha^{-1} yr^{-1}, equivalent to an increase of 0.9 % per year, exceeding the 0.4 % increase encouraged by the *4-per-mille* initiative.

The Highfield arable-ley experiment established in 1949 at Rothamsted Research Harpenden, UK, revealed that conversion of grassland to arable decreased SOC stocks by 30 %, whereas arable to grassland conversion only increased SOC stocks by 8 % (Jensen et al., 2020). These changes progressively slowed and tended towards new equilibria, indicating that rates of SOC loss and gain are greatest in the early years of management change. Evidence from long-term field trials, such as those held at Rothamsted Research, provides a warning that SOC content of soils under arable-ley rotations will eventually reach a new equilibrium or quasi-equilibrium where the total SOC will not change but the proportion of SOC in each pool will shift (Johnston et al., 2017). Once the new equilibrium is reached, tillage practices such as subsoil ripping may be useful to mix C rich topsoil with the subsoil as a form of occasional tillage where mixing from soil biota and fauna (e.g., earthworms) is not sufficient, allowing for C burial in the subsoil and establishment of a new equilibrium. Concerns over economic losses from the uptake of arable-ley rotations can be mitigated by utilizing livestock on the ley (Knight et al., 2019). However, this would require significant changes in policy, governance and stewardship schemes to encourage arable farmers to diversify their enterprise or engage with partnerships at a community level and establish grazing agreements with livestock farmers.

2.4.3. Biological N fixation and nitrification inhibitors

In most arable agriculture, mineral N fertilizers have largely replaced the use of legumes for increasing N content of soils (Crews and Peoples, 2004). However, restrictions on fertilizer usage to constrain water and air pollution, consumer demand for organically grown produce, and increases in mineral fertilizer costs has returned attention to legumes in cropping rotations (Crews and Peoples, 2004). The symbiotic relationship between legumes and rhizobacteria (such as *Rhizobium* or *Bradyrhizobium*) within root nodules allows for atmospheric N_2 fixation in return for plant generated carbohydrates (Wagner, 2011). This can also encourage the growth of grasses, leading to greater SOC inputs. For a 16-species diverse plant species mix, soil C and N increased by 70 ± 9 g C m^{-2} yr^{-1} and 3.5 ± 0.53 g N m^{-2} yr^{-1} respectively, compared to monocultures of the same species (14 ± 10 g C m^{-2} yr^{-1} and 0.59 ± 0.57 g N m^{-2} yr^{-1}) (Fornara and Tilman, 2008). Due to their N fixing abilities, legumes in leys can be used as a partial or full replacement for mineral fertilizers. Depending on abiotic conditions in the year of establishment, grass-clover leys plowed into the arable rotation saved 50 % to 83 % or 77 % to 92 % of fertilizer N typically applied during the arable phase of the rotation (Ten Berge et al., 2016). However, while grass-clover leys can reduce mineral fertilizer N inputs, plowing risks N leaching and subsequent eutrophication and nitrate (NO_3^-) pollution of watercourses (Nevens and Reheul, 2002), as well as release of the potent greenhouse gas N_2O. Under min-till management, these N losses may be greatly reduced.

Nitrification inhibitors (NIs) are used in agriculture to improve nitrogen use efficiency (NUE) by inhibiting the bacterial oxidation of ammonium (NH_4^+) to NO_3^-, thus reducing N losses through leaching, runoff and denitrification (Norton and Ouyang, 2019). Synthetic NIs such as DMPP (3,4-dimethylpyrazole phosphate) and DCD (dicyandiamide) are often used, however, these have varying levels of effectiveness and risk leaching into the environment and the contamination of water sources (Coskun et al., 2017; Zerulla et al., 2001), and in the case of DCD, potential contamination of the food-chain (MPI, 2013). Effectiveness of NIs is highly dependent on soil properties (e.g., pH and texture), crop type (e.g., cereals *vs*. forage crops) and management factors (e.g., N fertilizer rate, and irrigation *vs*. rainfed crops) (Abalos et al., 2014). When applied to cattle urine, DCD was effective at reducing N_2O emissions by 70 % but not NO_3^- leaching or ammonia (NH_3) emissions (Misselbrook et al., 2014) whereas DMPP was ineffective at reducing N_2O emissions from cattle and sheep urine patches (Marsden et al., 2017; Misselbrook et al., 2014).

Naturally occurring NIs, or biological nitrification inhibitors (BNIs), are excreted from root exudates by herbs such as plantain to inhibit nitrification in the surrounding soil in N limited environments (Subbarao et al., 2012). Plantain contains high levels of plant secondary metabolites such as aucubin, acteoside and catapol that act as BNIs of enzymes and nitrifying bacteria responsible for nitrification, *Nitrosomonas* and *Nitrobacter* (Dietz et al., 2013; Gardiner et al., 2018; Wu et al., 2017). A laboratory incubation study by Dietz et al. (Dietz et al., 2013) reported that the addition of plantain leaves resulted in reduced soil NO_3^- content for the 56 days in incubation. However, these plant secondary metabolites vary with growing season and climate. Aucubin concentrations in plantain increased from 3.8 to 6.9 mg g^{-1} DM in the first and second growing season (Navarrete et al., 2016). This can have implications for its efficacy as a BNI and the potential effect on NUE and N partitioning in livestock. Livestock grazing leys containing plantain can excrete plant secondary metabolites such as aucubin into the soil in urine, suppressing microbial activity within the urine patch (Gardiner et al., 2018). The effect of plant secondary metabolites on livestock is explored in detail in the following section.

2.4.4. Enteric CH_4 emissions

Ruminants, such as cattle and sheep, represent significant sources of CH_4 emissions in livestock production. In 2017, cattle and sheep in the UK produced 16.8 and 4.0 Mt CO_2e of CH_4 by enteric fermentation (Office for National Statistics, 2019). CH_4 is a powerful GHG, with a lifetime of 12.4 years and 100-year global warming potential (GWP) 28-36 times that of CO_2 (IPCC, 2014) and a 20-year GWP 86 times that of CO_2 (Liu et al., 2021). Recent revisions to GWP calculations have led to the development of GWP*, which accurately accounts for the reduced radiative forcing of short-lived climate pollutants such as CH_4 compared to long-lived climate pollutants such as N_2O and CO_2 (Allen et al., 2018). GWP* is currently used to increase the modeling accuracy assessing mitigation measures to reduce the future impacts of ruminant production, helping countries identify feasible methods to achieve the coveted Net Zero C emissions (Liu et al., 2021). In ruminants, CH_4 production can vary with diet, animal, rumen microbiome composition and health. Currently, the IPCC default tier 1 emission factor estimates enteric fermentation emissions from sheep in developed countries as 8 kg CH_4 head^{-1} yr^{-1} and requires refining (IPCC, 2006).

The use of plant secondary metabolites for enteric CH_4 mitigation in ruminants has been extensively reviewed in the literature (Aboagye and Beauchemin, 2019; Bodas et al., 2012; Martin et al., 2010; Ramírez-Restrepo and Barry, 2005). Key herbs used in herbal leys such as chicory, plantain, sainfoin and birdsfoot trefoil (*Lotus corniculatus*) contain high levels of plant secondary metabolites such as tannins, saponins and essential oils that can moderate the microbial production of CH_4 in the rumen (Bharanidharan et al., 2018). Saponins can suppress dihydrogen producing rumen protozoa, essential for the production of CH_4 (Wallace, 2004). Similarly, condensed or hydrolyzable tannins can reduce CH_4 production by preventing fiber degradation in the rumen by complexing with proteins that are released for degradation in the low pH of the abomasum (Hatew et al., 2015; Ramírez-Restrepo and Barry, 2005). Addition of tannins and saponins as a feed supplement, however, should be used with caution, as hydrolyzable tannins and saponins in the rumen can be toxic to the host animal as well as to methanogens (Aboagye et al., 2018; Martin et al., 2010; Wallace, 2004).

Naturally occurring tannins in chicory and sainfoin vary in concentration according to genotype, season, and management. Consequently, making measurements of tannin content within the forage is crucial for studies when investigating changes in rumen CH_4 production. Addition of tanniniferous crop species to ensiled or dried forages (e.g., hay) has recently been found to decrease CH_4 emissions (Chen et al., 2021). In contrast, tannin addition to beef cattle fed a basal diet of lucerne and barley (*Hordeum vulgare*) silage reduced rumen ammonia concentration but not daily CH_4 production (Aboagye et al., 2018). Similarly, no differences in daily CH_4 emissions were found for cattle fed sainfoin and birdsfoot trefoil hay (Stewart et al., 2019). This was attributed to the drying of plants inactivating the bioactive tannins in the forage (Stewart et al., 2019)$^-$. However, enteric CH_4 emissions were reduced from sheep fed either ensiled mixes of timothy (*Phleum pratense*) with either sainfoin (29.7 g CH_4 kg^{-1} DM intake) or red clover (*Trifolium pratense*) (30.5 g CH_4 kg^{-1} DM intake) containing high levels of condensed tannins and polyphenol oxidase respectively, compared to pure ensiled timothy (35.7 g CH_4 kg^{-1} DM intake) (Niderkorn et al., 2019). In fresh forage, no differences in daily CH_4 production was observed in sheep fed fresh chicory or ryegrass, with 24.1 and 21.4 g CH_4 kg^{-1} DM respectively (Sun et al., 2012). For sheep fed ryegrass or a herbal ley mixture containing clover and herbs (herb composition was unspecified), CH_4 production was lower in the herbal mix (16.1 g day^{-1} ryegrass *vs*. 12.9 g day^{-1} herbal) (Fraser et al., 2015).

The variable reports in the literature of plant secondary metabolites reducing CH_4 emissions within livestock systems indicates that much more work needs to be undertaken to explore the complex relationships between diet and CH_4 production. The effect of grazing ruminants on herbal leys with herbs containing plant secondary metabolites is relatively understudied but is thought to affect other aspects of the ruminant, such as N excretion and parasite burden (Niderkorn and Jayanegara, 2021). This indicates that a single focus on CH_4 and live weight gain also needs to be coupled with studies of other aspects of rumen functioning.

2.4.5. Urine-patch N_2O emissions

Increasing the available grazing area in the UK by reintroducing grazed leys into arable rotations risks increasing livestock N_2O emissions. As ruminants are relatively inefficient at N assimilation, only 5 % to 10 % of the N consumed is utilized in meat, milk and wool production, with the remainder excreted in urine and dung (Simon et al., 2019). Urine deposited to pasture contains 70 % to 75 % and 45 % to 60 % of N excreted by sheep and cattle, respectively, and represent significant sources of livestock N_2O emissions (López-Aizpún et al., 2020). N_2O is a potent GHG, with a GWP 298 times that of CO_2 that requires careful monitoring (Butterbach-Bahl et al., 2013). Between 1961 and 2014, 54 % of global annual N_2O emissions from grasslands were attributed to livestock excreta deposits, with only 13 % and 7 % attributed to manure N and mineral N respectively (Dangal et al., 2019). Urine in particular contains readily available C and N that produce hotspots of N_2O emissions within the grazing pasture, whereas dung contains more insoluble forms of N thus is more inert and slower to breakdown into N_2O (Butterbach-Bahl et al., 2013; Misselbrook et al., 2005; Van Groenigen et al., 2005).

Sheep urine composition and N content is heavily dependent on diet and animal health, and ranges from 1.2 to 13.0 g L^{-1} N (Marsden et al., 2020). 25 % to 90 % of N in urine consists of urea, followed by purine derivatives and non-urea compounds: hippuric acid, allantoin, creatine, creatinine, uric acid, xanthine and hypoxanthine as well as any plant secondary metabolites such as the aucubin derivative aucubigenin (Bristow et al., 1992; Dijkstra et al., 2013; Gardiner et al., 2016; Simon et al., 2019). Diet manipulation, for example, by introducing plants with particular secondary metabolites into the pasture, can have a diuretic effect, reducing the proportion of urea in the urine and increasing the content of less labile non-urea compounds (Gardiner et al., 2016). This has potential for use in swards containing high clover content to increase productivity and milk production, but exceeds the animal’s requirement for N resulting in increased N excretion to pasture (Dijkstra et al., 2013). Plant secondary metabolites, such as tannins, can also increase the proportion of N in dung, which is less vulnerable to N_2O and NH_3 losses than urine (Huyen et al., 2016; Misselbrook et al., 2005). However, the effect of plant secondary metabolite containing plants in temperate herbal leys on ruminant urine composition and subsequent N_2O emissions is relatively understudied. A recent meta-analysis found that the relationship between animal diet and urine composition were under-reported in the literature (López-Aizpún et al., 2020). Currently, the literature available for urine-patch N_2O emissions, and subsequent emission factors, is dominated by grass or grass-clover pastures with almost no information available for herbal leys.

In 2019, the IPCC announced the refinement of the 2006 Guidelines for National Greenhouse Gas Inventories, including refinements for the N_2O emission factor for livestock urine and dung on pasture, range and paddock (EF_{3PRP}) for sheep and cattle (IPCC, 2019). This reduced the 2006 EF_{3PRP} from 1 % to 0.3 % of the N applied to the soil in urine and dung emitted as N_2O (IPCC, 2019, 2006). There are currently no studies used by the IPCC to refine the EF_{3PRP} that use emissions reported from grazed herbs such as chicory. Although some studies did not report sward composition (Cardenas et al., 2016; Misselbrook et al., 2016; Tully et al., 2017), calculations were made from predominantly grass swards (Chadwick et al., 2018; Forrestal et al., 2017; Hoogendoorn et al., 2016; Hyde et al., 2016; Krol et al., 2016; O’Connor et al., 2016; Pelster et al., 2016; Thomas et al., 2017; Yamulki et al., 1998), grass-clover (Balvert et al., 2017; Di et al., 2016; Galbally et al., 2010; Hoogendoorn et al., 2016; Marsden et al., 2017, 2016; Owens et al., 2017, 2016) or forage crops (e.g., barley, lucerne, or brassicas such as rape, *Brassica napus,* or kale, *Brassica oleracea var. sabellica*) (Di et al., 2016; Hoogendoorn et al., 2016; van der Weerden et al., 2017). Currently, no estimates of direct livestock excreta N_2O emissions from grazed herbal leys are included in the IPCC calculations.

Although diet can alter the ratio of N in urine and dung, key soil properties such as pH, moisture, porosity, temperature, texture and microbial activity can affect N cycling in the urine patch (Bell et al., 2015; Loick et al., 2017; López-Aizpún et al., 2020; Wu et al., 2017). Microbial activity responsible for N_2O emissions by the processes of nitrification (NH_4^+ to NO_2^- then to N_2O and NO_3^-) and denitrification (NO_3^- and NO_2 to N_2O, NO_X and N_2) can be altered by plant secondary metabolites within a herbal ley (Allen et al., 1996; Gardiner et al., 2016). Urine-derived plant secondary metabolites such as acteoside, aucubin and isothiocyanates can act as natural NIs within the urine patch, suppressing the microbial activity of nitrate-oxidizing bacteria, ammonia-oxidizing archaea and ammonia-oxidizing bacteria (Gardiner et al., 2016; Liu et al., 2017; Navarrete et al., 2016). However, as with urine composition, the plant secondary metabolite content of urine and longevity in the soil is also inadequately studied. It is unclear what form plant secondary metabolites exist as when broken down in urine or how much is excreted. For a dairy system, Gardiner et al. (2018) estimated a potential aucubin urine excretion rate of 0.49-3.40 t ha^{-1} for cattle grazing pastures with variable proportions of plantain in the sward composition. Further, the persistence of these plant secondary metabolites in the subsequent crop remains unknown.

Several recent studies have investigated the effect of herbal leys on N_2O losses, particularly in dairy production systems. Di et al. (2016) reported a reduction of ~30 % in cattle urine N_2O emissions from lysimeters from lucerne grazed pasture compared to a ryegrass-white clover pasture, 7.1 and 10.9 kg N_2O-N ha^{-1}, respectively. In pastures containing 45 % plantain, cattle urine-patch N_2O emissions were observed to decrease from 6.9 to 1.8 mg N m^{-2} h^{-1} (Simon et al., 2019). This was observed to be a result of reductions in urine-N content from grazing key plants containing high levels of secondary metabolites. Cattle urine-N content was also observed to decrease from 6.1 g N L^{-1} for a simple pasture to 4.9 g N L^{-1} for a herbal ley containing chicory, plantain and lucerne (Edwards et al., 2015). Currently, there are no reports on sheep urine-patch N_2O emissions from grazed herbal leys under field conditions. Focus in the literature is predominantly on dairy production systems, as herbal leys can increase the proportion of N used in milk production in cattle (Woodward et al., 2013). This was observed in an indoor feeding trial; dairy cattle fed a herbal ley mixture containing perennial ryegrass, prairie grass (*Sporobolus cryptandrus*), white clover, chicory, plantain, and lucerne, had a higher milk yield (12.5 *vs*. 11.3 kg d^{-1} per cow) and higher percentage of N allocated to milk production (23 % *vs*. 15 %) compared to cows fed a grass-clover mixture (Woodward et al., 2012). Alongside the numerous ecosystem benefits herbal leys provide, plants containing high levels of secondary metabolites within a herbal ley may offer a potential mitigation option for livestock agriculture by reducing excreta patch N_2O emissions. However, it is important to note that the sward composition of herbal leys is crucial, as high proportions of grasses and clovers as well as other herbs with low levels of plant secondary metabolites may dilute the effects of plants with high levels of secondary metabolites and weaken potential benefits. Importantly, the presence of these plants within a herbal ley does not necessarily mean they are grazed by livestock. The astringency of plants such as chicory often reduces feed intake until livestock adjust to the taste difference or grazing management is changed to encourage consumption, for example, increased stocking density or rotational grazing.

2.5. Livestock health and productivity

In addition to reducing livestock GHG emissions, herbal leys have the potential to improve livestock productivity, particularly in grazing lambs. Due to the implications for soil structure, preference in arable-ley rotations should be given to sheep grazing over cattle.

Arable-ley rotations offer the potential for healthier grazing as newly established leys in arable rotations have reduced gastrointestinal nematode burdens than previously grazed permanent pasture (Coles, 2005). Gastrointestinal nematodes in livestock systems have significant economic impacts, costing the UK sheep industry 84 million GBP yr^{-1} (ADAS, 2011). Since the introduction of the first anthelminthic, phenothiazine, in the 1950s gastrointestinal nematodes in livestock have developed resistance to commonly used anthelminthics (Hoste and Torres-Acosta, 2011; Lazarus and Rogers, 1951). In the UK, increasing resistance to commonly used anthelminthics, such as benzimidazoles, levamisole and macrocyclic lactones, has pushed the UK livestock industry to consider alternative methods of gastrointestinal nematode management (AHDB, 2013). Gastrointestinal nematodes can pass between untreated animals grazing the same pasture and survive outside the host in the sward (Coles, 2005). Livestock productivity is affected by common gastrointestinal nematodes, such as *Nematodirus* and *Haemonchus contortus,* and results in livestock suffering from anemia, edema, weakness, reduced meat, milk and wool production (Marley et al., 2003; Roeber et al., 2013).

Plant secondary metabolites naturally occurring in species sown within herbal leys are promoted as natural anthelminthics and can reduce parasite burden in livestock (Marley et al., 2003). Studies including plantain, chicory, birdsfoot trefoil and lucerne in herbal leys found lower fecal egg counts in infected lambs than comparator grass or grass-clover pastures (Knight et al., 1996). Tannins in herbal leys can decrease motor activity of gastrointestinal nematodes, inhibit the transformation of eggs to larvae, and inhibit the energy metabolism of gastrointestinal nematodes (French, 2018). A study investigating plantain, chicory and grass swards versus permanent grass pasture found no differences in final fecal egg counts (Alomar et al., 2018). However, sparse and upright stems within the sward architecture in the plantain-chicory pasture was attributed to reduced adult (L3 parasite stage) parasite populations (Alomar et al., 2018). It is important to note that including plants with high levels of plant secondary metabolites in swards may not alleviate preexisting high-level gastrointestinal nematode burdens and should not replace effective pharmaceutical anthelminthic treatment on farms, but may reduce the frequency of anthelminthic use and increase the time between treatments. This was demonstrated by Grace et al. (2019), where lambs grazing a nine-species herbal ley required their second anthelminthic dose 59 days after the first treatment compared to lambs grazing a perennial ryegrass sward which needed another dose after 36 days.

As well as reducing parasite burdens, herbal leys can also increase live weight gains in livestock. Increased crude protein content in herbal leys can increase muscle mass and milk production, increasing livestock performance (Corner-Thomas et al., 2018a; Somasiri et al., 2016). In New Zealand, lambs grazing a herb-clover pasture experienced weight gains of 0.4 kg day^{-1} (Corner-Thomas et al., 2018b). Similarly, lambs grazing a grass-clover sward had a slower live weight gain and lighter carcass weight per ha over three years (1.27 t ha^{-1}) compared lambs grazing a herbal ley mixture of either red and white clover with plantain (1.71 t ha^{-1}) or chicory and plantain mixture (1.73 t ha^{-1}) (Kenyon et al., 2017). Reducing the time it takes to reach slaughter weight has, having implications for the carbon footprint and life cycle assessment of lowland meat production. In Ireland, lambs grazing a six- or nine-species pasture containing grasses, legumes and herbs were found to take 168 days to reach slaughter weight compared to lambs grazing pure grass pastures, which took 181 days (Grace et al., 2019).

Currently, the majority of published studies investigate the impact of an individual herb or legume species, e.g., chicory or sainfoin, on livestock productivity or livestock health. However, there is minimal evidence available for the benefits of herbal leys on livestock productivity. Future studies should carefully consider sward composition in their experimental design, as herbal ley mixtures vary between seed companies, ranging from four plant species selected from each plant group (grasses, legumes and herbs) to 16 plant species. Mixtures containing a higher diversity could potentially dilute the effect of plants containing high levels of secondary metabolites within the sward, negating their full potential and resulting in variable results across studies.

2.6. Sustainable agricultural intensification and resilience

Achieving sustainable agricultural intensification is a key cornerstone of environmental research. Arable-ley rotations have long been recognized for their potential for sustainable intensification, with evidence of increases in biodiversity (Albizua et al., 2015), yield (Taylor et al., 2006), soil nutrients (e.g., N) and organic matter (Jarvis et al., 2017), improvements in soil structure (Hallam et al., 2020) and in ungrazed rotations, subsequent crop performance including under drought and flood stresses (Berdeni et al., 2021). The recycling of nutrients and organic matter from livestock grazing has been observed to increase crop yields in integrated crop-livestock systems, but there is minimal data available for crop yields after the reversion of livestock-grazed leys back to arable. In a cattle grazed integrated crop-livestock system, dung inputs increased the availability of soil K and P by 122 % and 38 %, respectively, subsequently increasing the yield and number of pods per plant of the following soybean crop by 23 % and 20 % relative to the ungrazed control (Da Silva et al., 2014). However, Taylor et al. (2006) found that following a 3-year grass-clover ley grazed by sheep in Scotland, cereal crop yield was highest in the first year following ley reversion back to arable than the second year, producing in the first year and the second year 5.06 and 3.45 t ha^{-1} of grain and 3.60 t ha^{-1} and 2.26 t ha^{-1} of straw, respectively, demonstrating that potential increases in yield are relatively short-lived.

Due to the N fixing capacity of legume containing leys, arable-ley rotations have the potential to reduce the N requirement of the following crop. In the UK, use of mineral N fertilizer has increased by 7.4 % between 2008 and 2018, from 0.96 to 1.03 Mt, respectively (AIC, 2019). By 2030, global demand for mineral N fertilizer to maintain production is predicted to reach 135 Mt (Bell et al., 2015). The BNF ability of legumes within a herbal ley could help to reduce the mineral N fertilizer demand of conventional farms. However, there is limited information available on the effect of leys in rotation on the nitrogen fertilizer replacement value (NFRV) of plowed out leys on crop nitrogen requirements. In Belgium, after a 2-year grass-clover ley was plowed and reverted back to a forage maize (*Zea mays*) crop, the NFRV was highest in the first year at 177 kg N ha^{-1} but declined successively over the 3-year period, averaging 79 kg N ha^{-1} in the second year and 31 kg N ha^{-1} in the third (Cougnon et al., 2018). However, while a reduction in required fertilizer N was observed, Cougnon et al. (2018) noted that ley management (i.e., grazing or mowing) did not affect the NFRV. Currently, there is no estimate of the NFRV potential of herbal leys in rotation.

As well as their ability to reduce mineral N inputs and increase yield, herbal leys have greater resilience to environmental stresses and extreme weather events than their grass or grass-clover counterparts. The deep rooting capabilities of key species, for example, chicory, yarrow (*Achillea millefolium*), lucerne and sainfoin, can allow plants greater access to water during drought conditions and maintain biomass production (Somasiri et al., 2016). This could help to maintain productivity and resilience of the farm enterprise, as most countries are expected to experience more extreme weather events due to climate change; however, little is known about the effect of drought and flood events on herbal leys under field conditions. At the time of this review, there was no available literature on the effect on microbial activity, yield and C and N cycling on herbal leys under environmental stresses.

Deep rooted species may have the potential to access micronutrients in the subsoil and bring them to the surface to be made available to grazing livestock and cereal crops for human consumption. Micronutrient deficiencies in arable agriculture is often termed the ‘hidden hunger’, as deficiencies in iodine, iron and zinc content in cereal crops have implications for human health (Alloway, 2008; Gupta et al., 2008; Ritchie et al., 2018). This is also seen in livestock, as micronutrient deficiencies in the sward composition of grazing pastures can affect the reproductive system in livestock and subsequent meat and milk production, quality, and micronutrient content (Fisher, 2008; Gupta et al., 2008). Compared to grass pastures, herb-rich pastures containing chicory, plantain, white and red clover were found to have greater micronutrient content concentrations of cobalt, copper, zinc and iron but not molybdenum (Lindström et al., 2013). The grass species cocksfoot was found to have the greatest concentration of manganese (Lindström et al., 2013), however, this response is expected to be highly soil type specific. Pirhofer-Walzl et al. (2011) identified that herbs such as chicory, plantain, caraway, and salad burnet (*Sanguisorba minor*) in the herbal ley mixture had higher levels of sward macro- and micronutrients (e.g., zinc) than grasses and legumes. However, little is known of the effect of these herbal leys on the micronutrient content of meat and milk production. If herb-rich leys can increase the micronutrient content of livestock products, it may help to address the hidden hunger in modern food production.

Despite the benefits herbal leys can provide, it cannot be considered a magic bullet for many of the problems facing arable agriculture today. Under an organic farming scenario, if the UK, for example, was to shift to utilizing arable-ley rotations, GHG emissions and crop yield would reduce as production pressure is shifted overseas (Smith et al., 2019). If arable-ley rotations were used in conventional agriculture as well this may be avoided. However, arable-ley rotations also face socioeconomic barriers to uptake. A scoping study found that commonly cited reasons against utilizing leys were: (1) short-term economic losses, (2) lack of existing partnerships between arable and livestock farmers, (3) lack of skilled workers with animal husbandry skills, and (4) limited evidence of proven benefits for livestock and arable farmers (Knight et al., 2019). A recent review by Schut et al. (2021) highlighted that the socioeconomic limitations to reintroducing arable-ley rotations, and thus the recoupling of integrated crop-livestock system, in the EU was mainly driven by the lack of suitable infrastructure, for example, abattoirs and grazing agreements, to support arable-ley rotations. For farmers to overcome these barriers, a change in infrastructure, increased financial support, and improved evidence base evaluating the potential benefits and consequences of arable-ley rotations are needed to help farmers make informed decisions.

2.7. Summary

The use of ungrazed leys in arable-ley rotations is shown to increase ecosystem service delivery in agriculture through increasing C sequestration, symbiotic nitrogen fixation, water infiltration, biodiversity in soil fauna and microbial communities. However, currently there is insufficient literature available for arable soil improvement under grazed leys, particularly herbal leys. The majority of previous research has been conducted on grass or grass-clover leys in arable rotations, resulting in a limited evidence base to support and justify the use of herbal leys in cropping rotations.

This review has highlighted that due to their complexity and complementarity of species, herbal leys can potentially deliver greater ecosystem services than comparator grass or grass-clover ley. Increasing species diversity by utilizing a four- to eight-species herbal ley can offer greater multifunctionality and opportunities to improve soil quality than a monoculture grass or low diversity (e.g., two- to four-species) grass clover ley. This review has examined the available literature and identified key knowledge gaps in the current understanding of grazed arable-ley rotations. These are as follows:

1. There is a lack of evidence available on the effect of grazed herbal leys on AMF, soil biodiversity, soil microbial communities and functioning in degraded arable soils.
2. Further research is needed on the effect of plant secondary metabolites in fresh and ensiled herbal leys on rumen microbiome functioning, livestock enteric CH_4 emissions, livestock NUE, and gastrointestinal parasite burden.

3. Lack of information for the biological nitrification inhibitor potential of herbal leys in urine patches and after mineral N fertilizer applications.
4. There is a need to assess if a different N_2O EF_{3PRP} for excreta deposited by livestock grazing herbal leys is needed for national and international greenhouse gas inventories, as this is lacking in the IPCC calculations and in the available literature. Without an accurate N_2O EF_{3PRP} and enteric methane measurement this reduces the resolution of future carbon footprints and life cycle assessments.
5. No information is available for micronutrient sward content of herbal leys and the subsequent micronutrient content of meat and milk from grazed livestock.
6. There is minimal information available on the tolerance and resilience of herbal leys to extreme weather events (e.g., drought or flood) with respect to species persistence, pasture yield and quality, and ecosystem services.
7. Although there is ongoing research across the EU, there is a lack of published data on the effect of reversion from herbal leys to arable crop as part of the arable-ley rotation with regards to long-term SOC stocks, soil microbial community functioning, and soil N and C cycling.
8. Further research is needed to provide replicated long-term trials (10-25 years) to evaluate greater ecosystem services (e.g., flood reduction) and identify the best method of ley management (i.e., mowing *vs.* grazing, and sheep *vs*. cattle).
9. Socioeconomic research is needed to identify cultural barriers and evaluate the economic impacts of herbal leys and the reintegration of arable-ley rotations to provide an evidence base for on-farm and country-specific economic assessments.

Further research is required to support the development of new policies and legislation to encourage the use of livestock and herbal leys in arable rotations. Following new research, governments should support provide additional infrastructure (e.g., abattoirs) in predominately arable regions (e.g., eastern Europe) in addition to establishing national grazing networks linking arable and livestock farmers through grazing agreements. This may help overcome the socioeconomic barriers (e.g., skill gaps) that may be limiting the uptake of livestock and leys in arable rotations. Future agri-environment payment schemes should also consider payments for grazing livestock on arable land to encourage a reduction in mineral fertilizer use while improving soil quality. This may help to reduce short-term economic losses often incurred while adopting mixed farming methods. To enable farmers to make informed decisions for what is best for their land, research needs to fill these knowledge gaps and produce evidence-based recommendations.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 3

Herbal leys increase forage macro- and micronutrient content, spring lamb nutrition, liveweight gain, and reduce gastrointestinal parasites compared to a grass-clover ley

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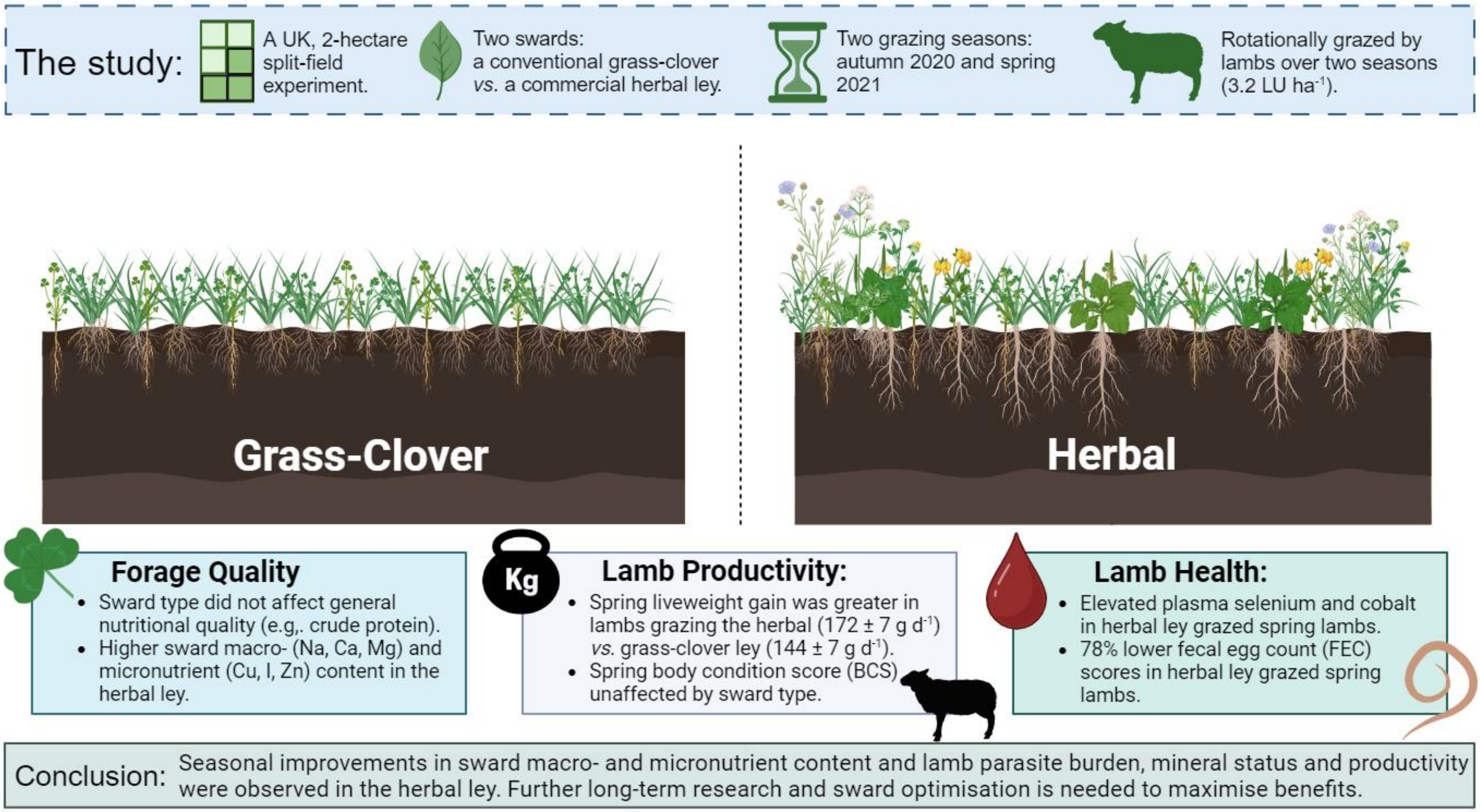

The study:

- A UK, 2-hectare split-field experiment.
- Two swards: a conventional grass-clover *vs.* a commercial herbal ley.
- Two grazing seasons: autumn 2020 and spring 2021
- Rotationally grazed by lambs over two seasons (3.2 LU ha^{-1}).

Forage Quality

- Sward type did not affect general nutritional quality (e.g,. crude protein).
- Higher sward macro- (Na, Ca, Mg) and micronutrient (Cu, I, Zn) content in the herbal ley.

Lamb Productivity:

- Spring liveweight gain was greater in lambs grazing the herbal (172 ± 7 g d^{-1}) *vs.* grass-clover ley (144 ± 7 g d^{-1}).
- Spring body condition score (BCS) unaffected by sward type.

Lamb Health:

- Elevated plasma selenium and cobalt in herbal ley grazed spring lambs.
- 78% lower fecal egg count (FEC) scores in herbal ley grazed spring lambs.

Conclusion: Seasonal improvements in sward macro- and micronutrient content and lamb parasite burden, mineral status and productivity were observed in the herbal ley. Further long-term research and sward optimisation is needed to maximise benefits.

Figure 3.0. *Chapter 3 graphical abstract.*

Highlights

- Increasing herb and legume content in grasslands can improve livestock productivity.
- Herbal ley had a greater sward macro- and micronutrient content.
- Spring lamb liveweight gain was higher in herbal ley grazed lambs.
- Faecal egg count scores were reduced by 78 % in herbal ley grazed lambs in spring.
- Higher plasma selenium and cobalt in spring lambs grazing the herbal ley.

Keywords

Sheep, gastrointestinal parasite burden, multispecies sward, grass quality, trace elements

Abstract

Commercial herbal leys (multispecies swards) are rapidly gaining popularity due to their potential to deliver an enhanced suite of ecosystem services. However, little is known about their impact on lamb production. A 2-ha split-field experiment using an herbal and grass-clover ley (0.33 ha paddock^{-1}, $n = 3$ per sward) aimed to evaluate the effect of sward-type on forage quality and lamb productivity. Lambs ($n = 40$ per sward) were rotationally grazed over two experimental seasons: autumn 2020 (males) and spring 2021 (females). Sward quality was measured at the start of each grazing season. Liveweight gain and faecal egg counts (FEC) were measured at week 0, 4 and 6 in autumn and week 0, 4, 9 and 11 in spring. Blood samples were analysed after 11-weeks in spring to assess mineral status. General sward nutritional quality (e.g., crude protein) did not improve under the herbal ley, however, higher sward macro- and micronutrient concentrations were observed in both seasons. Spring liveweight gain was significantly greater in lambs grazing the herbal (172 ± 7 g d^{-1}) *vs*. grass-clover ley (144 ± 7 g d^{-1}), while autumn liveweight gain showed no difference. Spring lambs grazing the herbal ley compared to the grass-clover ley had elevated plasma cobalt (2.0 ± 0.1 nmol l^{-1} *vs*. 1.6 ± 0.1 nmol l^{-1}) and selenium (0.7 ± 0.04 µmol l^{-1} *vs*. 0.5 ± 0.01 µmol l^{-1}), with lower blood urea (7.7 ± 0.3 nmol l^{-1} *vs*. 10.4 ± 0.4 nmol l^{-1}). Spring FEC scores were reduced by 78 % in herbal grazed lambs; there were no significant differences in autumn FEC from either sward. In conclusion, the herbal ley resulted in seasonal improvements in sward micronutrient content and lamb parasite burden, mineral status, and productivity.

3.1. Introduction

Globally, grazing sheep for meat and milk production produces 67.3 and 29.9 million tonnes CO_2-equivalent greenhouse gas (GHG) emissions per annum respectively, with sheep production representing approximately 6.5 % of emissions from the livestock sector (Gerber et al., 2013). Improving the efficiency of grazing sheep production may be achieved via feed additives and diet manipulation (e.g., altering sward botanical composition), improving their productivity and health, subsequently reducing GHG emissions and contributing towards achieving sustainable agricultural intensification (Gerber et al., 2013; Pulina et al., 2017).

In temperate regions, lowland sheep production systems are dominated by grass swards consisting of perennial ryegrass (*Lolium perenne*) and grass-clover swards utilising legumes such as white (*Trifolium repens*) and red clover (*Trifolium pratense*) due to their productivity, persistence under grazing, and high digestibility and sugar content (Fraser et al., 2022; Waghorn and Clark, 2004). However, perennial ryegrass swards typically require additional N inputs to maintain yields, resulting in increased on-farm economic constraints from fertiliser costs and environmental implications, e.g., soil acidification, watercourse eutrophication, and increased nitrous oxide emissions (Bell et al., 2015; Kidd et al., 2017). Herbal leys (also known as multispecies swards) consisting of grasses, legumes and herbs are rapidly increasing in popularity as a low N-input alternative to conventional pastures that can deliver greater ecosystem service benefits, such as improved sward productivity (Finn et al., 2013; Jordon et al., 2022), sward quality (Grace et al., 2018; Jing et al., 2017), drought tolerance (Grange et al., 2021; Hofer et al., 2016), and reduced nitrous oxide emissions (Cummins et al., 2021).

Improved livestock productivity has been reported when grazing a herbal ley due to the enhanced sward nutritional quality and dry matter intake (Golding et al., 2011; Grace et al., 2019; Jerrentrup et al., 2020; Somasiri et al., 2015). Unlike *Lolium perenne*, deep-rooting herb species such as chicory (*Cichorium intybus*) and ribwort plantain (*Plantago lanceolata*) can access subsoil nutrient reserves and often accumulate greater macro- and micronutrient concentrations than most grasses, which may reduce the risk of sub-clinical micronutrient deficiencies and improve productivity in grazing livestock (Barry, 1998; Darch et al., 2022; Darch et al., 2020; Kao et al., 2020; Watson et al., 2012). Herb and legume species often used within a herbal ley also contain high concentrations of plant secondary metabolites which can reduce the gastrointestinal parasite burden of livestock and subsequently the need for anthelminthic interventions (Marley et al., 2003; Peña-Espinoza et al., 2018). Gastrointestinal parasites can cause significant productivity and economic losses in livestock production, with the repeated use of synthetic anthelminthics contributing to the rise of anthelminthic resistance (Hoste and Torres-Acosta, 2011). Previous studies utilising a 6- or 9-speices herbal ley have shown a reduction in lamb faecal egg counts, increasing the duration between anthelminthic doses (Grace et al., 2019).

Operating a herbal ley system, however, is not without its challenges. For example, some herbs are vulnerable to selective grazing (Jing et al., 2017), and little is known about their impact on livestock productivity, health, persistence, and contribution to sward nutritional quality when included in a commercial herbal ley mixture (consisting of 10-18 species) under field conditions. Most grazing studies utilise low-diversity experimental herbal mixtures (e.g., 3-9 species) that do not fully encompass the complexity of commercial swards promoted via agri-environment schemes, resulting in a significant knowledge gap as farmers increase adoption of these swards.

This study aims to compare the impact of a commercial herbal ley on livestock productivity and health and sward productivity and quality compared to a conventional grass-clover ley. We hypothesised that i) sward productivity will be increased in the herbal ley due to plant species niche complementary; ii) sward nutritional quality and macro- and micronutrient content will be enhanced in the herbal ley due to greater accumulation of nutrients by herb species; iii) weaned lambs will have greater productivity due to consuming feed with high nutritional quality and anthelminthic properties.

3.2. Materials and Methods

3.2.1. Experimental Design

In July 2020, a 2-ha lowland field located at the Henfaes Research Centre, Abergwyngregyn, North Wales, UK (10 m a.s.l., 53.240329 N, -4.014574 W) was ploughed and reseeded with either a 18-species (19 cultivar) herb- and legume-rich multispecies ley (herbal) or a conventional 5-species (9-cultivar) grass-clover ley (grass-clover) (Table 3.1) supplied by Cotswold Seeds (Cotswold Seeds Ltd., Moreton-in-Marsh, UK). The field was previously grassland prior to reseeding. The soil at the field site was classified as a sandy clay loam textured free-draining Eutric Cambisol, with an average annual rainfall and temperature of 1060 mm and 10.8 °C, respectively.

One hectare was dedicated to each sward type and subsequently split into three independent permanently fenced ~0.33 ha paddocks, allowing for the rotational grazing of weaned lambs. Lambs were moved between each paddock every 4-5 days throughout their experimental grazing season, with each paddock having approximately 8-10 days' rest before grazing occurred again. The field received no nitrogen fertiliser in autumn-winter 2020, only receiving one application of 50 kg N ha^{-1} ammonium nitrate, 20 kg P_2O_5 ha^{-1}, 60 kg K_2O ha^{-1} fertiliser in March 2021 to encourage spring grass growth. Background soil characteristics and analytical methods of the field experiment have been evaluated in detail previously (see Chapter 4). Briefly, the soil pH (0-10 cm) was 6.9 and 7.0 with an electrical conductivity of 51 µS cm^{-1} and 38 µS cm^{-1} for the herbal and grass-clover leys, respectively ($p > 0.05$). Meteorological data were recorded every 30 minutes from an automated on-site weather station located approximately 200 m from the field site (Campbell Scientific Ltd., Leicestershire, UK). Meteorological data can be found in Supplementary Figure 3.1.

Table 3.1. *Species composition and seeding rate of the herbal ley and grass-clover ley sown in July 2020.*

Plant type	**Herbal ley**			**Grass-Clover ley**		
	Species and Cultivar	Proportion (%)	Seeding rate (kg ha^{-1})	Species and Cultivar	Proportion (%)	Seeding rate (kg ha^{-1})
Grass	Cocksfoot (*Dactylis glomerata*) cv. 'Amba'	11.5	3.75	Perennial ryegrass (*Lolium perenne*) cv. 'Glenstal'	30.8	10.0
	Festulolium cv. 'Lofa'	11.5	3.75	Perennial ryegrass (*Lolium perenne*) cv. 'AberMagic'	16.9	5.50
	Perennial ryegrass (*Lolium perenne*) cv. 'Oakpark'	7.7	2.50	Timothy (*Phleum pratense*) cv. 'Winnetou'	15.4	5.00
	Perennial ryegrass (*Lolium perenne*) cv. 'Glenstal'	3.8	1.25	Cocksfoot (*Dactylis glomerata*) cv. 'Amba'	15.4	5.00
	Timothy (*Phleum pratense*) cv. 'Winnetou'	4.6	1.50	Hybrid ryegrass (*Lolium perenne*) cv. 'Tetragraze'	11.5	3.75
	Tall fescue (*Festuca arundinacea*) cv. 'Kora'	3.8	1.25			
	Meadow fescue (*Festuca pratensis*) cv. 'Pardus'	3.1	1.00			
Legume	Sainfoin (*Onobrychis*)	19.2	6.25	Red clover (*Trifolium pratense*) cv. 'AberClaret'	3.9	1.25
	Sweet clover (*Melilotus*)	6.2	2.00	White clover (*Trifolium repens*) cv. 'AberDai'	3.1	1.00
	Red clover (*Trifolium pratense*) cv. 'Milvus'	5.4	1.75	White clover (*Trifolium repens*) cv. 'AberHerald'	2.3	0.75
	White clover (*Trifolium repens*) cv. 'AberHerald'	3.8	1.25	Wild white clover (*Trifolium repens*) cv. 'AberAce'	0.8	0.25
	Lucerne (*Medicago sativa*) cv. 'Luzelle'	2.3	0.75			
	Alsike clover (*Trifolium hybridum*) cv. 'Aurora'	1.5	0.50			
	Birdsfoot trefoil (*Lotus corniculatus*) cv. 'Bull'	1.5	0.50			
Herb	Burnet (*Sanguisorba minor*)	5.4	1.75			
	Chicory (*Cichorium intybus*) cv. 'Puna II'	4.6	1.50			
	Ribwort Plantain (*Plantago lanceolata*) cv. 'Endurance'	1.5	0.50			
	Sheep's Parsley (*Petroselenium crispum*)	1.5	0.50			
	Yarrow (*Achillea millefolium*)	0.8	0.25			

3.2.2. Livestock Management

Weaned Welsh mountain lambs (*Ovis aries*) (n = 40 per sward) at a stocking density of 3.2 LU ha^{-1} were grazed over two experimental seasons: autumn 2020 (late September to late October) and spring 2021 (late April to early July). The breed and sex of lamb grazed each experimental season represented seasonal differences in the local grazing system, with male ram lambs approximately 6-7 months old (average starting liveweight 22.1 ± 0.4 kg) being conditioned for slaughter grazing in autumn 2020, and female ewe lambs approximately 10-11 months old (average starting liveweight 23.0 ± 0.3 kg) being conditioned for breeding grazing in spring 2021. Each lamb was fitted with an EID ear tag at the start of the experiment to allow for identification and use of the electronic 3-way manual weighing crate (Prattley, Temuka, New Zealand) with liveweight gain (LWG) recorded digitally (Tru-Test XR5000, Auckland, New Zealand). At the start of each grazing season, lambs were weighed and assigned to each sward type based on their starting weight to ensure an even split across the grazing groups. Lambs received no additional dietary supplementation throughout the experiment. Mains supplied fresh water was freely accessible via communal water troughs in each paddock.

Following initial weighing at the start of grazing (week 0), lambs were weighed at week 4 and 6 in autumn 2020, and at week 4, 9 and 11 in spring 2021. Lambs used for excreta (urine and dung) collection (as described Chapter 4; ca. n = 6 lambs per sward) and faecal egg count sampling were excluded from the LWG dataset due to the grazing disruption experienced during collection periods. Liveweight gain was calculated for the duration each lamb spent on the grazing trial. Due to grass limitations in the autumn 2020 grazing experiment, lambs that reached their target condition (data not recorded) after 4-weeks were removed from the experiment to prevent overgrazing and damage to the sward.

Body condition score (BCS) of the older ewe lambs was assessed at each weighing session in the spring 2021 grazing experiment by a single trained worker assessing back fat using a scale of 1 – 5 with 0.5 increments (Russel et al., 1969). BCS was not measured in the autumn 2020 grazing experiment.

3.2.3. Fresh Sward Composition and Sward Standing Biomass

At the start of each grazing season, sward samples were obtained by cutting four randomly assigned 1 m^2 quadrat sward samples with hand shears across each paddock at an approximate grazing height (ca. 4 – 5 cm). These samples were then bulked into one sample per paddock for nutritional and chemical composition analyses. A subsample from each paddock was obtained to determine dry matter (DM) content by drying in a forced draught oven at 80 °C for 24 hours. Fresh sward samples were analysed by wet chemistry by Sciantec Analytical (Sciantec, Yorkshire, UK) to determine sward quality properties (dry matter, crude protein, sugar, metabolisable energy [ME], neutral-detergent fibre [NDF], D-value, ash, oil-A, nitrate-nitrogen and buffering capacity) and macro- and micronutrient content (calcium, magnesium, sodium, potassium, cobalt, copper, iodine, manganese, selenium, and zinc). A final sward composition measurement after all grazing trials had been completed was taken in autumn 2021 (15/09/2021) using methods described previously.

Sward standing biomass was measured regularly from each grazed paddock throughout each experimental season using a rising plate meter (Jenquip EC09 Plate Meter, Feilding, New Zealand) and taking 30 measurements in a ‘W’ shape transect across each paddock to determine average height and estimated yield. Sward standing biomass measurements were obtained by calibrating the height data from plate meter with the DM content of the sward at the start of each grazing season using the calibration equation provided by Farming Connect (2017). The equation is as follows:

Sward standing biomass (kg DM ha^{-1}) = compressed sward height (cm) × dry matter content (g kg^{-1}) + 500

The ‘+ 500’ value refers to the value added to compensate for the grass at the bottom of the sward that is not measured by the rising plate meter. Data is reported as tonnes DM ha^{-1}. Sward standing biomass at the start of each grazing season was 1.45 ± 0.06 t DM ha^{-1} and 1.75 ± 0.12 t DM ha^{-1} in autumn 2020 and 2.28 ± 0.10 t DM ha^{-1} and 2.18 ± 0.04 t DM ha^{-1} in spring 2021 for the herbal and grass-clover ley, respectively. However, as only limited DM measurements were conducted and no accurate calibration equation currently exists for utilising a rising plate meter on complex commercial herbal leys, the sward standing biomass data is only provided as an indicator of growth (Figure 3.1).

The sward was cut three times during the field experiment to maintain grass quality for grazing (Figure 3.1). Briefly, when the sward height exceeded ca. 10 cm and was not managed by grazing, this was then mown to ca. 5 cm with a Malone Procut 2.4 m disc mower (Malone Farm Machinery, Claremorris County Mayo, Ireland). The mown biomass was not removed from the field. Sward cuts occurred once during the spring 2021 grazing season (21/04/21), with the other cuts occurring to obtain a hay yield (17/07/21, see section 3.2.5) and after the grazing experiments had finished in autumn 2021 (22/09/21).

3.2.4. Sward Botanical Composition

The botanical composition of each sward was assessed in July 2021 at the end of the spring 2021 grazing experiment. Five 4 m^2 quadrats were evaluated in each paddock, accounting for spatial variability, with the resulting Domin scores then converted to percentage cover using the Domin 2.6 transformation described in Currall (1987). Sward botanical composition and overhead photographs of the sward are provided in Supplementary Figures 3.2 and 3.3, respectively.

3.2.5. Hay Cut and Composition

Following completion of the spring 2021 grazing trial and removal of sheep from the paddocks, the sward was allowed to recover for 19-days, reaching an approximate height of ca. 16 cm for the herbal ley and ca. 13 cm for the grass-clover prior to cutting both swards to ca. 5 cm using a Malone Procut 2.4 m disc mower (Malone Farm Machinery, Claremorris County Mayo, Ireland) on 17/07/21. The cut biomass was left *in-situ* in the field before turning and spreading evenly 24 and 48-hours after cutting to encourage drying using a PZ 300 Haybob. Hay was then baled 4-days post-cutting using a DANELANDER mini round baler (Haughton, England) and stored under cover at ambient air temperature.

A hay sample for analysis was obtained 26-days post-baling by randomly sampling from various points outside and inside bales from each paddock to obtain a representative sample per each replicate sward. Analysis and nutrient content (i.e., macro- and micronutrients) was conducted by Sciantec Analytical as described previously. Hay yield was verified by weighing randomly selected bales (n = 3) and obtaining an average bale weight per paddock.

3.2.6. Gastrointestinal Parasite Burden

Faecal samples were collected from selected lambs from each grazing group (n = 6 per sward, per season) at each liveweight gain measurement occasion using the excreta collection pens described previously (see Chapter 4) (Bangor University ethics committee approval code COESE2019EC01A). Lambs were held in individual collection pens with access to feed and fresh water until defecation (approximately 6 – 8 hours) to ensure individual sample identification. Upon natural deposition, samples were immediately collected and stored at 4 °C prior to analysis within 24-hours.

Individual faecal samples were homogenised prior to analysis using the modified McMaster technique (Zajac & Conboy, 2012) to determine faecal egg count (FEC). Briefly, 2 g of faecal matter was floated in 28 ml of saturated NaCl solution (ca. 6 M) before straining and adding to a glass two-chamber McMaster slide. Gastrointestinal parasitic eggs identified included *Moniezia*, *Nematodirus*, and *Strongylid*s. *Coccidia* oocysts were not included in the total FEC score.

Male lambs in autumn 2020 were dosed with Supaverm (10 mg kg^{-1}) and Levacur SC (2.5 ml kg^{-1}) two months and one month, respectively, before the start of grazing to treat any pre-existing gastrointestinal parasite burden. Female lambs in spring 2021 received Zolvix™ (2.5 mg kg^{-1}) 6 weeks prior to the start of grazing. If FEC scores exceeded 750 epg (eggs per gram) at any point in the grazing experiment, then lambs were dosed with Zolvix™ (2.5 mg kg^{-1}) to reduce the impact on LWG.

3.2.7. Spring 2021 Lamb Blood Analysis

Blood samples were obtained from spring ewe lambs (n = 10 per sward) at the end of the 11-week grazing period to determine differences in general haematology parameters and macro- and micronutrient content between the sward types. Blood was collected via jugular venepuncture under ASPA licence using a 20 g × 2.5 cm Vacuette® needle (Griener Bio One Ltd, Stonehouse, UK) and stored in a lithium heparin (LH) vacuum tube (BD vacutainer, Plymouth, UK) and a CAT serum clot activator vacuum tube (BD vacutainer, Plymouth, UK) at 4 °C until extraction.

Blood analysis was conducted by the Nottingham University Veterinary Nutritional Analysis (NUVetNA) lab (University of Nottingham, UK). General haematology parameters haemoglobin and haematocrit were determined by colorimetric assay (Randox Laboratories Ltd., UK) according to manufacturer's instructions on a clinical chemistry analyser (RX IOMA, Randox Laboratories Ltd., UK) and determined by capillary tube, respectively. Superoxide dismutase, caeruloplasmin, β-hydroxybutyrate, NEFA, urea, total protein, albumin, and globulin were determined either via colorimetric assay or using the RX series diagnostic kits (Randox Laboratories Ltd, UK). Glutathione peroxidase was determined following the addition of 25 µl of heparinised blood to 1 ml of RANsel diluent, then analysed using the RANsel kit (Randox Laboratories Ltd, UK). A subsample of plasma was analysed for Vitamin B12 at Axiom Laboratories (Axiom Veterinary Laboratories Ltd., Devon, UK).

Macro- and micronutrients were analysed by ICP-MS following a 1:20 dilution. Briefly, 0.5 ml serum in 10 ml of dilutant containing 0.5 % HNO_3 + 4 % MetOH + Internal standard: Sc (25 µg l^{-1}), Ge (10 µg l^{-1}), Rh (5 µg l^{-1}), and Ir (2.5 µg l^{-1}) was placed into 14 ml polypropylene tubes for multi-element analysis. The ICP-MS (Thermo-Fisher iCAP-Q, Thermo-Fisher Scientific, Loughborough, UK) was set to 'flatpole collision cell' mode charged upstream of the analytical quadrupole with helium gas for all elements except Se which was charged with hydrogen gas to reduce polyatomic interferences. Samples were introduced via a covered autosampler (Cetac ASX-520) through a 1317090 pfa-st nebulizer (ESI) (Thermo-Fisher Scientific, Loughborough, UK) with sample processing completed using the 'Qtegra' software (Thermo-Fisher Scientific, Loughborough, UK). Plasma inorganic iodine was determined via ICP-MS using an adapted method by Aumont and Tressol (1987).

3.2.8. Statistical Analysis

Data were analysed in R Studio (version 4.2.1) with graphical images produced using the ‘ggplot2’ package (version 3.3.6, Wickham (2016)). Prior to analysis, all data was tested for normality using the Shapiro Wilks test (R core stats package) and homogeneity of variance using the Levene’s test (‘car’ package, version 3.1.1). Sward (autumn 2021: cobalt, selenium and oil-A) and livestock (blood: caeruloplasmin, NEFA, β-hydroxybutyrate, vitamin B12, plasma selenium and plasma inorganic iodine) data that did not meet these assumptions were log transformed. If the log transformation did not work then an alternative non-parametric test (e.g., Kruskal-Wallis test) was conducted where appropriate.

Livestock measurements (liveweight gain and blood characteristics) were analysed within each season only using a t-test; no comparisons were made between grazing seasons due to seasonality differences in lamb sex used in the experiment. Spring lamb body condition score was assessed using a Mann-Whitney-Wilcoxon test. Gastrointestinal parasite burden (i.e., FEC scores) were assessed at the end of each grazing experiment using either a t-test (autumn 2020) or a Kruskal-Wallis test (spring 2021). As the autumn 2021 sward composition data were not part of the grazing experiments, these were analysed separately using a t-test. Hay composition and yield data were also analysed using a t-test. Sward standing biomass was analysed using a two-way mixed measures ANOVA, followed by a post-hoc pairwise comparison. The two-way mixed measures ANOVA model included sward type and date as the fixed effects, paddock as the random effect, and explored sward type × date as the interaction. A two-way ANOVA was used to analyse general sward properties and macro- and micronutrient content. The model of the two-way ANOVA assessed sward chemical composition (e.g., crude protein content) and season as the fixed effects, with sward chemical composition × season as the interaction. Significance level was set at $p < 0.05$ for all statistical tests. Values presented are means ± SEM unless otherwise stated.

3.3. Results

3.3.1. Sward Growth

A significant difference in sward standing biomass was only identified 66-days after the spring grazing experiment had started on 17/06/21 ($F_{(35,140)} = 36.650$, $p < 0.001$; Figure 3.1), where the herbal ley had a greater residual biomass than the grass-clover ley ($p = 0.035$).

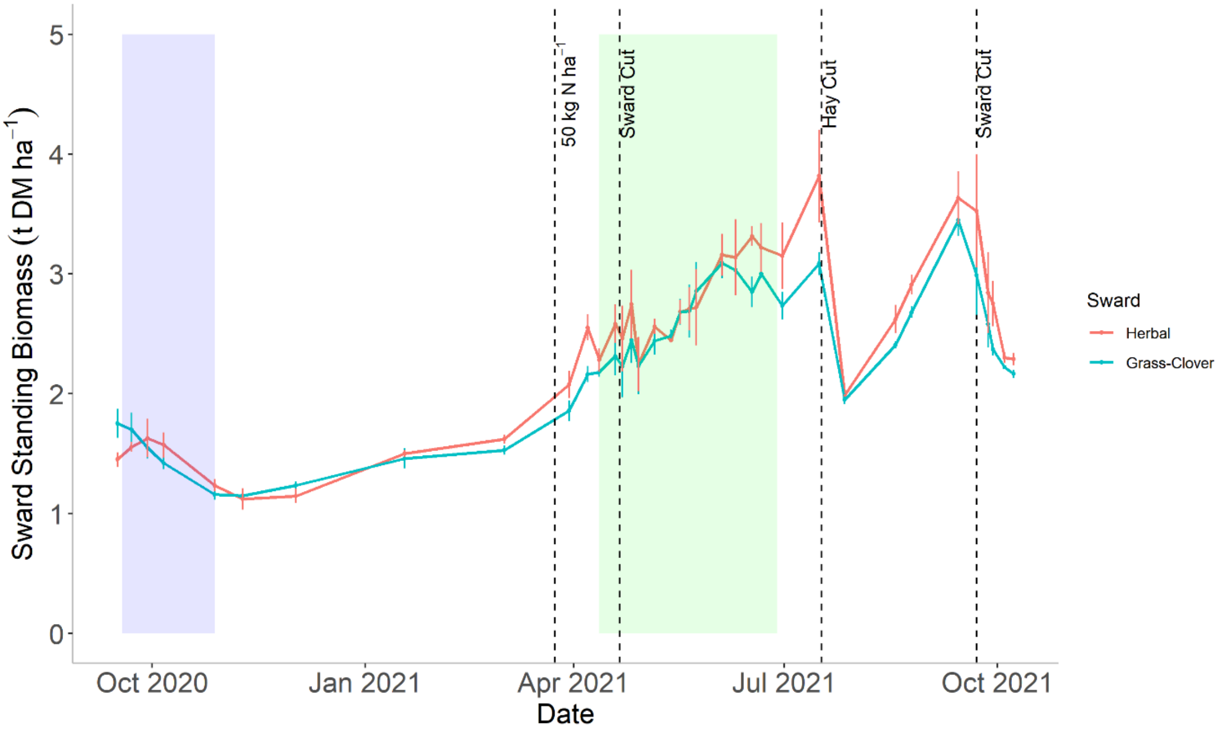

Figure 3.1. *Sward standing biomass (t DM ha^{-1}) measured from 16/09/20 to 8/10/21. The blue shaded area indicates the autumn 2020 grazing period (18/09/20 to 28/10/20) and the green shaded area indicates the spring 2021 grazing period (12/04/21 to 28/06/21). Dashed lines represent field management, such as grass and hay cuts and a 50 kg N ha^{-1} ammonium nitrate application. Data represents mean ± SEM, n = 3 per sward type.*

3.3.2. Fresh Sward Quality

3.3.2.1. Autumn 2020 and Spring 2021

General sward properties were not influenced by sward type within either grazing season ($p > 0.05$, Table 3.2 and S3.1). However, a two-way ANOVA indicated that season had a significant impact on sward properties such as dry matter ($F_{(1,8)} = 174.5$, $p < 0.001$), crude protein ($F_{(1,8)} = 13.661$, $p = 0.006$), ash ($F_{(1,8)} = 33.937$, $p < 0.001$), and sugar content ($F_{(1,8)} = 23.123$, $p = 0.001$). Dry matter and sugar concentration of both swards were higher in spring 2021 than autumn 2020, where concentrations of crude protein and ash were highest.

Macronutrient concentrations were strongly affected by both season and sward type, with a two-way ANOVA finding a significant interaction between sward and season for sodium, calcium, magnesium, and potassium (Table 3.2 and S3.1). Concentrations of sodium ($F_{(1,8)} = 27.780$, $p < 0.001$), calcium ($F_{(1,8)} = 48.270$, $p < 0.001$), and magnesium ($F_{(1,8)} = 77.540$, $p < 0.001$) were greater in the herbal ley than the grass-clover ley in both seasons (Table 3.2 and S3.1). Highest levels were measured in autumn 2020, where concentrations of sodium, calcium, and magnesium were 229 %, 100 %, 58 % greater in the herbal ley, respectively. Potassium concentrations were only affected by season ($F_{(1,8)} = 1933.335$, $p < 0.001$), sward type did not have an effect.

A significant interaction between sward and season was found for iodine ($F_{(1,8)} = 8.245$, $p = 0.021$) and manganese ($F_{(1,8)} = 8.856$, $p = 0.018$) concentrations following a two-way ANOVA (Table 3.3 and S3.1). Sward type influenced copper ($F_{(1,8)} = 16.472$, $p = 0.004$), iodine ($F_{(1,8)} = 34.127$, $p < 0.001$), and zinc ($F_{(1,8)} = 6.644$, $p = 0.033$), where concentrations were greater in the herbal ley than the grass-clover in both seasons. However, copper ($F_{(1,8)} = 155.46$, $p < 0.001$), cobalt ($F_{(1,8)} = 25.063$, $p = 0.001$), iodine ($F_{(1,8)} = 84.794$, $p < 0.001$), selenium ($F_{(1,8)} = 6.670$, $p = 0.033$), and zinc ($F_{(1,8)} = 37.705$, $p < 0.001$) were also influenced by seasonal differences, with higher concentrations of each micronutrient found in autumn 2020. Sward micronutrient content was above the recommended content by Kao et al. (2020) for growing lambs in autumn 2020 for cobalt, manganese, and selenium in both swards, but below this recommendation in spring 2021. Copper and zinc only exceeded the recommended level in autumn 2020 in the herbal ley, the grass-clover ley did not reach the recommendation. Iodine did not meet the recommended nutritional content in either season or sward type.

3.3.2.2. Autumn 2021

A repeat autumn sward composition measurement was taken on 15/09/2021 despite no grazing taking place. Overall, general sward properties did not statistically differ between sward types ($p > 0.05$, Table S3.2), however, crude protein concentration was lower in the herbal (154 ± 21 g kg^{-1}) than the grass-clover ley (242 ± 23 g kg^{-1}) ($T_{(4)} = 2.876$, $p = 0.045$). As with previous seasons, the herbal ley contained higher concentrations of macronutrients calcium (17.9 ± 0.3 g kg^{-1} DW herbal *vs*. 14.2 ± 0.9 g kg^{-1} DW grass-clover) and magnesium (3.0 ± 0.1 g kg^{-1} DW herbal *vs*. 2.5 ± 0.1 g kg^{-1} DW grass-clover) ($T_{(4)} = -3.734$, $p = 0.020$ calcium; $T_{(4)} = -3.411$, $p = 0.027$ magnesium).

Most measured micronutrients, e.g., cobalt, copper, manganese, and selenium, were not affected by sward type. Sward zinc, however, was 30 % higher in the herbal ley (28.5 ± 1.0 mg kg^{-1} DW) than the grass-clover ley (21.9 ± 04 mg kg^{-1} DW) ($T_{(4)} = -6.408$, $p = 0.003$). Sward iodine was also greater in the herbal ley ($T_{(4)} = -2.982$, $p = 0.041$). With the exception of manganese, which was greater than the recommended dietary micronutrient concentrations provided by Kao et al. (2020), none of the remaining measured micronutrients met the recommended levels for growing lambs in autumn 2021.

Table 3.2*. General sward composition measured from a herbal or a grass-clover ley in autumn 2020 (06/10/20) or spring 2021 (18/05/21). Data represents mean ± SEM, n = 3 per sward type, expressed on a dry-weight basis. Lowercase and uppercase letters indicate statistical differences within and between seasons, respectively, following a two-way ANOVA, $p < 0.05$ = significance level, indicated by *.*

Sward Properties	Autumn 2020		Spring 2021		Interaction between Sward and Season	
	Grass-Clover	Herbal	Grass-Clover	Herbal	$F_{(1,8)}$	*p*-value
Dry matter (g kg^{-1})	131.0 ± 3.5^{aA}	133.0 ± 9.7^{aC}	259.3 ± 13.3^{aB}	293.3 ± 5.7^{aD}	1.534	0.251
Crude protein (g kg^{-1})	252.3 ± 3.28^{aA}	206.7 ± 33.1^{aC}	126.3 ± 14.7^{aB}	172.3 ± 23.7^{aC}	4.465	0.068
Sugar (g kg^{-1})	110.3 ± 8.0^{aA}	138.3 ± 37.8^{aC}	213.7 ± 11.1^{aB}	235.7 ± 11.3^{aC}	1.436	0.265
Metabolisable energy (MJ kg^{-1})	12.0 ± 0.1^{aA}	11.5 ± 0.2^{aA}	12.0 ± 0.1^{aA}	12.0 ± 0.1^{aA}	3.658	0.092
Neutral-detergent fibre (g kg^{-1})	401.7 ± 18.6^{aA}	419.3 ± 52.2^{aA}	361.3 ± 14.1^{aA}	351.7 ± 16.6^{aA}	0.018	0.896
D-value (%)	76.5 ± 0.5^{aA}	73.0 ± 1.6^{aA}	76.5 ± 0.8^{aA}	76.8 ± 0.6^{aA}	3.780	0.088
Ash (g kg^{-1})	109.3 ± 3.5^{aA}	108.0 ± 8.5^{aC}	78.7 ± 5.2^{aB}	74.0 ± 3.2^{aC}	0.292	0.604
Oil-A (g kg^{-1})	35.3 ± 0.01^{aA}	31.3 ± 0.2^{aA}	30.3 ± 1.9^{aA}	24.7 ± 2.2^{aA}	2.612	0.145
Nitrate-nitrogen (%)	0.1 ± 0.1^{aA}	0.5 ± 0.1^{aA}	0.1 ± 0.01^{aA}	0.02 ± 0.1^{aA}	1.921	0.203
Buffering capacity (meq kg^{-1})	417.0 ± 1.7^{aA}	411.0 ± 4.2^{aA}	381.3 ± 11.3^{aA}	402.7 ± 16.3^{aA}	1.796	0.217
Calcium (g kg^{-1})	7.2 ± 1.0^{aA}	14.4 ± 0.3^{bC}	4.2 ± 0.3^{aB}	7.8 ± 1.0^{bD}	39.37	<0.001*
Magnesium (g kg^{-1})	1.9 ± 0.1^{aA}	3.0 ± 0.1^{bC}	1.1 ± 0.1^{aB}	1.5 ± 0.1^{bD}	66.540	<0.001*
Potassium (g kg^{-1})	40.9 ± 1.3^{aA}	37.0 ± 1.0^{aC}	25.7 ± 0.7^{aB}	27.1 ± 0.6^{aD}	6.004	0.040*
Sodium (g kg^{-1})	2.1 ± 0.4^{aA}	6.9 ± 0.8^{bC}	0.4 ± 0.1^{aB}	0.9 ± 0.1^{bD}	26.730	<0.001*

***Table 3.3**. Sward trace element content measured from a herbal or a grass-clover ley in autumn 2020 (06/10/20) or spring 2021 (18/05/21). Data represents mean ± SEM expressed on a dry-weight basis. Lowercase letters indicate statistical differences within seasons, uppercase letters denote between season statistical differences following a two-way ANOVA, significance level is set at $p < 0.05$, $n = 3$ per sward type. [1] = Recommended micronutrient content values and typical levels in UK pastures are obtained from Kao et al. (2020). † Degrees of freedom = 1,2.*

Micronutrient content (mg kg^{-1} DW)	Recommended micronutrient content for growing lambs[1] (mg kg^{-1} DW)	Typical micronutrient content of UK pastures[1] (mg kg^{-1} DW)	Autumn 2020		Spring 2021		Interaction between Sward and Season	
			Grass-Clover	Herbal	Grass-Clover	Herbal	F$_{(1,8)}$	*p*-value
Cobalt	0.2	0.05 – 0.25	0.42 ± 0.12^{aA}	0.31 ± 0.02^{aC}	0.07 ± 0.006^{aB}	0.07 ± 0.003^{aD}	0.871	0.377
Copper	11.0	2.0 – 15.0	10.37 ± 0.59^{aA}	12.27 ± 0.15^{bC}	4.73 ± 0.03^{aB}	6.53 ± 0.68^{bD}	0.012	0.915
Iodine	0.8	0.1 – 0.5	0.31 ± 0.02^{aA}	0.46 ± 0.01^{aC}	0.20 ± 0.01^{aB}	0.25 ± 0.02^{aD}	8.245	0.021*
Manganese	40	25.0 – 250	90.77 ± 8.68^{aA}	73.13 ± 4.19^{aA}	63.67 ± 2.8^{aA}	80.57 ± 5.8^{aA}	8.856	0.018*
Selenium	0.2	0.02 – 0.15	0.30 ± 0.06^{aA}	0.36 ± 0.16^{aC}	0.08 ± 0.01^{aB}	0.12 ± 0.02^{aD}	†0.022	0.885
Zinc	33	20.0 – 60.0	29.87 ± 1.95^{aA}	35.77 ± 3.12^{aA}	18.06 ± 0.32^{aB}	22.63 ± 1.68^{aA}	0.108	0.751

3.3.3. Hay Quality and Yield

General hay properties were not affected by sward type ($p > 0.05$, Table S3.3). However, as found with the macronutrient content measured in the fresh sward, concentrations of calcium (5.2 ± 0.6 g kg^{-1} DW grass-clover *vs*. 7.2 ± 0.2 g kg^{-1} DW herbal) and sodium (0.6 ± 0.1 g kg^{-1} DW grass-clover *vs*. 0.9 ± 0.03 g kg^{-1} DW herbal) in hay were greater in the herbal ley than the grass-clover ley ($T_{(4)} = 3.074$, $p = 0.037$ calcium; $T_{(4)} = 4.000$, $p = 0.016$ sodium). Other micronutrients within hay such as cobalt, iodine, selenium and zinc were not affected by sward type ($p > 0.05$). Copper concentration of hay, however, was 24 % greater in the herbal ley (5.1 ± 0.2 mg kg^{-1} DW) than the grass-clover ley (4.1 ± 0.2 mg kg^{-1} DW) ($T_{(4)} = 4.330$, $p = 0.012$).

Hay yield following the spring 2021 grazing season did not significantly differ between the two sward types ($T_{(4)} = -1.821$, $p = 0.143$), with an average yield of 748 ± 135 kg DM ha^{-1} and 490 ± 42 kg DM ha^{-1} from the herbal and grass-clover ley, respectively.

3.3.4. Lamb Health and Productivity

3.3.4.1. Daily Liveweight Gain

Sward type only had a significant effect on spring ewe lamb liveweight gain (Figure 3.2). In spring 2021, ewe lambs consuming the herbal ley had a 15 % increase in liveweight gain compared to lambs consuming the grass-clover ley (172 ± 7 g d^{-1} herbal *vs*. 144 ± 7 g d^{-1} grass-clover; $T_{(65)} = -2.8677$, $p = 0.006$). However, in autumn 2020, despite a 23 % increase in liveweight gain in ram lambs consuming the herbal ley compared to their grass-clover counterparts this was not statistically significant (146 ± 15 g d^{-1} herbal *vs*. 119 ± 9 g d^{-1} grass-clover; $K_{(1)} = 0.981$, $p = 0.322$).

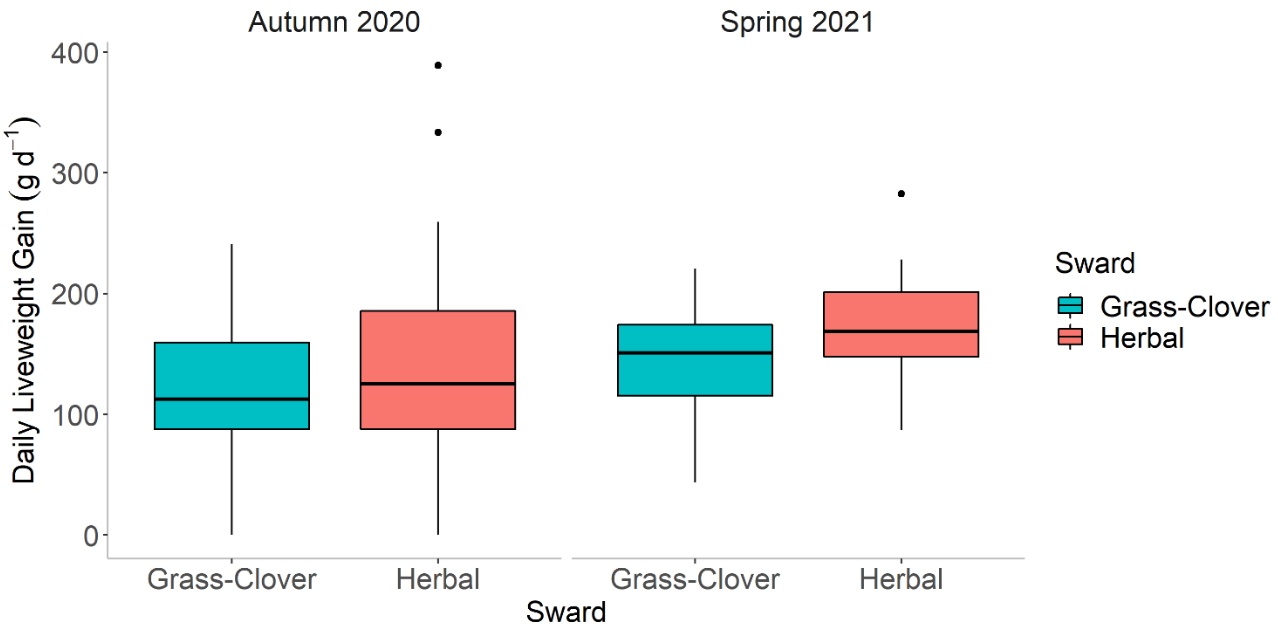

Figure 3.2. *Daily liveweight gain of Welsh mountain lambs consuming either a grass-clover or herbal ley, measured from ram lambs over 6 weeks in autumn 2020 (n = 34 grass-clover, n = 32 herbal) and ewe lambs over 11 weeks in spring 2021 (n = 36 grass-clover, n = 32 herbal). Boxplot displays median and interquartile range, with whiskers showing minimum and maximum values in the data, and dots indicating potential outliers.*

3.3.4.2. Body Condition Score

Spring ewe lamb BCS was not affected by sward type ($p > 0.05$, Supplementary Table 3.4). Lambs consuming either the herbal ($n = 32$) or the grass-clover ley ($n = 36$) only experienced a 0.6 ± 0.1 unit increase in BCS over the 11-week grazing period (W = 556.5, $p = 0.810$). Final lamb BCS was 3.6 ± 0.1 and 3.5 ± 0.1 units in the grass-clover and herbal ley, respectively. BCS was not measured in autumn ram lambs.

3.3.4.3. Spring 2021 Lamb Blood Analysis

General haematology parameters (haematocrit and haemoglobin) did not differ significantly and remained within the normal range for ewe lambs consuming either sward ($p > 0.05$, Table 3.4 and S3.5). Diet did not affect energy indicators (e.g., Non-Esterified Fatty Acids (NEFAs) or β-hydroxybutyrate) measured at the end of the 11-week grazing period ($p > 0.05$, Table 3.4). However, concentrations of blood urea were significantly higher in lambs grazing the grass-clover ley ($T_{(18)} = 5.340$, $p < 0.001$; Table 3.4), with levels 46.4 % and 8.4 % above the normal reference range for lambs grazing a grass-clover and herbal sward, respectively, which may indicate a potential energy-protein imbalance in both diets. Albumin was also elevated above normal ranges in both swards. This was significantly higher in grass-clover ley grazed lambs than the herbal ley lambs ($T_{(18)} = 2.132$, $p = 0.047$), indicative of either potential dehydration or a high protein diet.

Glutathione peroxidase (GSHPx) concentrations were 76 % higher in lambs consuming the herbal ley, placing them within the normal reference range compared to grass-clover grazed lambs ($T_{(18)} = -3.765$, $p = 0.001$). Levels of GSHPx in grass-clover lambs indicated a potential decline in functional selenium status, which was reflected in borderline deficient plasma selenium levels (Table 3.4). However, despite no statistical difference in concentrations of sward selenium or cobalt, levels of plasma selenium and cobalt in herbal ley grazed lambs were 40 % ($T_{(18)} = -5.183$, $p < 0.001$) and 25 % greater ($T_{(18)} = -2.642$, $p = 0.017$), respectively, than their grass-clover counterparts. Despite higher levels of sward copper in spring 2021, there was no effect on plasma copper ($p > 0.05$, Table 3.4).

Table 3.4. *General haematology, serum biochemistry, and mineral parameters for Welsh mountain ewe lambs measured after 11 weeks of grazing either a herbal (n = 10) or grass-clover (n = 10) ley in Spring 2021. Results reported as mean ± SEM. * = indicates a significant result, significance level is $p < 0.05$. ^{1} = normal reference range values provided by NUVetNA lab, ^{2} = normal reference range values obtained from MSD Veterinary Manual (Merck & Co., 2022).*

Blood Characteristic	Normal Reference Range for Sheep	Grass-Clover	Herbal	*p*-value
Haematocrit (%)	-	38.7 ± 1.1	37.7 ± 1.0	0.507
Haemoglobin (g dl^{-1})	-	13.2 ± 0.3	13.3 ± 0.4	0.837
Glutathione peroxidase (U ml^{-1} PCV)	80 – 150 ^{1}	47.8 ± 6.2	84.4 ± 7.5	0.001*
Plasma selenium (µmol l^{-1})	0.5 – 1.0 ^{1}	0.5 ± 0.01	0.7 ± 0.04	< 0.001*
Caeruloplasmin (mg dl^{-1})	15.0 – 35.0 ^{1}	27.3 ± 3.2	26.7 ± 1.5	0.919
Plasma copper (µmol l^{-1})	12.0 – 19.0 ^{1}	15.4 ± 1.2	15.4 ± 1.0	1.000
Caeruloplasmin/plasma copper (ratio)	1.7 – 2.0 ^{1}	1.7 ± 0.1	1.7 ± 0.1	0.995
Superoxide dismutase (U g^{-1} Hb)	> 2000 ^{1}	1672 ± 170	1528 ± 101	0.070
β-hydroxybutyrate (mmol l^{-1})	0.47 – 0.63 ^{1}	0.38 ± 0.03	0.33 ± 0.02	0.214
Non-esterified fatty acids (mmol l^{-1})	< 0.4 ^{1}	1.1 ± 0.2	0.8 ± 0.1	0.052
Urea (mmol l^{-1})	2.8 – 7.1 ^{2}	10.4 ± 0.4	7.7 ± 0.3	< 0.001*
Total protein (g l^{-1})	67 – 88 ^{2}	84 ± 1.2	83 ± 1.9	0.539
Albumin (g l^{-1})	24 – 30 ^{2}	35 ± 0.4	34 ± 0.6	0.047*
Globulin (g l^{-1})	35 – 57 ^{2}	49 ± 1.4	49 ± 2.0	0.968
Plasma calcium (mmol/l)	2.88 – 3.2 ^{2}	2.4 ± 0.02	2.4 ± 0.04	0.787
Plasma cobalt (nmol l^{-1})	> 5.0 ^{1}	1.6 ± 0.1	2.0 ± 0.1	0.017*

Vitamin B12 (pmol l^{-1})	> 400 [1]	353 ± 38.4	453 ± 61.4	0.261
Plasma inorganic iodine (µg l^{-1})	> 100 [1]	48 ± 5.8	43 ± 3.5	0.562
Plasma magnesium (mmol l^{-1})	0.9 – 1.31 [2]	1.1 ± 0.03	1.0 ± 0.04	0.227
Plasma manganese (µmol l^{-1})	> 0.9 [1]	0.03 ± 0.001	0.05 ± 0.01	0.706
Plasma potassium (mmol l^{-1})	3.9 – 5.4 [2]	7.9 ± 0.8	7.1 ± 0.7	0.545
Plasma sodium (mmol l^{-1})	139 - 152 [2]	136.3 ± 1.9	138.9 ± 1.7	0.315
Plasma zinc (µmol l^{-1})	12.3 – 18.5 [1]	12.2 ± 0.3	11.6 ± 0.8	0.821

3.3.4.4. Gastrointestinal Parasite Burden

FEC measurements throughout each grazing season are presented in Figure 2. In autumn 2020, herbal ley sheep #5 was identified as an extreme outlier in week 0 with a starting FEC score of 4550 epg, therefore was removed from the dataset prior to data analysis. By week 4, FEC scores in both grazing groups exceeded the 750 epg dosing threshold, requiring intervention with Zolvix™. Statistical analysis was only conducted on week 4 prior to dosing where there was no difference between grazing groups ($T_{(10)} = -0.348$, $p = 0.735$) and the herbal ley did not suppress gastrointestinal parasite burden. No further FEC measurements were included in the analysis for autumn 2020 as samples measured after week 4 were under both the influence of artificial and natural anthelminthic effects.

Anthelminthic intervention was not required during the grazing experiment in spring 2021. However, sward type did affect the final FEC score in week 11, where the herbal ley reduced gastrointestinal parasite burden by 78 % relative to the grass-clover control ($K_{(1)} = 4.246$, $p = 0.039$).

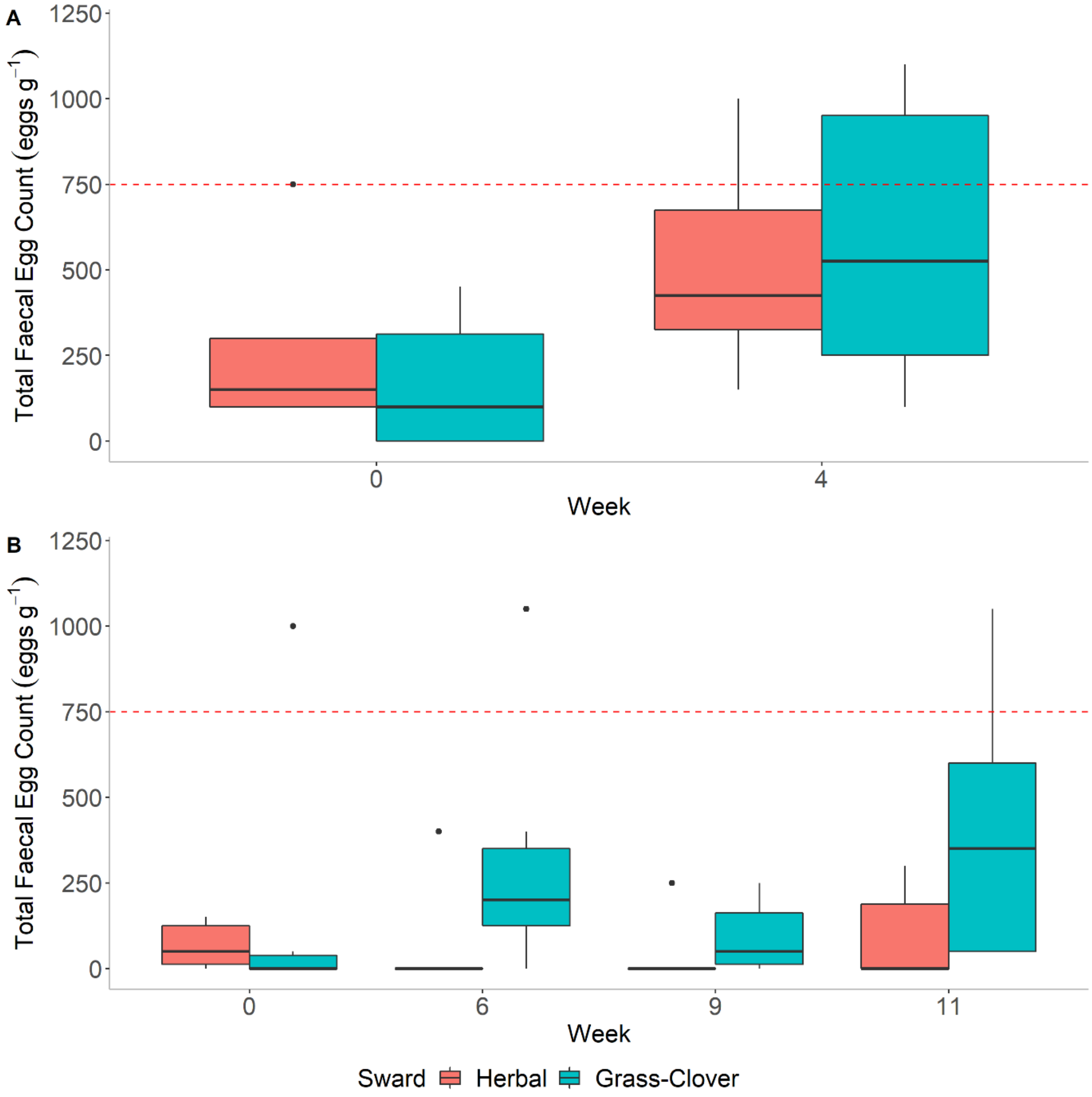

Figure 3.3. *Total faecal egg count (FEC) measured in autumn 2020 (male lambs, n = 6 per sward; panel A) and spring 2021 (female lambs, n = 6 per sward; panel B) respectively. Total FEC (eggs per gram) includes identified Moniezia, Nematodirus and Strongylids eggs. Dashed line indicates anthelminthic dosing threshold, set at 750 epg. Boxplot displays median and interquartile range, with whiskers showing minimum and maximum values in the data, and dots indicating potential outliers.*

3.4. Discussion

3.4.1. Sward Annual Growth

Previous research has shown that increasing species diversity in grasslands results in yield improvements due to the complementarity of species between plant functional groups (Cong et al., 2018; Finn et al., 2013; Grange et al., 2021; Jordon et al., 2022). The inclusion of forage herbs such as *Cichorium intybus*, *Plantago lanceolata*, *Lotus corniculatus* and *Achillea millefolium* to traditional grass-legume leys is proposed to deliver greater ecosystem service benefits while improving livestock health (Cong et al., 2018; Jordon et al., 2022). However, there are limited data on the productivity of herbal leys when subjected to livestock grazing, particularly by sheep. Under continuous cattle grazing, sward productivity was 9 % higher compared to mowing of a 10- or 12-species herbal ley, however, the proportions of herbs and legumes present in the herbal ley declined over time due to selective grazing pressure (Jing et al., 2017). Ultimately, this resulted in no differences in herbage yield between the mixtures and the grass-clover control under grazing by the end of the 3-year trial (Jing et al., 2017). Grace et al. (2018) also reported a decline in forage herb abundance under rotational grazing of a 6- or 9-species herbal ley receiving 90 kg N ha^{-1} yr^{-1} due to selective grazing by sheep (stocked at 12.5 ewes ha^{-1}), but found no difference between seasonal or annual sward dry matter production when compared to a *Lolium perenne* sward receiving 163 kg N ha^{-1} yr^{-1}. Our research has shown that under low N inputs (one annual application of 50 kg N ha^{-1} in spring) and seasonal rotational grazing (at a grazing intensity of 3.2 LU ha^{-1}), there was no difference in sward standing biomass between the herbal and grass-clover ley until 66-days into the spring grazing experiment (Figure 3.1), thus our first hypothesis is only partially accepted.

The lack of overall seasonal differences on sward biomass under either forage mixture was unexpected in this study, and conflicts with previous research showing an overyielding effect of a herbal ley when compared to a monoculture perennial ryegrass or grass-clover mixture throughout the year (Finn et al., 2013). For example, a recent meta-analysis of herbal leys found that dry matter yield increased by 1.6 t ha^{-1} per meter increase in average sward rooting depth, with this effect greatly enhanced when legumes are present allowing non-leguminous species to overcome resource limitations, e.g., water and nitrogen (Jordon et al., 2022). Similar findings were reported in a 3-year continental-scale experiment across 31-sites using plot-trials managed by mowing, where four-species mixtures outyielded monocultures in ca. 97 % of site comparisons due to the synergistic effect of combining functional plant groups (Finn et al., 2013). This may explain the 36 % greater sward standing biomass in the herbal ley compared to the grass-clover ley towards the end of the spring grazing experiment in our study (66-days after the grazing experiment started). In June 2021, the field experiment was under moderate water stress where average monthly rainfall and temperature only reached 1.21 mm and 17 °C respectively (Supplementary Figure 3.1). Although changes in seasonal sward species diversity and abundance was not measured in this study, it can be assumed that the deep-rooting plants in the herbal ley accessed subsoil resources and increased sward biomass under borderline drought conditions.

Previous studies have reported that following a two-month experimental drought, a 6-species mixture containing deep-rooting herbs (*Cichorium intybus* and *Plantago lanceolata*) had similar yields to a rainfed *Lolium perenne* sward receiving 300 kg N ha^{-1} (Grange et al., 2021). Similarly, Hofer et al. (2016) showed that when subjecting a mown 4-species mixture containing a deep-rooting herb *Cichorium intybus* and legume *Trifolium pratense* to drought conditions, it yielded approximately 50 % more than the rainfed monocultures. However, these drought responses were shown to be both site and soil type specific (sandy soils *vs*. silt and clay dominant soils) and heavily dependent on the subsequent water retention capacity of each location (Hofer et al., 2016). Skinner (2008) noted that adding *Cichorium intybus* to grass-legume mixtures only resulted in increased drought resilience when the herb comprised 24-39 % of the forage biomass, with herb persistence declining in the second year.

Each of the deep-rooting plant species previously mentioned were sown in the herbal ley in our study and were visually observed to persist into the second year. However, limited information exists on the annual productivity of commercial herbal ley mixtures (as used here), with most previous experiments comparing smaller mixtures (e.g., 4- to 9-species) as opposed to the commercial 12- to 18-species mixtures typically used by farmers. However, the results of this study should be interpreted with some caution as we only present sward standing biomass, rather than yield, from its establishment to one year old under a grazing and cutting regime. Consequently, further investigation is needed to measure productivity as the sward ages under a range of different climate conditions and environmental stresses. Although the stocking density was the same on each sward, potential changes in lamb grazing behaviour with an increase in dry matter intake due to the improved sward palatability may have contributed to the minimal differences in sward standing biomass between leys and requires further investigation (Jordon et al., 2022).

3.4.2. Sward Nutritional Quality

The nutritional quality of grasslands is intrinsically linked to its botanical composition, with the inclusion of grasses primarily increasing sward digestibility and fiber content (Belesky et al., 2001; Wilson et al., 2020) and legumes and herbs increasing crude protein and macro- and micronutrient concentrations (Darch et al., 2020; Sanderson, 2010; Scales et al., 1995). Previous research has shown that increasing sward complexity through herbal leys can improve key elements of sward quality, such as metabolisable energy, crude protein content and digestibility (Deak et al., 2009; Komainda et al., 2022). Our study has shown that despite no difference in general nutritional quality (e.g., crude protein content, sugar, metabolisable energy, digestibility) between the herbal and grass-clover ley, the herbal ley increased concentrations of certain macro- and micronutrients in fresh forage and hay samples (Table 3.2 and 3.3), allowing us to partially accept our second hypothesis.

The lack of differences in general nutritional quality between sward types was not expected in this study but may be explained by the low proportion of herbs (13.8 %) sown in the herbal ley relative to the proportion of grasses (46 %) and legumes (39.9 %), whereas in the grass-clover ley grasses and legumes comprised 90 % and 10 % of the mixture respectively. Although species presence and absence will vary seasonally, a sward botanical survey conducted in July 2021 revealed that of the 18 species sown in the herbal ley, only 11 species persisted after two grazing seasons (Supplementary Figure 3.2), with key deep-rooting herbs such as *Plantago lanceolata*, *Achillea millefolium* and *Cichorium intybus* comprising 4.9 ± 1.2 %, 6.2 ± 0.7 %, and 21.1 ± 1.3 % of the sward composition, respectively. While the proportion of herbs present in the mixture in this study reflects the typical botanical composition of a commercial herbal ley, the higher proportions of grasses and legumes combined with selective grazing pressure and poor establishment of some species, e.g., *Onobrychis*, can dilute the potential nutritional benefits, resulting in a similar composition to the grass-clover ley (Grace et al., 2018; Jing et al., 2017). Herb-clover swards predominately containing *Cichorium intybus*, *Plantago lanceolata*, *Trifolium pratense* and *Trifolium repens* have shown higher organic matter digestibility and greater metabolisable energy for grazing lambs than herb-grass (containing only *Plantago lanceolata* as the herb), grass-clover, or grass-only mixtures (Golding et al., 2011).

Seasonal differences where sward dry matter, sugar, metabolisable energy and digestibility (D-value) were higher in spring than in autumn was likely driven by changes in plant maturity and botanical composition, where maturing plants cause a decline in nutritive quality (Sanderson, 2010). Plant maturity, seasonal differences in botanical composition and nutritional quality of fresh forage reflects on the summer 2021 hay quality. However, it is difficult to compare our findings to previous research as there is little or no information on the hay production and the subsequent nutritional quality of herbal leys. This needs further investigation.

The herbal ley consistently produced higher concentrations of key macro- and micronutrients than the grass-clover ley across both grazing seasons in this study, with the effect observed to persist after hay production. Individual plant species within the herbal ley, e.g., *Cichorium intybus*, are known to accumulate greater concentrations of beneficial and antagonistic nutrients than other common grassland species, e.g., *Lolium perenne* (Belesky et al., 2001; Scales et al., 1995). Higher concentrations of sward sodium, calcium and magnesium within the herbal ley despite no differences in topsoil macronutrient content agrees with previous research, and is likely driven by the dominance of *Cichorium intybus* in the sward in this study and its greater nutrient accumulation ability (Barry, 1998; Høgh-Jensen et al., 2006). *Cichorium intybus* also has deep-rooting capabilities, allowing it to access subsoil nutrients which may explain the higher concentration of sward copper in the herbal ley across both grazing seasons (Belesky et al., 2001). However, the increase in sward copper content in the herbal ley was subject to seasonal variability, only meeting the grazing requirements for lambs in autumn 2020, not spring 2021. While promising, the low frequency of sward chemical composition measurements in this study only provides a snapshot of the nutritional benefits of using herbal leys, as these results are influenced by seasonal factors and require further investigation (Kao et al., 2020).

It is difficult to compare our results to previous research as in addition to the botanical composition of the sward varying between studies, the macro- and micronutrient content can be heavily influenced by geographical location (e.g., distance from the sea), field management (e.g., grazing *vs*. mowing), seasonal nutrient availability (e.g., soil moisture and temperature), soil factors (e.g., pH, redox potential, organic matter, microbial activity), and geochemical abundance in the underlying parent material (Darch et al., 2022; Kao et al., 2020; Watson et al., 2012). In this study, the field experiment had a neutral soil pH of ca. 6.9-7.0 which did not limit nutrient availability but was located approximately 450 m from the sea, so may have been subjected to aerosol deposition of salt spray providing an additional external selenium source (Watson et al., 2012). Due to the underlying soil condition, it was not possible to measure soil micronutrient content or subsoil (10–100 cm) macronutrient content in this study. To our knowledge, this is the first study reporting the macro- and micronutrient content of a commercial herbal ley under field conditions, as previous studies have often only examined the chemical composition of species in isolation (e.g., Hamacher et al. (2021)) or following pot-scale trials (e.g., Darch et al. (2022); Darch et al. (2020); Lindström et al. (2013)). A key limitation of this study that future research should explore is the soil macro- and micronutrient content in greater detail throughout the soil profile to better understand the relationship between root depth, nutrient availability and uptake in herbal leys using a conventional grass-clover ley as a comparison.

3.4.3. Lamb Productivity, Gastrointestinal Parasite Burden, and Blood Characteristics

It was anticipated that the herbal ley would increase lamb liveweight gain and reduce gastrointestinal parasite burden due to the improved sward nutritional quality, greater dry matter intake, and higher plant secondary metabolite content providing anthelminthic properties (Grace et al., 2019; Jordon et al., 2022; Kenyon et al., 2010). In this study, liveweight gain was only significantly greater in the herbal ley than grass-clover grazed lambs in spring 2021, with ewe lambs gaining an additional 28 g d^{-1} (Figure 3.2). This agrees with previous research, where ewes consuming a 6- or 9-species herbal ley had greater liveweight gains than those consuming a perennial ryegrass sward (Grace et al., 2019). Similarly, lambs consuming a herb-clover or pure herb stands frequently had a greater liveweight gain (Golding et al., 2011; Jerrentrup et al., 2020; Somasiri et al., 2015). This is likely due to the improved sward nutritional quality combined with higher concentrations of plant secondary metabolites present in key plant species (e.g., *Cichorium intybus*, *Plantago lanceolata*) within a herbal ley reducing rumen protein degradation, therefore increasing absorption in the intestines and overall protein utilisation in grazing ruminants (Jordon et al., 2022). This mechanism of improving protein utilisation, combined with a potential increase in dry matter intake and improved macro- and micronutrient content, may explain the greater liveweight gain in herbal ley grazed lambs in spring 2021.

Plant secondary metabolites, e.g., sesquiterpene lactones, condensed tannins and saponins, can also provide anthelminthic properties when consumed. Further, livestock have been observed to self-medicate by selectively grazing certain plant species to reduce gastrointestinal parasite burden while carefully balancing any unintended toxicity effects (Costes-Thiré et al., 2019; Peña-Espinoza et al., 2018). Suppression of the naturally acquired gastrointestinal parasite burden was observed in spring 2021, where the herbal ley reduced FEC scores by 78 % relative to the grass-clover control in week 11 (Figure 3.3). Although research into the gastrointestinal parasite burden of livestock consuming herbal leys is limited, this finding supports previous research by Grace et al. (2019), which showed lambs consuming a 6- or 9-species herbal ley required less anthelminthic interventions than their grass or grass-clover counterparts. In addition to the plant secondary metabolite content reducing parasite motility, egg production and survival *in vivo*, utilising grassland management options such as increasing the sward height and including plants such as *Cichorium intybus* can inhibit parasitic larvae migration up the plant towards the leaves, thus preventing consumption by the next host (French, 2018; Hoste et al., 2006; Jordon et al., 2022; Marley et al., 2003; Peña-Espinoza et al., 2018). This may partially explain why the parasite burden was suppressed in the herbal ley in the spring 2021 grazing period, as sward height ranged between 10–13 cm and 8–11 cm for the herbal and grass-clover ley respectively.

The lack of differences in both lamb liveweight gain and the reduction in parasite burden in the autumn 2020 grazing season may be due to the high initial FEC scores at the start of the experiment that did not respond to natural anthelminthic intervention when consuming the herbal ley. This required intervention with a broad spectrum synthetic anthelminthic as lambs on both diets hosted a mixed infection of *Moniezia*, *Nematodirus*, and *Strongylids*. This would have subjected lambs to additional physiological stress, with the resulting nutrient depletion and potential reduced voluntary dry matter intake arising from the high burden diminishing any additional nutritional benefit of grazing the herbal ley (Houdijk et al., 2017). Combined with the high pre-existing burden, potential reduced dry matter intake, seasonal differences in plant secondary metabolite availability and abundance, along with a shorter sward height during the autumn 2020 grazing period of 4–7 cm and 5–9 cm for the herbal and grass-clover ley respectively, may have contributed to the insignificant increase in liveweight gain (Gilleard et al., 2021). However, the small grazing groups (ca. *n* = 40 per sward) used each season due to field experiment limitations may have hidden any natural variation in liveweight gain. Similarly, the plant secondary metabolite content of both swards and dry matter intake of grazing lambs was not measured in this study. To our knowledge, there is no available data of the plant secondary metabolite content of commercial herbal ley mixtures that account for the potential dilution effect not explored in single species pot-scale experiments, e.g., Hamacher et al. (2021).

Although no blood measurement was obtained in autumn 2020, the spring 2021 samples obtained after 11-weeks of grazing from the ewe lambs showed an overall positive effect of grazing the herbal ley, with higher concentrations of GSHPx, plasma cobalt and plasma selenium bringing lambs closer to the normal range for their growth stage (Table 3.4). These improvements were surprising as there was no significant increase in the sward cobalt or selenium content in the herbal ley in spring 2021 (Table 3.3), however, it is important to note that the bioavailability of these micronutrients was not assessed and may vary with seasonality. It is therefore likely that the increase in plasma selenium and cobalt may be driven by a potential higher dry matter intake in herbal grazed lambs combined with reduced parasitism (Jordon et al., 2022), or lower cobalt requirements due to slower growth rates in older ewe lambs (ca. 12-13 months old) after 11-weeks of grazing. The reduced blood urea content in herbal grazed lambs despite a similar sward crude protein (126-172 g kg^{-1}), sugar (214 – 236 g kg^{-1}), and metabolizable energy (12 MJ kg^{-1}) content between diets may be indicative of increased urine urea excretion caused by plant secondary metabolites such as aucubin (Deaker et al., 1994), or condensed tannins increasing urea ‘recycling’ within the animal (Azuhnwi et al., 2013).

Alternatively, the higher blood urea content in the grass-clover lambs may be due to a dietary protein and energy imbalance, driven by higher gastrointestinal parasite burden and potential differences in voluntary dry matter intake. However, the mechanisms driving this are complex, and as it was only possible to measure one timepoint from a limited number of lambs (n = 10 per sward), any temporal changes in sward and plasma macro- and micronutrient content may have been missed. While there is currently no literature available on the haematological or plasma biochemical composition of livestock grazing herbal leys to compare our study to, research into lambs grazing single herb species has shown a significant decline in serum urea when lambs consume *Plantago lanceolata* (7.42 mmol/l) compared to *Lolium perenne* (9.41 mmol/l) (Deaker et al., 1994). Similar findings were also reported in dairy heifers consuming a pasture mixture or pure *Cichorium intybus*, where plasma urea concentrations declined from 0.32 g/l to 0.24 g/l, reducing overall nitrogen excretion (Cheng et al., 2017).

Optimising the plasma cobalt and selenium status in lambs and reducing the need for synthetic anthelminthic interventions through grazing herbal leys has wider economic and environmental implications. Altering the grazing pasture with herbal leys can reduce on-farm greenhouse gas emissions associated with grazing and consequently the carbon footprint of the finished livestock product by removing barriers to improving livestock efficiency, such as micronutrient deficiencies and parasitic challenges (Houdijk et al., 2017). Diet manipulation can reduce the risk of clinical (e.g., white muscle disease, anaemia) and sub-clinical (e.g., limited growth, reduced fertility) diseases arising from micronutrient deficiencies and thus can improve overall livestock health and tolerance to stressors (e.g., gastrointestinal parasite burden) (Kao et al., 2020). This may also reduce additional production costs through reducing the need for additional supplementation (e.g., mineral licks, boluses) and veterinary interventions (Kao et al., 2020). However, while the lambs in our study consuming the herbal ley did have a reduced deficiency risk for vitamin B12 and selenium compared to grass-clover grazed lambs, lambs on both diets were below the normal range for multiple macro- and micronutrients such as manganese, iodine, cobalt, and calcium which is likely caused by the sward quality not meeting nutrient requirements. Additional supplementation was not provided in our study to allow us to evaluate sward-only effects in a typical lowland grazing system. These nutrient deficiencies are known to be highly soil and site specific. Therefore, future research or farm adoption of herbal leys may face similar challenges and further research is needed across multiple sites to account for botanical and geographical variations and their impact on livestock health. Following these results, we can accept our third hypothesis.

3.5. Conclusion

Improving lamb productivity and health through diet manipulation is vital to reducing the environmental impacts of lowland meat production. This study has shown the seasonal potential for herbal leys to improve ewe lamb liveweight gain, reduce gastrointestinal parasite burden, reduce the risk of micronutrient deficiency disease through increasing plasma cobalt and selenium, and reduce nutrient losses by reducing blood urea content. These differences were potentially driven by higher dry matter intake and plant secondary metabolite content but require further investigation. Despite an increase in sward macro- and micronutrient content in the herbal ley, no differences were found in general sward nutritional quality or productivity between diets.

To our knowledge, this is the first study to show a commercial herbal ley under field conditions, their effect on sward macro- and micronutrient content and the subsequent impact on lamb health and productivity. While these results show the promise of adopting herbal leys, further research is needed using longer-term studies across multiple geographical locations to account for variations in sward productivity, composition, climate, and soil type to fully capture the benefits and challenges of wide-scale adoption of herbal leys for livestock production.

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Ethical Statement

Liveweight gain and faecal egg count measurements were approved by the Bangor University Ethics Committee (code COESE2019EC01A). Blood samples were obtained under ASPA licence.

CRediT authorship statement

Funding acquisition, J.R.L. and D.L.J.; Conceptualisation, experimental design, sampling, data curation, formal analysis, and writing – original draft E.C.C., N.R.K., D.R.C, D.L.J; Writing – review and editing E.C.C. N.R.K., D.L.J, D.R.C. and J.R.L.; Supervision, D.L.J. and D.R.C.

Declaration of interest

The authors declare they have no competing interests.

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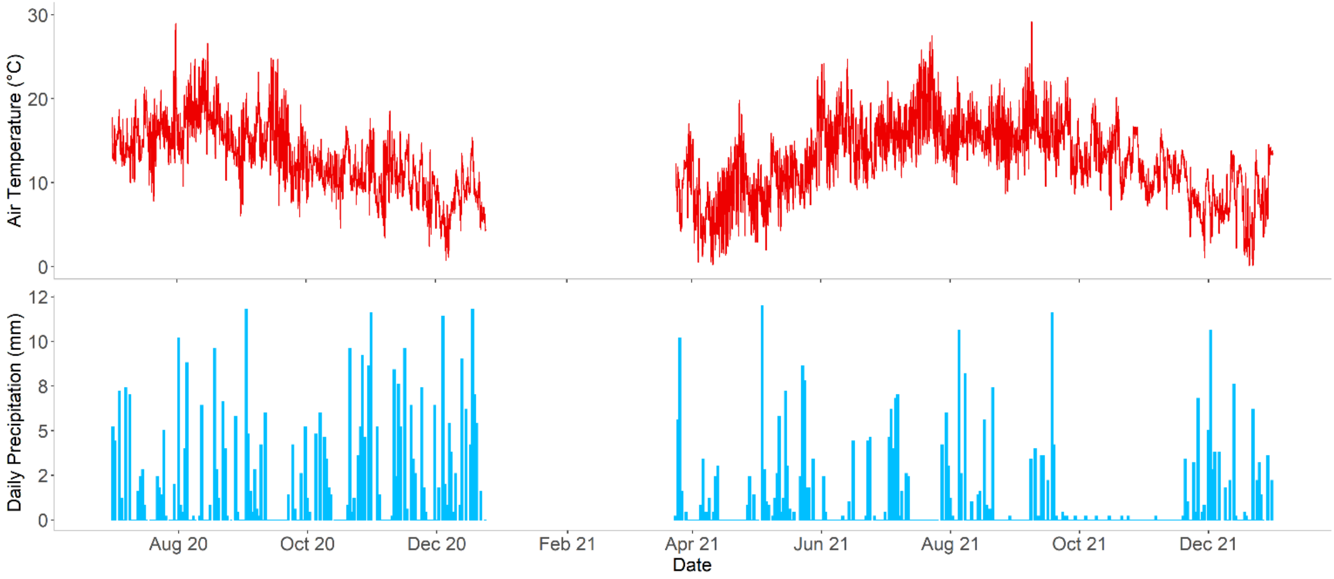
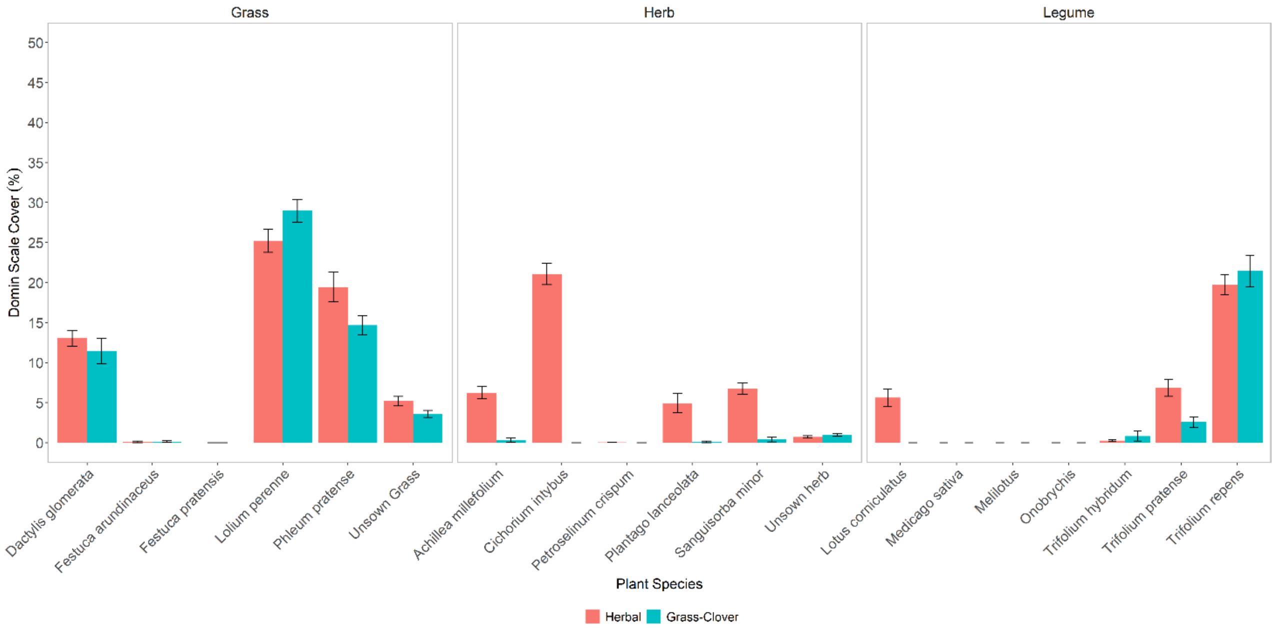
3.7. Supplementary Materials



Supplementary Figure 3.1. *Daily air temperature (°C) and precipitation (mm) obtained at the same altitude 200 m from the field experiment at Henfaes Research Centre, Abergwyngregyn, (UK) between July 2020 and December 2021. Data is not reported between 24/12/20 and 24/03/21 due to a malfunction with the metrological apparatus.*



Supplementary Figure 3.2: *Domin score cover (%) of sown and unsown species in the herbal and the grass-clover ley assessed in July 2021. Data represents mean ± SEM, n = 3 per sward.*

Supplementary Figure 3.3: *Overhead photography of the herbal ley (left) and grass-clover ley (right) in summer 2021.*

Supplementary Table 3.1*. Two-way ANOVA results of sward type, season, or the interaction between sward and season on measured sward characteristics. * indicates statistically significant values, significance level was determined as* $p < 0.05$*, degrees of freedom are presented in brackets. † Degrees of freedom = 1,2.*

Sward Characteristic	Effect of sward type		Effect of season		Interaction between Sward × Season	
	$F_{(1,8)}$	*P*-value	$F_{(1,8)}$	*P*-value	$F_{(1,8)}$	*P*-value
Dry matter	1.027	0.341	174.5	<0.001*	1.534	0.251
Crude protein	0.000	0.994	13.661	0.006*	4.465	0.068
D-value	2.689	0.140	3.649	0.093	3.780	0.088
Metabolisable energy	3.658	0.092	3.658	0.092	3.658	0.092
NDF	0.211	0.658	3.295	0.107	0.018	0.896
Ash	0.090	0.772	33.937	<0.001*	0.292	0.604
Oil-A	0.078	0.788	3.804	0.087	2.612	0.145
Sugar	0.021	0.889	23.123	0.001*	1.436	0.265
Sodium	27.780	<0.001*	90.590	<0.001*	26.730	<0.001*
Nitrate	0.397	0.546	3.571	0.096	1.921	0.203
Buffering capacity	0.565	0.474	4.655	0.063	1.796	0.217
Potassium	5.183	0.052	1933.335	<0.001*	6.004	0.040*
Calcium	48.27	<0.001*	348.550	<0.001*	39.37	<0.001*
Magnesium	77.54	<0.001*	1221.410	<0.001*	66.540	<0.001*
Cobalt	0.769	0.4068	25.063	0.001*	0.871	0.377
Copper	16.472	0.004*	155.456	<0.001*	0.012	0.915
Iodine	34.127	<0.001*	84.794	<0.001*	8.245	0.021*
Manganese	0.004	0.951	2.872	0.129	8.856	0.018*
Selenium	0.315	0.590	6.670	0.033*	†0.022	0.885
Zinc	6.644	0.033*	37.705	<0.001*	0.108	0.751

Supplementary Table 3.2. *General sward properties with macro- and micro-nutrients measured from either a conventional grass-clover ley or herbal ley (n = 3) in autumn 2021 (15/09/21). Data represents mean ± SEM, presented alongside statistical results. * = indicates statistically significant result, significance level is set to $p < 0.05$. Data is expressed on a dry weight basis.*

T-test					
Sward Properties	Grass-Clover	Herbal	Degrees of Freedom	T	*P*-value
Dry matter (g kg^{-1})	172.0 ± 18.0	187.0 ± 0.6	4	-0.832	0.452
Crude protein (g kg^{-1})	242.0 ± 22.6	153.7 ± 20.8	4	2.876	0.045*
Sugar (g kg^{-1})	63.3 ± 14.4	77.0 ± 1.5	4	-0.941	0.400
Neutral-detergent fibre (g kg^{-1})	483.0 ± 30.5	526.7 ± 46.5	4	-0.786	0.476
Ash (g kg^{-1})	110.0 ± 9.5	96.0 ± 4.2	4	1.441	0.223
Oil-A (g kg^{-1})	28.0 ± 4.2	19.3 ± 1.5	4	1.990	0.118
Nitrate-nitrogen (%)	0.03 ± 0.1	0.01 ± 0.1	4	2.085	0.105
Calcium (g kg^{-1})	14.2 ± 0.9	17.9 ± 0.3	4	-3.734	0.020*
Magnesium (g kg^{-1})	2.5 ± 0.1	3.0 ± 0.1	4	-3.411	0.027*
Sodium (g kg^{-1})	0.8 ± 0.3	1.1 ± 0.1	4	-1.108	0.330
Cobalt (mg kg^{-1})	0.14 ± 0.009	0.13 ± 0.003	4	1.018	0.366
Copper (mg kg^{-1})	8.1 ± 0.3	8.5 ± 0.3	4	-1.095	0.335
Iodine (mg kg^{-1})	0.5 ± 0.04	0.6 ± 0.02	4	-2.982	0.041*
Manganese (mg kg^{-1})	115.3 ± 21.9	113.2 ± 7.8	4	0.087	0.935
Selenium (mg kg^{-1})	0.1 ± 0.04	0.01 ± 0.01	4	0.345	0.747
Zinc (mg kg^{-1})	21.9 ± 0.4	28.5 ± 1.0	4	-6.408	0.003*
Kruskal-Wallis Test					
Sward Properties	Grass-Clover	Herbal	Degrees of Freedom	Chi-squared	*P*-value
D-value (%)	66.9 ± 2.4	65.0 ± 0.5	1	0.049	0.825
Metabolisable energy (MJ kg^{-1})	10.5 ± 0.4	10.2 ± 0.1	1	0.054	0.817
Buffering capacity (meq kg^{-1})	404.3 ± 20.5	370.0 ± 0.0	1	2.400	0.121
Potassium (g kg^{-1})	33.8 ± 4.8	27.2 ± 0.5	1	1.191	0.275

Supplementary Table 3.3. *Hay composition including micro- and macronutrient content measured after the spring 2021 grazing season. Hay was cut on 17/07/21 and sampled on 16/08/21. Data represents mean ± SEM. * = indicates statistically significant result, significance level set at $p < 0.05$. $n = 3$ measurements per sward type. Data is expressed on a dry weight basis.*

Hay Properties	Grass-Clover	Herbal	Degrees of Freedom	T	*P*-value
Dry matter (g kg^{-1})	857.3 ± 2.8	861.3 ± 0.3	4	1.395	0.236
Crude protein (g kg^{-1})	103.0 ± 5.0	112.0 ± 7.2	4	1.021	0.365
Sugar (g kg^{-1})	98.0 ± 1.5	103.0 ± 1.7	4	2.165	0.096
Metabolisable energy (MJ kg^{-1})	8.6 ± 0.1	8.5 ± 0.2	4	-0.781	0.479
Neutral-detergent fibre (g kg^{-1})	567.7 ± 4.3	569.7 ± 4.1	4	0.337	0.753
D-value (%)	53.7 ± 0.3	53.0 ± 1.2	4	-0.555	0.609
Ash (g kg^{-1})	63.3 ± 1.2	65.0 ± 1.7	4	0.791	0.473
Oil-B (g kg^{-1})	24.3 ± 0.3	26.0 ± 0.6	4	2.500	0.067
Digestible energy (MJ kg^{-1})	9.3 ± 0.02	9.2 ± 0.1	4	-0.555	0.609
Calcium (g kg^{-1})	5.2 ± 0.6	7.2 ± 0.2	4	3.074	0.037*
Magnesium (g kg^{-1})	1.2 ± 0.2	1.4 ± 0.1	4	2.121	0.101
Potassium (g kg^{-1})	20.8 ± 0.8	23.8 ± 0.9	4	2.481	0.068
Sodium (g kg^{-1})	0.6 ± 0.1	0.9 ± 0.03	4	4.000	0.016*
Copper (mg kg^{-1})	4.1 ± 0.2	5.1 ± 0.2	4	4.330	0.012*
Iodine (mg kg^{-1})	0.4 ± 0.03	0.3 ± 0.02	4	-2.646	0.057
Selenium (mg kg^{-1})	0.11 ± 0.01	0.14 ± 0.01	4	1.706	0.163
Zinc (mg kg^{-1})	18.0 ± 1.5	21.8 ± 2.5	4	1.323	0.257
Kruskal-Wallis Test					
Hay Properties	Grass-Clover	Herbal	Degrees of Freedom	Chi-squared	*P*-value
Acid-detergent fibre (g kg^{-1})	344.0 ± 5.6	338.0 ± 1.2	1	0.784	0.376
Cobalt (mg kg^{-1})	0.1 ± 0.01	0.3 ± 0.2	1	1.226	0.268
Manganese (mg kg^{-1})	89.8 ± 7.1	98.2 ± 14.5	1	0.048	0.827

***Supplementary Table 3.4**. Spring lamb body condition score (BCS) measured at the start and end of an 11-week grazing period in spring 2021 from lambs consuming either a herbal (n = 32) or grass-clover diet (n = 36). Data represents mean ± SEM. No superscripted statistical significant letters are presented as all results were insignificant (p > 0.05).*

BCS, scale 1-5	Grass-Clover	Herbal
Starting BCS	3.0 ± 0.1	2.9 ± 0.1
Final BCS	3.6 ± 0.1	3.5 ± 0.1
Change in BCS		
BCS increase (units)	0.5 ± 0.1	0.6 ± 0.1

Supplementary Table 3.5. *Statistical results of blood characteristics detailed in Table 4 following either a t-test (T-value) if data was parametric or if non-parametric then a Kruskal-Wallis test (Chi-squared value). Statistical significance was set at* $p < 0.05$*, * indicates significant results.*

T-test			
Blood Characteristic	T-value	Degrees of Freedom	*P*-value
Haematocrit	0.678	18	0.507
Haemoglobin	-0.288	18	0.837
Glutathione peroxidase	-3.765	18	0.001*
Caeruloplasmin	-0.103	18	0.919
Caeruloplasmin/plasma copper ratio	-0.006	18	0.995
β-hydroxybutyrate	1.288	18	0.214
Non-esterified fatty acids	2.086	18	0.052
Urea	5.340	18	< 0.001*
Total protein	0.626	18	0.539
Albumin	2.132	18	0.047*
Globulin	-0.041	18	0.968
Plasma calcium	0.274	18	0.787
Plasma cobalt	-2.642	18	0.017*
Vitamin B12	-1.161	18	0.261
Plasma inorganic iodine	0.591	18	0.562
Plasma selenium	-5.183	18	< 0.001*
Plasma sodium	-1.034	18	0.315

Kruskal-Wallis test			
Blood Characteristic	Chi-squared	Degrees of Freedom	*P*-value
Plasma potassium	0.366	1	0.545
Plasma magnesium	1.463	1	0.227
Plasma manganese	0.143	1	0.706
Plasma zinc	0.051	1	0.821
Plasma copper	0.000	1	1.000
Superoxide dismutase	3.291	1	0.070

Chapter 4

Grazing lambs on herbal leys increases urinary sodium excretion but does not affect excreta N concentration or nitrous oxide emissions compared to a grass-clover ley

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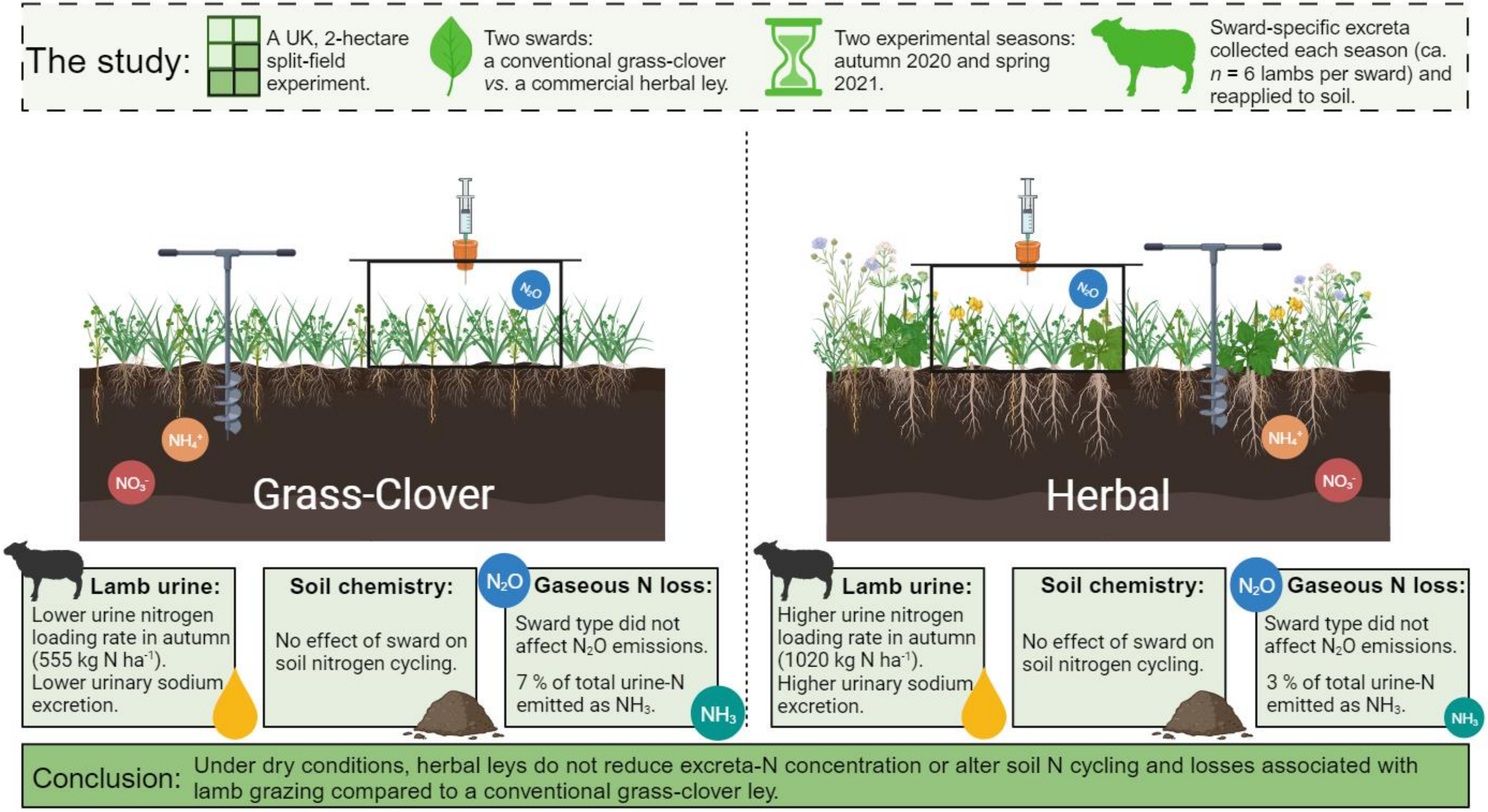
This chapter is currently under review at Agriculture, Ecosystems and Environment.



The study:

A UK, 2-hectare split-field experiment.

Two swards: a conventional grass-clover *vs.* a commercial herbal ley.

Two experimental seasons: autumn 2020 and spring 2021.

Sward-specific excreta collected each season (ca. n = 6 lambs per sward) and reapplied to soil.

Grass-Clover

Lamb urine: Lower urine nitrogen loading rate in autumn (555 kg N ha^{-1}). Lower urinary sodium excretion.

Soil chemistry: No effect of sward on soil nitrogen cycling.

Gaseous N loss: Sward type did not affect N_2O emissions. 7 % of total urine-N emitted as NH_3.

Herbal

Lamb urine: Higher urine nitrogen loading rate in autumn (1020 kg N ha^{-1}). Higher urinary sodium excretion.

Soil chemistry: No effect of sward on soil nitrogen cycling.

Gaseous N loss: Sward type did not affect N_2O emissions. 3 % of total urine-N emitted as NH_3.

Conclusion: Under dry conditions, herbal leys do not reduce excreta-N concentration or alter soil N cycling and losses associated with lamb grazing compared to a conventional grass-clover ley.

Figure 4.0. *Chapter 4 graphical abstract.*

Highlights

- Increasing the herb and legume content in grasslands can alter livestock GHGs.
- Excreta N concentration was not altered in herbal ley grazed lambs.
- Urinary sodium excretion was significantly elevated in lambs grazing the herbal ley.
- Sward-specific urine ammonia volatilisation was reduced by 140% in the herbal ley.
- Sward type had no effect on urine, dung, or synthetic N fertiliser N_2O emissions.

Keywords

Multispecies sward, ammonia, N leaching, sheep, bioactive sward

Abstract

Herbal leys (multispecies swards) can deliver multiple agronomic and environmental benefits; however, little is known about their effect on nitrogen (N) losses associated with lowland sheep production. A UK split-field experiment utilising a herbal or grass-clover ley (*n* = 3 per sward) aimed to quantify differences in lamb excreta composition and develop a seasonal urine-patch nitrous oxide (N_2O) emission factor (EF_{3PRP}). Lamb excreta obtained in autumn (*n* = 7 herbal, *n* = 6 grass-clover) and spring (*n* = 6 per sward) was reapplied to the soil of each respective pasture. N_2O measurements and soil (0-10 cm depth) samples were taken from each treatment (urine, dung, 50 kg N ha^{-1} ammonium nitrate, control) over 103 and 78 days in autumn and spring, respectively. Urine N concentration did not differ between sward types ($p > 0.05$), however, seasonal variations in urination volume resulted in a higher urine N loading rate in the herbal ley in autumn (1020 *vs*. 555 kg N ha^{-1}, $p < 0.001$) but not spring ($p > 0.05$). Urinary sodium excretion was greater in herbal ley grazed lambs in autumn (439 *vs*. 71 mg Na l^{-1}, $p < 0.001$) and spring (389 *vs*. 47 mg Na l^{-1}, $p = 0.002$). Dung composition was not affected by sward ($p > 0.05$). Sward type did not affect N_2O emissions or emission factors ($p > 0.05$). However, N_2O fluxes were low due to dry soil conditions (ca. 45-52 % WFPS) experienced in both seasons. In a bench-scale experiment, NH_3 volatilisation from sward-specific urine was lower from the herbal (3.05 %) than the grass-clover ley (7.33 %) ($p < 0.05$). Overall, this study has shown that under dry conditions herbal leys do not reduce excreta-N concentration or alter soil N cycling and losses associated with lamb grazing compared to a conventional grass-clover ley.

4.1. Introduction

Livestock excreta nitrogen (N) deposition to pasture represents a major source of nitrous oxide (N_2O) emissions in grazing systems, contributing to ca. 54 % (1.31 Tg N_2O-N yr^{-1}) of global N_2O emissions from grasslands annually (Dangal et al., 2019). Ruminants, such as sheep, are inefficient at utilising consumed N, excreting 70-95 % in urine and dung (López-Aizpún et al., 2020). This can greatly exceed plant N uptake when deposited, with excess N vulnerable to losses through the soil profile via nitrate (NO_3^-) leaching or via gaseous losses through ammonia (NH_3) volatilisation (Laubach et al., 2013), nitric oxide (NO) (Clough et al., 2020), dinitrogen (N_2) (Skiba et al., 2011), or N_2O emissions (Luo et al., 2018), leading to negative impacts on human and environmental health (Adhikari et al., 2021; López-Aizpún et al., 2020). As N_2O is a potent, ozone depleting greenhouse gas (GHG), pressure is growing to identify economically viable mitigation strategies that can reduce N_2O emissions associated with livestock grazing systems (de Klein et al., 2020).

In grazed grasslands, N_2O emissions are dependent on soil (e.g., moisture, pH), plant (e.g., sward composition), climatic (e.g., temperature), and excreta composition (e.g., N concentration, urination volume) factors (Dangal et al., 2019; Dijkstra et al., 2013; López-Aizpún et al., 2020). Due to the highly heterogenous spatial and temporal nature of livestock excreta deposition, current N_2O abatement strategies target emissions either directly from the ruminant (e.g., by reducing dietary protein intake, grazing bioactive forages) (de Klein et al., 2020) or at the pasture scale (e.g., by altering the sward composition, application of synthetic nitrification inhibitors) (Adhikari et al., 2021; Niklaus et al., 2016). Synthetic nitrification inhibitors, such as dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP), have been shown to reduce N_2O emissions and NO_3^- leaching from urine patches with varying efficacy (Adhikari et al., 2021; Marsden et al., 2017; Minet et al., 2016). However, evidence of DCD entering the food chain via milk products has since led to its voluntary withdrawal in

New Zealand, increasing pressure to identify natural alternatives (Adhikari et al., 2021; de Klein et al., 2022; MPI, 2013).

Herbal leys (multispecies swards) consist of a mixture of grasses, legumes and herb species that can deliver greater agronomic and environmental benefits than conventional grass or grass-clover pastures (Jordon et al., 2022). Key herbs and legumes frequently sown in the sward composition, such as ribwort plantain (*Plantago lanceolata*) and chicory (*Cichorium intybus*), contain high levels of plant secondary metabolites that can improve livestock productivity (Grace et al., 2019), sward quality (Darch et al., 2020), and reduce rumen protein degradation (Minneé et al., 2017), subsequently altering the ratio of N excretion in urine and dung (Bryant et al., 2018; Cheng et al., 2017; Totty et al., 2013).

Previous studies have shown a reduction in urine-N concentration and an increase in faecal-N excretion in livestock grazing high proportions of *Plantago lanceolata* in low-diversity (e.g., 3-6 species) herbal leys (Cheng et al., 2017; Totty et al., 2013), with reports of biological nitrification inhibition (BNI) compounds, such as aucubin, excreted in urine (Gardiner et al., 2018; Peterson et al., 2022). BNI excretion and dilution of urine has been shown to reduce NO_3^- leaching and N_2O emissions from cattle grazing a low-diversity herbal ley (Simon et al., 2019). However, to-date, no studies have investigated the effect of a high-diversity (e.g., 9-18 species) commercial herbal ley promoted via agri-environment schemes on livestock excreta composition or N_2O emissions. Consequently, sward-specific N_2O emission factors have not been developed, thus herbal leys are currently not included in national greenhouse gas inventory calculations, presenting a significant research gap.

This study aims to investigate if a commercial herbal ley mixture can reduce urine N excretion and N_2O emissions associated with lowland lamb grazing compared to a conventional grass-clover ley. We hypothesised that i) urine N concentration will be reduced in herbal ley grazed lambs due to lambs consuming herbs with high levels of plant secondary metabolites; ii) subsequent N_2O emissions from excreta deposition will be lower in the herbal ley due to reduced urine N excretion, increased plant N uptake, and potential biological nitrification inhibition from *Plantago lanceolata*.

4.2. Materials and Methods

4.2.1. Site Description

In July 2020, a 2-ha split-field experiment was established at Bangor University's Henfaes Research Centre, Abergwyngregyn, North Wales, UK (10 m a.s.l., 53.240329 N, -4.014574 W). The soil type at the field site was classified as a Eutric Cambisol, with an average annual rainfall and temperature of 1060 mm and 10.8°C, respectively. The field was ploughed and reseeded with either a 5-species (9 cultivar) conventional grass-clover mixture (grass-clover) or an 18-species (19 cultivar) herb- and legume-rich multispecies (herbal) mixture (Table 4.1) supplied by Cotswold Seeds (Cotswold Seeds Ltd., Moreton-in-Marsh, UK). The field received one fertiliser application of 50 kg N ha^{-1} ammonium nitrate, 20 kg P ha^{-1} and 60 kg K ha^{-1} in March 2021 to encourage grass and herb growth prior to spring grazing. Exclusion areas in each paddock were used for soil and greenhouse gas sampling and were protected from fertiliser applications to prevent contamination.

Each sward type covered 1 ha and was split into three permanently fenced paddocks ($n = 3$) approximately 0.33 ha in size to allow Welsh mountain lambs (*Ovis aries*) ($n = 40$ per sward) to rotationally graze at a stocking density of 3.2 LU ha^{-1}. The breed and sex of lamb used in this experiment was reflective of those typically grazed in the area for each season. Lambs were moved between each paddock on their respective swards every 4-5 days to encourage grass regrowth. Exclusion areas for soil and gas sampling, approximately 13.4 m^2

in size, were established two months prior to grazing to prevent livestock access and additional deposition of urine and dung that could affect subsequent measurements. Aerial imagery of the field experiment design can be seen in Supplementary Figure 4.1. Meteorological data was recorded every 30 minutes from an automated on-site weather station located approximately 200 m from the field site (Campbell Scientific Ltd., Leicestershire, UK).

Sward composition (i.e., dry matter, crude protein, sugar, metabolisable energy, digestibility, and sodium content) was determined by wet chemistry by Sciantec Analytical (Sciantec, Yorkshire, UK). The full sward composition is reported in Chapter 3. Briefly, at the start of each grazing season, sward samples were obtained by cutting four randomly assigned 1 m^2 quadrat sward samples with hand shears across each paddock at an approximate grazing height (ca. 4 – 5 cm). These samples were then bulked into one sample per paddock for nutritional and chemical composition analyses.

The botanical composition of each sward was assessed in July 2021 at the end of the spring 2021 grazing experiment. Five 4 m^2 quadrats were evaluated in each paddock, accounting for spatial variability, with the resulting Domin scores then converted to percentage cover using the Domin 2.6 transformation described in Currall (1987). Sward botanical composition and overhead photographs of the sward are provided in Supplementary Figures 4.2 and 4.3, respectively.

Table 4.1. *Species composition and seeding rate of the herbal ley and grass-clover ley sown in July 2020.*

Plant type	**Herbal ley**			**Grass-Clover ley**		
	Species and Cultivar	Proportion (%)	Seeding rate (kg ha^{-1})	Species and Cultivar	Proportion (%)	Seeding rate (kg ha^{-1})
Grass	Cocksfoot (*Dactylis glomerata*) cv. 'Amba'	11.5	3.75	Perennial ryegrass (*Lolium perenne*) cv. 'Glenstal'	30.8	10.0
	Festulolium cv. 'Lofa'	11.5	3.75	Perennial ryegrass (*Lolium perenne*) cv. 'AberMagic'	16.9	5.50
	Perennial ryegrass (*Lolium perenne*) cv. 'Oakpark'	7.7	2.50	Timothy (*Phleum pratense*) cv. 'Winnetou'	15.4	5.00
	Perennial ryegrass (*Lolium perenne*) cv. 'Glenstal'	3.8	1.25	Cocksfoot (*Dactylis glomerata*) cv. 'Amba'	15.4	5.00
	Timothy (*Phleum pratense*) cv. 'Winnetou'	4.6	1.50	Hybrid ryegrass (*Lolium perenne*) cv. 'Tetragraze'	11.5	3.75
	Tall fescue (*Festuca arundinacea*) cv. 'Kora'	3.8	1.25			
	Meadow fescue (*Festuca pratensis*) cv. 'Pardus'	3.1	1.00			
Legume	Sainfoin (*Onobrychis*)	19.2	6.25	Red clover (*Trifolium pratense*) cv. 'AberClaret'	3.9	1.25
	Sweet clover (*Melilotus*)	6.2	2.00	White clover (*Trifolium repens*) cv. 'AberDai'	3.1	1.00
	Red clover (*Trifolium pratense*) cv. 'Milvus'	5.4	1.75	White clover (*Trifolium repens*) cv. 'AberHerald'	2.3	0.75
	White clover (*Trifolium repens*) cv. 'AberHerald'	3.8	1.25	Wild white clover (*Trifolium repens*) cv. 'AberAce'	0.8	0.25
	Lucerne (*Medicago sativa*) cv. 'Luzelle'	2.3	0.75			
	Alsike clover (*Trifolium hybridum*) cv. 'Aurora'	1.5	0.50			
	Birdsfoot trefoil (*Lotus corniculatus*) cv. 'Bull'	1.5	0.50			
Herb	Burnet (*Sanguisorba minor*)	5.4	1.75			
	Chicory (*Cichorium intybus*) cv. 'Puna II'	4.6	1.50			
	Ribwort Plantain (*Plantago lanceolata*) cv. 'Endurance'	1.5	0.50			
	Sheep's Parsley (*Petroselenium crispum*)	1.5	0.50			
	Yarrow (*Achillea millefolium*)	0.8	0.25			

4.2.2. Experimental Design

Within the exclusion area on each paddock, treatment sub-plots (1.26 m^2) were established to allow for soil sampling (0-10 cm) and greenhouse gas measurements. For each sampling season, one week prior to treatment application the sward within each plot was cut to ca. 7 cm and the biomass removed.

Autumn-winter 2020

In autumn 2020, treatments included i) urine, ii) dung, and iii) control. Urine and dung collected from lambs grazing each sward type was reapplied to its respective sward. The average volume or weight of urine and dung collected from each sward was reapplied in their respective plot. One urine or dung patch was applied within each greenhouse gas chamber with six additional urine patches and four additional dung patches outside the chamber to allow for soil sampling (Supplementary Figure 4.4). Background soil and gas measurements were taken from each plot 5 days before treatment application. After treatment application (16/10/20), soil and gas samples were taken on day 0, 2, 3, 5, 7, 10, 12, 14, 17, 20, 24, 27, 31, 48, and 103.

Spring-summer 2021

In spring 2021, the exclusion plots were moved to a different area of the paddock to avoid repeated applications on the same area and to match the bulk density of the rest of the field after a season of grazing. A new treatment of ammonium nitrate (50 kg N ha^{-1}) was added to stimulate soil N and C cycling and subsequent greenhouse gas emissions when a low N fertiliser rate is applied in the spring to encourage grass and herb growth. Thus, the treatments applied in spring were i) urine, ii) dung, iii) control, and iv) 50 kg N ha^{-1} ammonium nitrate. To maximise the potential for greenhouse gas emissions, within each urine or dung gas chamber two patches were applied instead of one. Background soil and gas measurements were taken from each plot 4 days before treatment application. After application (10/05/21), soil and gas samples were taken on day 0, 2, 4, 7, 9, 11, 14, 16, 18, 21, 24, 30, 38, 51, and 78.

4.2.3. Lamb Excreta Collection and Analysis

Welsh mountain lambs not suitable for breeding or market when gathered from the uplands were selected to improve on the lowland swards described in this study. Sex of the weaned lambs used in the study varied with each season to represent a typical lowland Welsh finishing system, with male ram lambs approximately 6-7 months old (average liveweight 22.1 ± 0.4 kg) grazing in autumn-winter (late September to early December) and female ewe lambs approximately 10-11 months old (average liveweight 23.0 ± 0.3 kg) grazing from spring-summer (late April to early July). The field was rested when lambs were not present.

Lambs (n = 40 per sward) were introduced to their respective swards at the start of the 6-week or 11-week grazing period in autumn 2020 and spring 2021, respectively, and allowed to graze *ad libitum* with free access to water. No additional supplementation was provided. After a week of acclimatisation, urine and dung was collected over two weeks from lambs (n = 6) from each sward using the excreta collection pen method described in detail in Marsden et al. (2020) approved by the Bangor University ethics committee (approval code COESE2019EC01A).

Briefly, the excreta collection pens were constructed from 7-rail galvanised steel interlocking sheep hurdles (LM Bateman, Staffordshire, UK) fitted with a wire mesh ‘roof’ to create individual stalls for twelve lambs (n = 6 per sward). Each stall was fitted with slatted livestock flooring (Rimco Ltd., Yorkshire, UK) raised ca. 10 cm above the ground to enable a plastic tray covered with a muslin mesh screen to be placed beneath each animal. Urine events could then freely collect in the plastic tray below without contamination from faeces or other debris (e.g., wool or refused feed), with each urination event volume corrected for the liquid absorbed to the muslin mesh screen or the excreta collection apparatus (see Marsden et al. 2020 for correction factor). The urine collection trays were cleaned with DI H_2O and dried after each urine event. Each dung event was collected from the mesh screen and the slatted floor. The excreta collection pens were cleaned regularly to prevent faecal contamination of collected urine. If any faecal contamination of urine in the collection trays was observed, then urine was discarded, and the collection trays and mesh screens cleaned.

Lambs were provided with freshly cut feed from their respective swards and had access to water throughout the excreta collection period, with the collection period lasting approximately 6 hours a day (occurring between 10:00-16:00). Lambs were then returned to the field to graze overnight before collection the following morning. As it was not possible to conduct a 24-hour excreta collection period to capture variations in excretion frequency and composition due to ethical and health and safety limitations, the chemical composition of collected urine and dung is expressed on an individual event or per animal (bulked) basis only. An estimation of daily urination frequency, volume, and N content per sheep, assuming that the 6-hour collection period is representation of a 24-hour period, is calculated for the spring 2021 urine dataset only.

Individual urine events were immediately collected in sterile polypropylene bottles and measured to determine volume then filtered through Whatman No. 1 filter papers (11 μm pore size) to remove any debris. Each event was then subsampled for analysis (referred to an individual urine events) before homogenising and bulking into a sterile glass bottle per individual animal (referred to as the bulked urine). Once filtered, urine subsamples were immediately chilled before freezing at -20°C prior to analysis. pH and electrical conductivity (EC) were determined for each urine event using standard electrodes upon sample collection prior to freezing. Total nitrogen and carbon content was determined for each urine event using a Multi N/C 2100S analyser (AnalytikJena, Jena, Germany). Ammonium (NH_4^+) and nitrate (NO_3^-) and content was determined colorimetrically for each urine event as described in Mulvaney (1996) and Miranda et al. (2001), respectively. Urine urea was determined colorimetrically on bulk urine samples per animal each season as described in Orsonneau et al. (1992). An Agilent 5800 ICP-OES (Agilent, USA) was used to determine cations calcium (Ca), sodium (Na), phosphorus (P), and potassium (K) in bulk urine samples for both seasons (see supplementary for more details). Urine non-urea nitrogenous compounds, e.g., allantoin, hippuric acid, creatinine, uric acid, and benzoic acid, were also determined in the bulk urine samples for each season using high-performance liquid chromatography using a HyperClone™ 5 µm ODS (C18) 120 Å, (250 x 4.6 mm) column (Phenomenex Inc., Cheshire, UK) (Marsden et al., 2020). Urine patch area was determined by applying the sward-specific average urination event equivalent volume of ‘Brilliant Blue Dye’ for each season to its respective sward and measuring the visible wetted area.

Individual dung event samples were immediately collected upon deposition and weighed before bulking into bags per individual animal prior to analysis. The bulked dung was chilled prior to field application and subsamples extracted in 0.5 M K_2SO_4 (1:5 w/v dung:solution) for NO_3^-, NH_4^+, and total extractable organic carbon and total extractable nitrogen analysis as described previously. Dung was also extracted in 1 M CH_3COOH (acetic acid) for phosphate and exchangeable cation analysis and DI H_2O (1:2.5 w/v) for dung pH and EC. To determine moisture content, bulked dung samples per individual animal was dried at 105 °C for 24 h. ICP-OES was used to determine Ca, Na, P and K using methods described previously.

4.2.4. Soil characteristics

Background soil samples (0-10 cm depth, *n* = 3 per sward) were taken to determine soil characteristics (Table 4.2). 10 soil samples per paddock were sampled and homogenised to produce one replicate to account for spatial variability across the field.

Table 4.2. *Soil characteristics (0-10 cm depth) of the grass-clover and herbal ley used in the field experiment. Results are expressed on a dry weight basis and as mean ± SEM, with n = 3 per treatment. * = bulk density was sampled 0-5 cm.*

Soil characteristics	Grass-Clover	Herbal
pH	6.99 ± 0.06	6.90 ± 0.08
Electrical conductivity (µS cm^{-1})	38 ± 5	51 ± 14
Moisture content (g g^{-1})	0.31 ± 0.01	0.33 ± 0.02
Organic matter (g kg^{-1})	56.4 ± 3.7	54.5 ± 3.1
Bulk density (g cm^{-3}) *	0.94 ± 0.04	0.97 ± 0.06
Microbial biomass carbon (g C kg^{-1})	1.67 ± 0.02	2.02 ± 0.44
Microbial biomass nitrogen (mg N kg^{-1})	124.0 ± 17.7	115.4 ± 14.5
N mineralisation rate (mg N kg^{-1} day^{-1})	5.87 ± 2.45	6.68 ± 0.93
Total extractable nitrogen (mg N kg^{-1})	29.75 ± 9.68	20.35 ± 1.69
Total dissolved carbon (mg C kg^{-1})	74.26 ± 2.52	71.66 ± 3.18
Extractable ammonium (mg NH_4^+-N kg^{-1})	13.97 ± 11.85	5.58 ± 0.77
Extractable nitrate (mg NO_3-N kg^{-1})	0.91 ± 0.27	0.94 ± 0.04
Extractable phosphorus (mg P kg^{-1})	11.17 ± 2.96	13.35 ± 4.96
Exchangeable sodium (mg Na kg^{-1})	0.55 ± 0.01	0.65 ± 0.12
Exchangeable calcium (mg Ca kg^{-1})	105.1 ± 18.9	85.2 ± 4.2
Exchangeable potassium (mg K kg^{-1})	2.74 ± 1.40	1.83 ± 0.39

Soil pH and EC was determined by methods described previously. Gravimetric soil moisture content was determined by drying soil at 105 °C for 24 h. Following drying, soil organic matter was determined by loss-on-ignition (450 °C; 16 h; Ball, 1964). Soil bulk density was determined by inserting stainless steel rings (100 cm^3) into the soil at a depth of 0-5 cm and removing intact cores. Cores were then oven dried at 105 °C and sieved to < 2 mm to remove stones and large roots, then corrected for stone weight and volume to determine bulk density. Soil NO_3^-, NH_4^+, total extractable nitrogen and total extractable organic carbon was determined from 0.5 M K_2SO_4 extracts (1:5 w/v soil:solution) as described previously. Available P was determined colorimetrically from soil extracted in 1 M acetic acid (1:5 w/v) using the method described in Murphy and Riley (1962). Exchangeable cations (Ca, Na, and K) were analysed using ICP-OES as described previously. Soil microbial biomass C and N was determined by the chloroform fumigation-extraction method described in Voroney et al. (2008), with a k_{EC} and k_{EN} correction factor of 0.45 and 0.54 applied, respectively. Soil nitrogen mineralisation rate was determined through measuring soil NH_4^+ concentration before and after an anaerobic incubation at 40°C for 7 days then extracting soils in 1 M KCl (1:1 w/v) (Keeney, 1982).

4.2.5. Nitrous Oxide Emissions

N_2O measurements were taken alongside soil samples using the manual sampling static gas chamber technique. Static chambers were inserted into the soil of each plot at a depth of 5 cm a minimum of two weeks prior to treatment application. Chambers had a basal area of 0.16 m^2 with a headspace volume of 0.03 m^3. Soil samples and background gas samples were collected 5 and 4 days prior to treatment application in autumn and spring, respectively, to determine background GHG emissions. Gas and soil samples were taken between 0900 hrs and 1100 hrs. Gas samples were taken from each gas chamber at lid closure (T_0) and after 1 hour (T_{60}) to determine the increase in headspace N_2O concentration and stored in pre-evacuated 20 mL glass vials prior to analysis. Samples were analysed using a Perkin Elmer 580 Gas

Chromatograph (GC), fitted with a Turbo Matrix 110 autosampler (Perkin Elmer Inc., Beverly, CT, USA). Samples were passed through two Elite-Q mega bore columns via a split injector, with the ECD column determining N_2O content, and FID determining CO_2 and CH_4. Due to an instrumental error with the FID, CO_2 and CH_4 data are not reported in this study. Cumulative N_2O required for the emission factor calculations was determined for each treatment by trapezoidal integration. Emission factors for urine and 50 kg N ha^{-1} fertiliser application are expressed as a % of N applied emitted as N_2O-N. A seasonal dung emission factor was not determined in this study as it was not possible to measure lamb dung N loading rate.

4.2.6. Bench-scale Ammonia Volatilisation Measurements

NH_3 volatilisation from urine addition to each sward type was determined using the bench scale measurement method described in Misselbrook et al. (2005) (Supplementary Figure 4.5). Intact vegetated soil cores (0-12 cm depth, 9 cm diameter, 763.41 cm^3) were collected from each sward type ($n = 6$) two weeks prior to urine application and acclimatised indoors at 20°C under natural light conditions. Cores were maintained at field moisture ca. 25 % water filled pore space (WFPS) throughout the experiment to replicate field conditions. Three days prior to application, cores were placed in individual chambers with a headspace of 495 cm^3 to monitor background NH_3 emissions. Air was continuously circulated through the system using a vacuum pump at 3 litres min^{-1} with the intake and outtake air passed through a 200 ml acid trap of 0.0125 M orthophosphoric acid (H_3PO_4) to capture volatilised NH_3. This flow rate constantly replaced the headspace and reduced NH_3 build-up. NH_3 recovery tests were conducted prior to starting the experiment as described in Misselbrook et al. (2005), with NH_3 recovery per chamber ranging between 96 – 113 %.

Sward-specific urine was applied evenly to the core surface area (63.6 cm^2, $n = 3$ per sward type) based on the average urination volume and subsequent urine patch area (ml cm^{-2})

of ram lambs grazing each sward in autumn 2020 (0.5 ml cm^{-2} herbal, 0.9 ml cm^{-2} grass-clover). After urine application, acid traps were sampled and replaced on day 1, 2, 3, 4, 5, 8 and 12. NH_3-N in the traps was determined using the colorimetric NH_4^+-N analysis method described previously. Control cores (*n* = 3 per sward) did not receive urine; thus no detectable NH_3-N was found during the sampling period. NH_3 was not detected in the pre-measurement period (data not shown).

NH_3 emissions are presented as a percentage of total urine-N emitted as NH_3-N to account for differences in sward-specific urine composition and urine N loading rate (Figure 4.6, panel A), on an area basis (as mg N m^{-2} h^{-1}; Figure 4.6, panel B) calculated using the equation provided in Misselbrook et al. (2005), and as a percentage of urine urea-N emitted as NH_3-N (Figure 4.6, panel C).

4.2.7. Statistical Analysis

Each season was subject to independent statistical analyses; comparisons between seasons were not made due to variability arising from seasonal differences and sex differences of the lambs used. Data were analysed in R studio (version 4.2.1) with graphical images produced using the ‘ggplot2’ package (version 3.3.6, Wickham (2016)). Prior to analysis, data were tested for normality using a Shapiro-Wilks test (R core package) and homogeneity of variance using a Levene’s Test (‘car’ package). Data that passed were then analysed using an independent samples t-test (background soil characteristics), Welch t-test (lamb excreta composition, daily urination frequency, volume and N content), or a two-way ANOVA (sward composition, cumulative N_2O emissions and emission factors). For the two-way ANOVA, sward composition, cumulative N_2O emissions and emission factors and season were used as the fixed factors, with their interaction assessed. A linear mixed effects model was used to assess the ammonia volatilisation dataset, using the ‘lme4’ package in R (version 1.1-30; Bates et al. (2015)). Sward and day were used as the fixed factors, with their interaction explored (sward × day) using chamber as the random factor.

If a statistically significant interaction was found following the two-way ANOVA or the linear mixed effects model, then a pairwise post-hoc comparison test was conducted to identify the significant pairs. If assumptions were not met following $\log_{10}$-transformation, then data were analysed using a non-parametric (e.g., Kruskal-Wallis) test where appropriate. The significance level was set at $p < 0.05$. Values presented in the text represent mean ± SEM unless otherwise stated.

4.3. Results

4.3.1. Meteorological Conditions

Average air temperature and precipitation were similar between the two experimental seasons (Figure 4.1). In the first three weeks following treatment application (urine and dung addition to soil) in autumn (16/10/20), average air temperature and daily precipitation were 11.6 °C and 1.5 mm d^{-1} in week 1, 10.9 °C and 12.4 mm d^{-1} in week 2, and 10.1 °C and 8.2 mm d^{-1} in week 3, respectively. Similarly, in spring, after treatment application (10/05/21) air temperature was also relatively cool with low average daily precipitation in week 1 (10.4 °C, 2.75 mm d^{1}), week 2 (10.6 °C, 10 mm d^{-1}), and week 3 (12.6 °C, 0.95 mm d^{-1}).

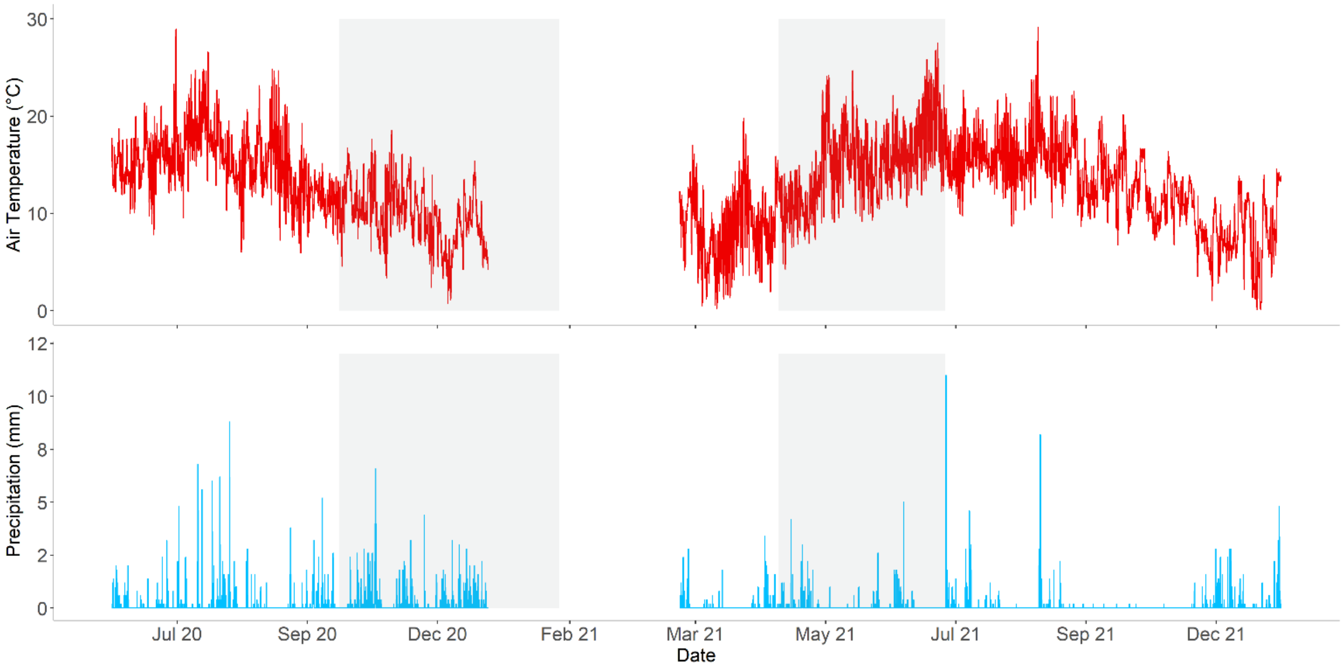


Figure 4.1. *Daily air temperature (°C) and precipitation (mm) obtained 200 m from the field experiment at Henfaes Research Centre, Abergwyngregyn, (UK) between July 2020 and December 2021. Data are not reported between 24/12/20 and 24/03/21 due to a malfunction with the metrological apparatus. Grey shaded regions indicate autumn (16/10/20 – 27/01/21) and spring (10/05/2021 – 27/07/21) experimental sampling periods.*

4.3.2. Sward Composition

General sward composition (dry matter, crude protein, sugar, metabolisable energy and D-value) did not significantly differ between sward types within each season ($p > 0.05$) (Table 4.3 and S4.1). Seasonal differences, however, did significantly ($p < 0.05$) drive differences in sward composition where dry matter content, sugar, metabolisable energy, and D-value were highest in spring than autumn (Table S4.2). Crude protein content was greatest in autumn in both swards. Sodium concentrations were consistently higher in the herbal ley in both seasons ($F_{(1,8)} = 26.730$, $p < 0.001$), with a 229 % and 125 % greater sodium content in autumn and spring, respectively, affecting subsequent lamb urine sodium content.

***Table 4.3**. General sward composition measured from a herbal or a grass-clover ley in autumn 2020 (06/10/20) or spring 2021 (18/05/21). Data represents mean ± SEM, n = 3 per sward type, expressed on a dry-weight basis. Lowercase and uppercase letters indicate statistical differences within and between seasons respectively following a two-way ANOVA, significance level is determined as p < 0.05.*

Sward Properties	Autumn 2020		Spring 2021		Interaction between Sward and Season	
	Grass-Clover	Herbal	Grass-Clover	Herbal	F	*p*-value
Dry matter (g kg^{-1})	131 ± 4^{aA}	133± 10^{aC}	259 ± 13^{aB}	293 ± 6^{aD}	1.534	0.251
Crude protein (g kg^{-1})	252 ± 3^{aA}	207 ± 33^{aC}	126 ± 15^{aB}	172 ± 24^{aC}	4.465	0.068
Sugar (g kg^{-1})	110 ± 8^{aA}	138 ± 38^{aC}	214 ± 11^{aB}	236 ± 11^{aC}	1.436	0.265
Metabolisable energy (MJ kg^{-1})	12.0 ± 0.1^{aA}	11.5 ± 0.2^{aA}	12.0 ± 0.1^{aA}	12.0 ± 0.1^{aA}	3.658	0.092
D-value (%)	76.5 ± 0.5^{aA}	73.0 ± 1.6^{aA}	76.5 ± 0.8^{aA}	76.8 ± 0.6^{aA}	3.780	0.088
Sodium (g kg^{-1})	2.1 ± 0.4^{aA}	6.9 ± 0.8^{bC}	0.4 ± 0.1^{aB}	0.9 ± 0.1^{bD}	26.730	< 0.001*

4.3.3. Lamb Excreta Composition

4.3.3.1. Urine characteristics

Urine characteristics of both bulked and individual urine events are given in Table 4.4. There was no significant effect ($p > 0.05$, Table S4.2) of sward type on general urine characteristics (electrical conductivity, N content and total organic C), cations (potassium and calcium), and non-urea nitrogen compounds (hippuric acid, allantoin, creatinine, benzoic acid and uric acid) in either season. Urine pH, nitrate, ammonium, and phosphorus was only influenced by sward type in autumn, with a higher pH ($T_{(173)} = 6.912$, $p < 0.001$), levels of nitrate ($K_{(1)} = 4.195$, $p = 0.041$), ammonium ($K_{(1)} = 7.186$, $p = 0.007$), and phosphorus ($T_{(10.4)} = 2.730$, $p = 0.021$) in urine produced from the grass-clover ley than the herbal ley.

Table 4.4. *Urine properties of individual urine events and bulked urine collected from Welsh mountain lambs in autumn 2020 (males) and spring 2021 (females) grazing either a grass-clover ley or a herbal ley. Data represents mean ± SEM, different superscripted letters within seasons indicate statistical difference (p < 0.05).*

Individual Urine Events				
Urine properties	Autumn 2020		Spring 2021	
	Grass-Clover (*n* = 80)	Herbal (*n* = 97)	Grass-Clover (*n* = 63)	Herbal (*n* = 71)
pH	8.93 ± 0.02^{a}	8.72 ± 0.02^{b}	8.07 ± 0.09^{a}	8.12 ± 0.08^{a}
Electrical conductivity (mS cm^{-1})	17.8 ± 0.6^{a}	16.3 ± 0.5^{a}	15.0 ± 1.1^{a}	14.7 ± 1.1^{a}
Urination event volume (ml)	181± 12^{a}	237 ± 11^{b}	140 ± 9^{a}	115 ± 8^{b}
Urine patch area (cm^{2})	185 ± 16^{a}	501 ± 10^{b}	326 ± 32^{a}	227 ± 30^{a}
Total N (g N l^{-1})	5.3 ± 0.3^{a}	4.8 ± 0.2^{a}	6.4 ± 0.6^{a}	6.6 ± 0.6^{a}
Total organic C (g C l^{-1})	7.6 ± 0.4^{a}	7.5 ± 0.3^{a}	11.7 ± 1.2^{a}	12.0 ± 1.2^{a}
N loading rate (kg N ha^{-1})	555 ± 33^{a}	1020 ± 46^{b}	1484 ± 131^{a}	1305 ± 127^{a}

Nitrate (mg NO_3^--N l^{-1})	0.3 ± 0.03^a	0.2 ± 0.02^b	1.3 ± 0.3^a	1.2 ± 0.3^a
Ammonium (mg NH_4^+-N l^{-1})	69 ± 3^a	59 ± 2^b	192 ± 15^a	241 ± 26^a
Bulked Lamb Urine				
Urine Properties	Autumn 2020		Spring 2021	
	Grass-Clover (*n* = 6)	Herbal (*n* = 7)	Grass-Clover (*n* = 6)	Herbal (*n* = 6)
Urea (g l^{-1})	9.2 ± 0.5^a	10.3 ± 0.6^a	9.9 ± 0.9^a	10.2 ± 2.3^a
Sodium (mg Na l^{-1})	71 ± 26^a	439 ± 62^b	47 ± 18^a	389 ± 150^b
Potassium (g K l^{-1})	2.1 ± 0.1^a	1.9 ± 0.1^a	2.2 ± 0.2^a	2.2 ± 0.1^a
Calcium (mg Ca l^{-1})	3.7 ± 0.9^a	5.0 ± 1.8^a	2.3 ± 0.5^a	4.8 ± 2.0^a
Phosphorus (mg P l^{-1})	2.4 ± 0.6^a	1.1 ± 0.1^b	1.9 ± 0.4^a	2.5 ± 0.6^a
Hippuric acid (mg l^{-1})	329 ± 73^a	265 ± 73^a	88 ± 42^a	29 ± 13^a
Allantoin (mg l^{-1})	292 ± 43^a	343 ± 31^a	646 ± 199^a	749 ± 197^a
Creatinine (mg l^{-l})	164 ± 22^a	146 ± 10^a	178 ± 51^a	229 ± 79^a
Benzoic acid (mg l^{-1})	77 ± 11^a	69 ± 12^a	193 ± 18^a	213 ± 20^a
Uric acid (mg l^{-1})	344 ± 32^a	374 ± 67^a	937 ± 91^a	1147 ± 197^a

Sward type had a variable effect on urination volume. Male lambs grazing the herbal ley in autumn had a significantly higher average urine volume (237 ± 11 ml) than those grazing the grass-clover ley (181 ± 12 ml) ($T_{(156)} = -3.718$, $p < 0.001$), with individual event volume ranging between 38 ml to 565 ml for the herbal ley (*n* = 97) and 42 ml to 540 ml for the grass-clover ley (*n* = 80). Whereas in spring, female lambs grazing the herbal ley had a significantly lower average urine volume (115 ± 8 ml) than their grass-clover counterparts (140 ± 9 ml) ($T_{(132)} = 2.328$, $p = 0.021$), with individual event volumes ranging from 32 ml to 321 ml for the herbal ley (*n* = 71) and 36 ml to 429 ml for the grass-clover ley (*n* = 63).

Despite no difference in urine-N concentration in autumn (4.8 ± 0.2 g N L^{-1} herbal, 5.3 ± 0.3 g N L^{-1} grass-clover, $p > 0.05$) and spring (6.6 ± 0.6 g N L^{-1} herbal, 6.4 ± 0.6 g N L^{-1} grass-clover, $p > 0.05$), seasonal variations in N loading rate were largely driven by inconsistent effects on urine volume. In autumn, urine N loading rate was 84 % greater in the herbal ley than the grass-clover ley (1020 ± 46 kg N ha^{-1} *vs*. 555 ± 33 kg N ha^{-1}; $T_{(136)} = -8.433$, $p < 0.001$) due to the 77 % increase in average urination volume. However, despite the slightly lower urine volume in herbal grazed lambs in spring, no significant reduction in urine N loading rate was observed ($T_{(125)} = 1.594$, $p = 0.113$) (Table 4.4).

When urine collected from ewe lambs in spring 2021 is expressed as a daily rate, assuming the 6-hour collection period is representative of a 24-hour period, urination frequency (6.9 ± 0.9 *vs*. 7.7 ± 1.1 urine events $sheep^{-1}$ day^{-1}; $T_{(9.702)} = -0.569$, $p = 0.582$), volume (0.77 ± 0.10 *vs*. 1.15 ± 0.10 L $sheep^{-1}$ day^{-1}; $T_{(6.631)} = -1.158$, $p = 0.287$), and N content (5.1 ± 0.6 *vs*. 6.9 ± 1.2 g N $sheep^{-1}$ day^{-1}; $T_{(7.169)} = -1.151$, $p = 0.287$) excreted did not significantly differ between the herbal and grass-clover ley, respectively. As time and date was not recorded during the autumn 2020 excreta collection, similar results cannot be calculated.

Due to the higher sward sodium content, urine sodium excretion was significantly greater in herbal ley grazed lambs in both seasons (Table 4.4). Urine obtained from herbal grazed lambs had a 521% greater sodium concentration in autumn ($T_{(7.93)} = -5.458$, $p < 0.001$) and a 727% increase in spring ($T_{(9.99)} = -4.068$, $p = 0.002$). To account for variations in urination volume arising from sward and seasonal differences, urine sodium content can be expressed as a loading rate (Figure 4.2). As a result, male lambs grazing the herbal ley in autumn had a 1229 % higher sodium loading rate (9.3 ± 1.3 g Na m^{-2}) than lambs grazing the grass-clover ley (0.7 ± 0.3 g Na m^{-2}) ($T_{(6.29)} = -6.386$, $p < 0.001$). This was also found in spring, where female lambs grazing the herbal ley had a 601 % higher sodium loading rate (7.7 ± 3.0 g Na m^{-2}) than grass-clover ley grazed lambs (1.1 ± 0.4 g Na m^{-2}) ($T_{(9.99)} = -3.746$, $p = 0.004$).

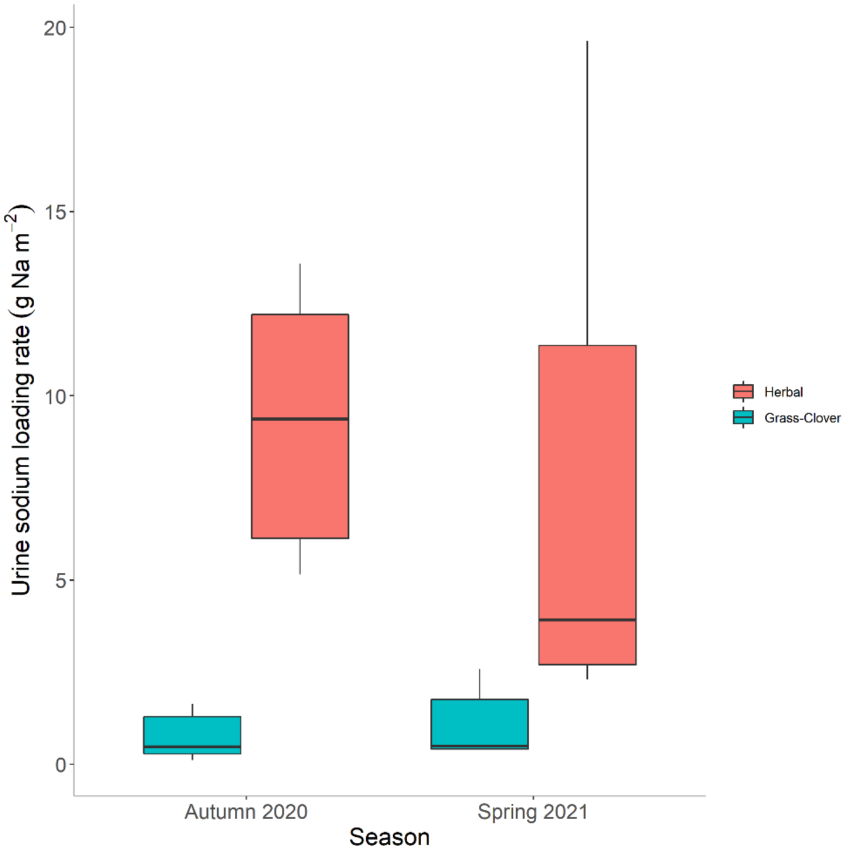


Figure 4.2. *Urine sodium loading rate excreted by lambs grazing either a conventional grass-clover ley or an herbal ley in autumn 2020 (male lambs, n = 6 per ley) and spring 2021 (female lambs, n = 6 per ley). Data are expressed on a grams per metre squared basis to represent the potential sodium loading in a urine-patch. Boxplot displays median and interquartile range, with whiskers showing minimum and maximum values in the data.*

4.3.3.2. Dung characteristics

Sward type did not have any significant effect on general dung characteristics or N content collected from Welsh mountain lambs over both seasons ($p > 0.05$) (Table 4.5 and S4.3). However, individual dung event weight was heavier in herbal ley grazed lambs in autumn ($K_{(1)} = 5.73$, $p = 0.017$) but not in spring, where grass-clover lambs had greater individual dung weight ($T_{(102)} = 2.370$, $p = 0.020$).

Table 4.5. *Bulked lamb dung characteristics collected in autumn 2020 (males) and spring 2021 (females). Data represents mean ± SEM. Chemical characteristics are reported on a dry weight basis. Different superscripted letters within season indicate statistical differences. n.d. = not determined. * average dung event weight is determined from individual events deposited in the collection pens in autumn (herbal n = 53, grass-clover n = 33) and spring (herbal n = 57, grass-clover n = 48).*

Bulked Dung Characteristics				
Dung Properties	Autumn 2020		Spring 2021	
	Grass-Clover (n = 6)	Herbal (n = 7)	Grass-Clover (n = 7)	Herbal (n = 6)
pH	7.33 ± 0.09^a	7.42 ± 0.08^a	7.18 ± 0.11^a	7.06 ± 0.08^a
Electrical conductivity (mS cm^{-1})	3.22 ± 0.26^a	2.84 ± 0.07^a	1.96 ± 0.12^a	2.01 ± 0.06^a
Moisture content (g g^{-1})	4.35 ± 0.76^a	3.60 ± 0.76^a	0.78 ± 0.01^a	0.81 ± 0.04^a
Dunging event weight (g)*	33.2 ± 4.4^a	48.6 ± 4.7^b	42.5 ± 3.1^a	34.3 ± 2.4^b
Total N (g N kg^{-1})	4.6 ± 0.5^a	5.8 ± 0.5^a	4.8 ± 0.9^a	13.0 ± 7.6^a
Total dissolved C (g C kg^{-1})	28.8 ± 4.5^a	39.1 ± 3.3^a	37.8 ± 8.4^a	82.4 ± 37.1^a
Extractable nitrate (mg NO_3^--N kg^{-1})	155 ± 16^a	173 ± 12^a	306 ± 77^a	297 ± 101^a
Extractable ammonium (mg NH_4^+-N kg^{-1})	95 ± 15^a	111 ± 20^a	811 ± 146^a	988 ± 162^a
Extractable phosphorus (mg P kg^{-1})	n.d.	n.d.	0.40 ± 0.06^a	0.36 ± 0.07^a
Exchangeable sodium (g Na kg^{-1})	n.d.	n.d.	0.02 ± 0.01^a	0.04 ± 0.01^a
Exchangeable calcium (g Ca kg^{-1})	n.d.	n.d.	0.65 ± 0.08^a	0.75 ± 0.05^a
Exchangeable potassium (g K kg^{-1})	n.d.	n.d.	0.31 ± 0.07^a	0.22 ± 0.03^a

4.3.4. Soil Characteristics and Nitrous Oxide Emissions

4.3.4.1. Soil pH, Electrical Conductivity and Water-filled Pore Space

Background soil physical and chemical properties measured before the first treatment application in autumn showed no significant difference between either ley ($p > 0.05$) (Table 4.2). There was no distinguishable change in soil pH in any treatment throughout each season (Supplementary Figure 4.6, panel A).

Soil electrical conductivity is displayed in Supplementary Figure 4.6, panel B. In both seasons, the urine treatment had a greater soil electrical conductivity in the herbal ley, peaking 3-days after application in autumn (188 ± 28 µS cm^{-1} herbal *vs*. 153 ± 18 µS cm^{-1} grass-clover) and on the day of application in spring (91 ± 13 µS cm^{-1} herbal *vs*. 82 ± 13 µS cm^{-1} grass-clover). Soils receiving the dung application had a similar electrical conductivity to the urine treatment, peaking 7-days post-application in both swards in autumn (176 ± 30 µS cm^{-1} herbal *vs*. 158 ± 38 µS cm^{-1} grass-clover), whereas in spring the grass-clover peaked on the day of application (150 ± 95 µS cm^{-1}). However, the peak in the herbal ley receiving dung was smaller (83 ± 38 µS cm^{-1}) and delayed until day 11. Soil electrical conductivity in the control and 50 kg N ha^{-1} ammonium nitrate fertiliser treatment in both sward types was much smaller than the previous treatments, with peaks ranging between 69 and 99 µS cm^{-1}.

Soil WFPS in the 0-10 cm soil layer was relatively similar in both sward types throughout each experimental season, with a higher average WFPS of 52.0 ± 0.3 % during the autumn measurement period, and a slightly lower average WFPS in spring of 44.9 ± 0.4 % (Supplementary Figure 4.7). There was no difference in average WFPS in the first three weeks following treatment application in both seasons, with the WFPS remaining at 51.3 ± 0.4 % and 50.8 ± 0.3 % in autumn and spring, respectively.

4.3.4.2. Soil Mineral N Dynamics

Soil ammonium concentrations peaked on the day of application following the addition of sward-specific urine, with differences primarily driven by the seasonal variations in urine N loading rate (Figure 4.3, panel A). In autumn, the soil ammonium peak was 33 % greater in the herbal ley (130 ± 23 mg NH_4^+-N kg^{-1} DW) compared to the grass-clover ley (97 ± 19 mg NH_4^+-N kg^{-1} DW), with the reverse found in spring where the peak was 22 % lower in the herbal ley (82 ± 19 mg NH_4^+-N kg^{-1} DW) than the grass-clover (100 ± 20 mg NH_4^+-N kg^{-1} DW). Peaks in soil ammonium following urine addition were relatively short-lived, returning to control levels in both swards by day 14. Except for a brief peak in soil ammonium 4-days post-application in the 50 kg N ha^{-1} ammonium nitrate treatment in spring (44 mg NH_4^+-N kg^{-1} DW) in the herbal ley, the addition of fertiliser or sward-specific dung did not induce a noticeable soil response throughout each season, with concentrations of soil ammonium following these treatments, remaining within the range and SEM of the control (6 – 33 mg NH_4^+-N kg^{-1} DW).

Peaks in soil nitrate following urine addition appeared between 5-21 days post-application, with appearance of the peak affected by sward type and season (Figure 4.3, panel B). Despite a similar magnitude of peaks between sward types in autumn, the grass-clover peaked on day 5 (17 ± 3 mg NO_3^--N kg^{-1} DW) whereas in the herbal ley this was delayed until day 12 (19 ± 3 mg NO_3^--N kg^{-1} DW). In spring, soil nitrate was noticeably higher in the herbal ley, peaking 21-days following urine application (13 ± 10 mg NO_3^--N kg^{-1} DW) whereas in the grass-clover the peak appeared on day 14 (6 ± 1 mg NO_3^--N kg^{-1} DW), however, this was much smaller and within the range of the control. For much of the measurement period, soil nitrate concentrations following fertiliser or sward-specific dung application often did not exceed the range for the control in autumn (5 – 7 mg NO_3^--N kg^{-1} DW) and spring (8 – 22 mg NO_3^--N kg^{-1} DW). However, in spring, a sharp peak in nitrate was observed in the herbal ley for the fertiliser treatment on day 4 (33 ± 13 mg NO_3^--N kg^{-1} DW) and in the grass-clover ley for the dung treatment on day 21 (32 ± 26 mg NO_3^--N kg^{-1} DW) before returning to control levels.

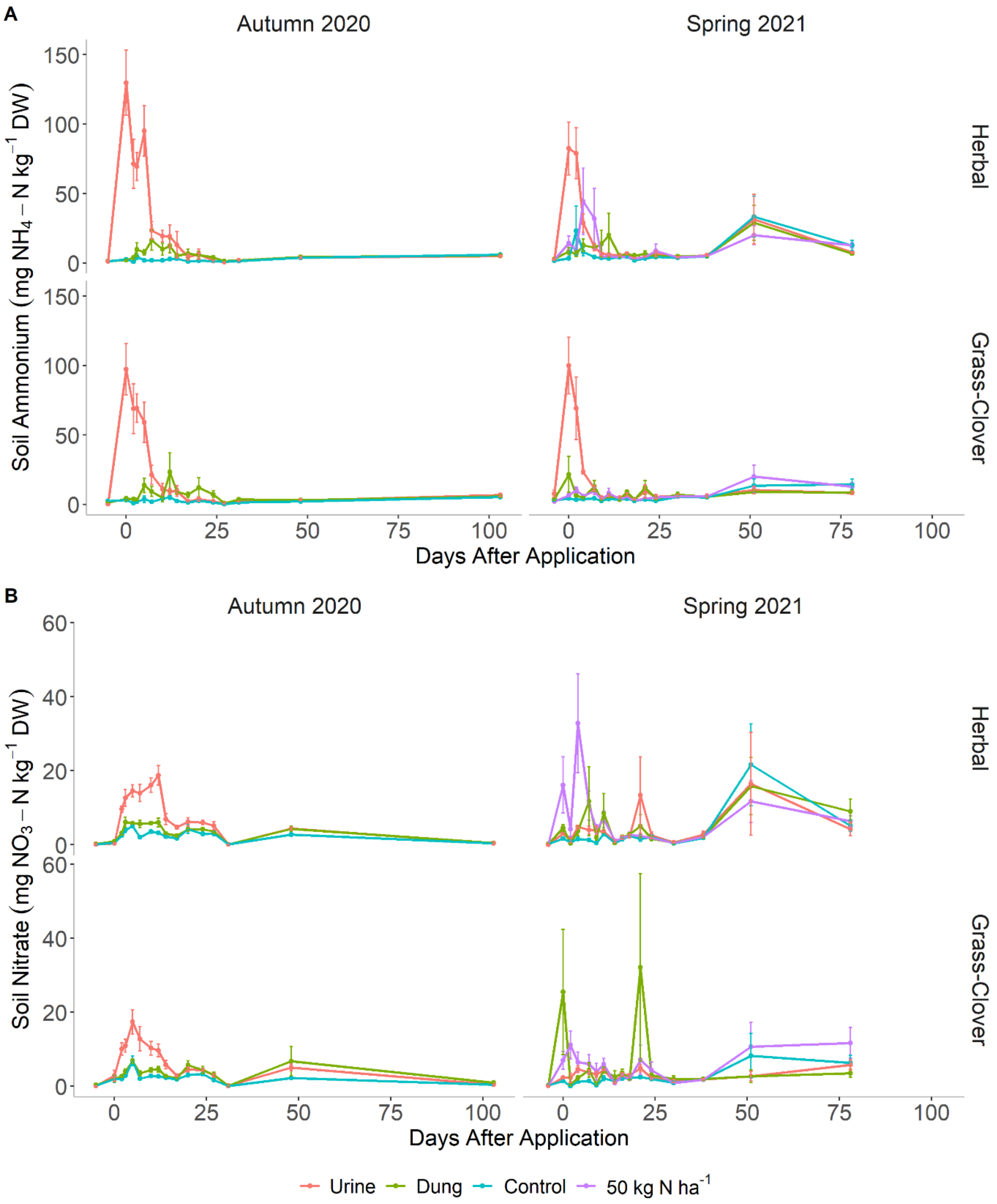


Figure 4.3. *Soil extractable ammonium-N (panel A) and nitrate-N (panel B) measured following application of sward-specific lamb urine, dung, or 50 kg N ha^{-1} ammonium nitrate to either a grass-clover or herbal ley in autumn 2020 (16/10/2020) and spring 2021 (10/05/2021). Data represents mean ± SEM, n = 3 per treatment per ley. Legend applies to both panels.*

4.3.4.3. Soil Total Nitrogen and Total Organic Carbon

Sward-specific urine addition induced a greater response of extractable dissolved soil total nitrogen (TN) in autumn than spring (Figure 4.4, panel A). In autumn, levels of TN were 16% higher in the herbal ley (128 ± 22 mg N kg^{-1} DW) than the grass-clover ley (110 ± 23 mg N kg^{-1} DW) likely driven by the higher urine N loading rate in the herbal ley, with concentrations of TN peaking on the day of application and 3-days post-application, respectively. Levels of TN in the urine treatments remained elevated for approximately three weeks, before returning to control levels (2 – 51 mg N kg^{-1} DW) in both swards by day 27. Following addition of sward-specific dung, TN levels were 64 % higher in the herbal ley and peaked on day 7 (64 ± 35 mg N kg^{-1} DW) whereas the grass-clover ley peaked on day 12 (39 ± 23 mg N kg^{-1} DW). At their peak, levels of dung TN exceeded the control by 56 mg N kg^{-1} DW and 30 mg N kg^{-1} DW in the herbal and grass-clover ley, respectively.

In spring, despite the high urine N loading rate in both swards (1305 ± 127 kg N ha^{-1} herbal *vs*. 1484 ± 131 kg N ha^{-1} grass-clover), there was a minimal response of soil TN following urine addition. TN concentrations were abnormally low, peaking on day 2 in the herbal ley (14 ± 2 mg N kg^{-1} DW) and the day of application in the grass-clover ley (17 ± 4 mg N kg^{-1} DW). These peaks of TN following urine addition only exceeded the control by 12 mg N kg^{-1} DW in the herbal ley and 16 mg N kg^{-1} DW in the grass-clover ley, before quickly returning to control levels (1 – 8 mg N kg^{-1} DW) by day 7. Similar to the urine treatment, the addition of sward-specific dung only induced a minimal soil response, with TN peaking at day 11 in the herbal ley (9 ± 7 mg N kg^{-1} DW) and on the day of application in the grass-clover ley (23 ± 15 mg N kg^{-1} DW). There was no increase in TN concentrations beyond the control following the addition of 50 kg N ha^{-1} ammonium nitrate.

Soil extractable total dissolved organic carbon (TOC) exceeded the control in the sward-specific dung treatment in both autumn and spring (Figure 4.4, panel B). There was no noticeable peak or increase from the control in urine or fertiliser treatments in either season or sward. TOC levels in the control in autumn and spring ranged between 6 – 197 mg C kg^{-1} DW and 56 – 212 mg C kg^{-1} DW, respectively. In autumn, soil TOC levels in the dung treatment were higher in the herbal ley (367 ± 179 mg C kg^{-1} DW) than the grass-clover ley (294 ± 167 mg C kg^{-1} DW), peaking on day 7 and day 12 respectively, before returning to control concentrations 24-days post-application. In spring, soil TOC levels were extremely high in the grass-clover ley, peaking on the day of application at 1189 ± 799 mg C kg^{-1}, whereas at soil TOC only peaked in the herbal ley at 314 ± 185 mg C kg^{-1} on day 11. Peaks in both swards returned to control levels by day 24.

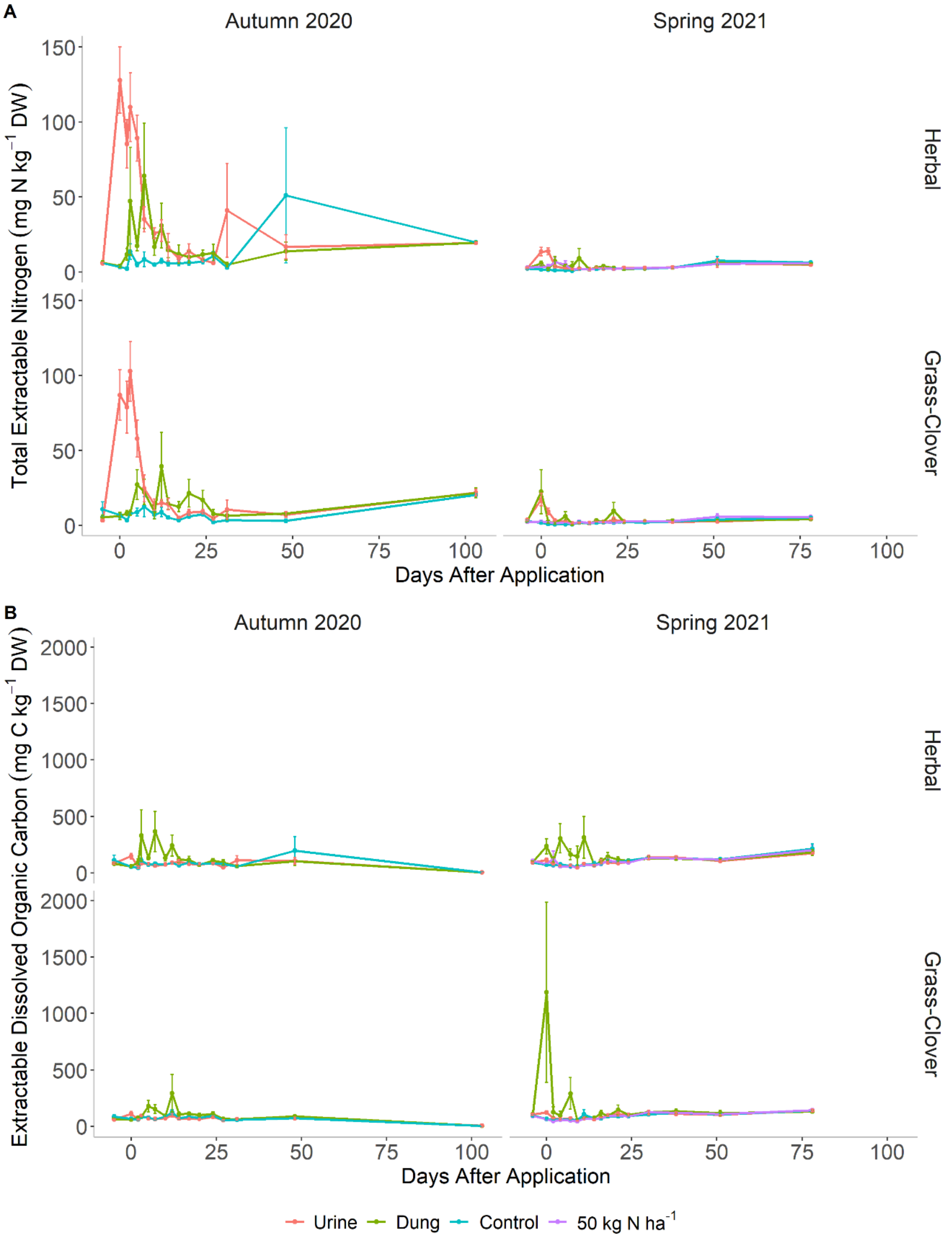


Figure 4.4. *Soil total extractable nitrogen (panel A) and total extractable dissolved organic carbon (panel B) measured following application of sward-specific lamb urine, dung, or 50 kg N ha^{-1} ammonium nitrate to either a grass-clover or herbal ley in autumn 2020 (16/10/2020) and spring 2021 (10/05/2021). Data represents mean ± SEM, n = 3 per treatment per ley. Legend applies to both panels.*

4.3.4.4. Nitrous Oxide Emissions

Nitrous oxide emissions during the two experimental seasons are displayed in Figure 4.5. Addition of sward-specific urine, dung, or 50 kg N ha^{-1} ammonium nitrate (spring only) had no significant effect on N_2O emissions emitted in either season or from either sward type ($p > 0.05$). Unusually, the magnitude of emissions emitted during the sampling campaign were extremely small and did not exceed 53.6 ± 3.4 µg N_2O-N m^{-2} h^{-1} in autumn or 46.5 ± 5.3 µg N_2O-N m^{-2} h^{-1} in spring. Negative fluxes of a similar magnitude were observed on several sampling dates in autumn in each treatment, which may have been driven by higher emissions at lid closure of the static gas chamber or caused by variability in low headspace N_2O concentrations below the limit of detection (± 18 µg N_2O-N m^{-2} h^{-1}). Consequently, no distinguishable peaks in N_2O emissions were observed in any treatment or either sward type throughout the measurement period.

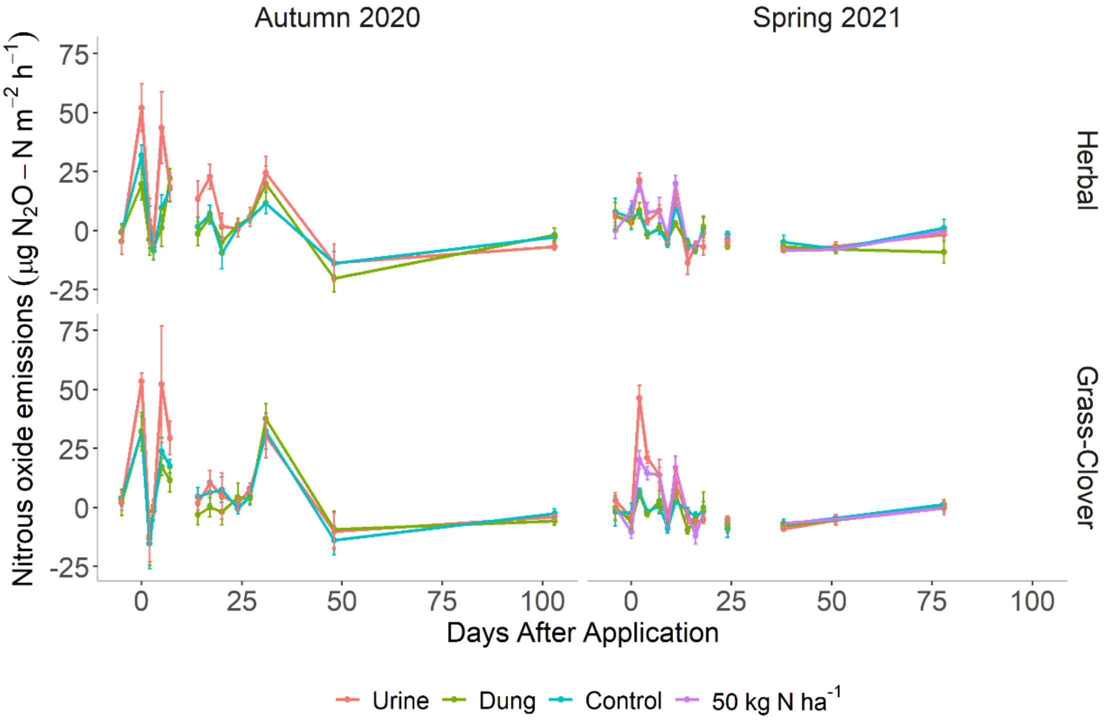


Figure 4.5. *Soil nitrous oxide emissions emitted following the application of sward-specific urine, dung or 50 kg N ha^{-1} ammonium nitrate to either a herbal ley or a grass-clover ley on day 0 in autumn 2020 (16/10/2020) or spring 2021 (10/05/2021). The control received no*

additions. Data denotes mean ± SEM, n = 3 per treatment per sward. Line breaks indicate omitted data due to instrumental errors.

Sward type had no significant effect on cumulative N_2O emissions or emission factors arising from the various treatments ($p > 0.05$) (Table 4.6). The low and negative cumulative emissions produced smaller than anticipated emission factors (EF_{3PRP}) for the urine treatment and the 50 kg N ha^{-1} ammonium nitrate treatment emission factor (EF) in spring. No difference between sward types was found following the addition of sward-specific urine in autumn (0.008 ± 0.01 % EF_{3PRP} herbal *vs*. 0.008 ± 0.01 % EF_{3PRP} grass-clover, $p > 0.05$) and spring (-0.001 ± 0.001 % EF_{3PRP} herbal *vs*. 0.001 ± 0.001 % EF_{3PRP} grass-clover, $p > 0.05$). Similarly, sward type did not influence emission factors for the 50 kg N ha^{-1} ammonium nitrate treatment in spring (-0.002 ± 0.001 % EF herbal *vs*. 0.03 ± 0.03 % EF grass-clover, $p > 0.05$).

Table 4.6. *Cumulative nitrous oxide emissions determined in autumn 2020 and spring 2021 following addition of sward-specific urine, dung, ammonium nitrate (50 kg N ha^{-1}) or no addition (control) to soil. Emissions were measured over 103 days in autumn 2020 and 78 days in spring 2021. Data represents mean ± SEM. No significant differences were found between sward-types in each season; thus no superscripted letters are presented ($p > 0.05$). n.d. indicates not determined.*

Sward	Treatment	Autumn 2020 Cumulative N_2O Emissions (mg N_2O-N m^{-2} 103 days)	Spring 2021 Cumulative N_2O Emissions (mg N_2O-N m^{-2} 78 days)
Grass-Clover	Urine	5.92 ± 6.17	-3.55 ± 1.49
	Dung	8.71 ± 5.92	-7.42 ± 1.62
	50 kg N ha^{-1}	n.d.	-4.87 ± 1.88
	Control	1.40 ± 4.86	-6.26 ± 1.33
Herbal	Urine	7.44 ± 4.09	-7.03 ± 1.05
	Dung	-7.83 ± 5.24	-10.17 ± 1.50
	50 kg N ha^{-1}	n.d.	-4.67 ± 1.38
	Control	-0.50 ± 4.89	-4.58 ± 1.12

4.3.5. Ammonia Volatilisation

Sward-specific urine applied to the herbal ley resulted in significantly lower NH_3 volatilisation (3.05 ± 0.10 % of total urine-N) than urine applied to the grass-clover ley (7.33 ± 0.56 % of total urine-N) emitted over the 12-day measurement period (Figure 4.6, panel A). This amounted to a 140 % reduction in NH_3 volatilisation from the intact soil cores, with emissions plateauing on day 12. In the initial 24-hours following urine addition, NH_3 volatilisation from the hydrolysis of urine urea was low in both leys, with 1.00 ± 0.02 % of the total urine-N applied lost as NH_3 in the herbal ley and 1.28 ± 0.15 % lost in the grass-clover ley ($p > 0.05$). Sward type and day had a significant interaction on NH_3 volatilisation ($F_{(1,34)} = 14.42$, $p < 0.001$), with a post-hoc pairwise comparison showing a significant reduction in NH_3 volatilisation arising from the herbal ley on days 4 ($p = 0.023$), 5 ($p = 0.020$), 8 ($p = 0.024$) and 12 ($p = 0.025$).

When expressed on an area basis, the cumulative NH_3-N volatilised over 12-days was equivalent to 5.35 ± 0.12 mg N m^{-2} h^{-1} from the herbal ley compared to 27.90 ± 2.47 mg N m^{-2} h^{-1} from the grass-clover ley (Figure 4.6, panel B), with significantly lower NH_3-N emissions from the herbal ley measured on days 1 ($p = 0.024$), 2 ($p = 0.039$), 4 ($p = 0.014$) and 5, 8 and 12 ($p = 0.012$).

Similarly, if evaluated as a percentage of the total urine urea-N applied emitted as a NH_3-N (Figure 4.6, panel C), where urea-N comprises 90.8 ± 4.7 % and 80.3 ± 3.6 % of the total urine-N in the herbal and grass-clover ley respectively, then the NH_3-N subsequently emitted is equivalent to 4.95 ± 0.19 % and 13.10 ± 1.00 % in the herbal and grass-clover ley. A significant interaction was found between sward and day ($F_{(1,34)} = 18.61$, $p < 0.001$), with a post-hoc test identifying significantly lower NH_3-N emissions from the herbal ley on days 4 ($p = 0.019$), 5 ($p = 0.016$), 8 ($p = 0.018$) and 12 ($p = 0.018$).

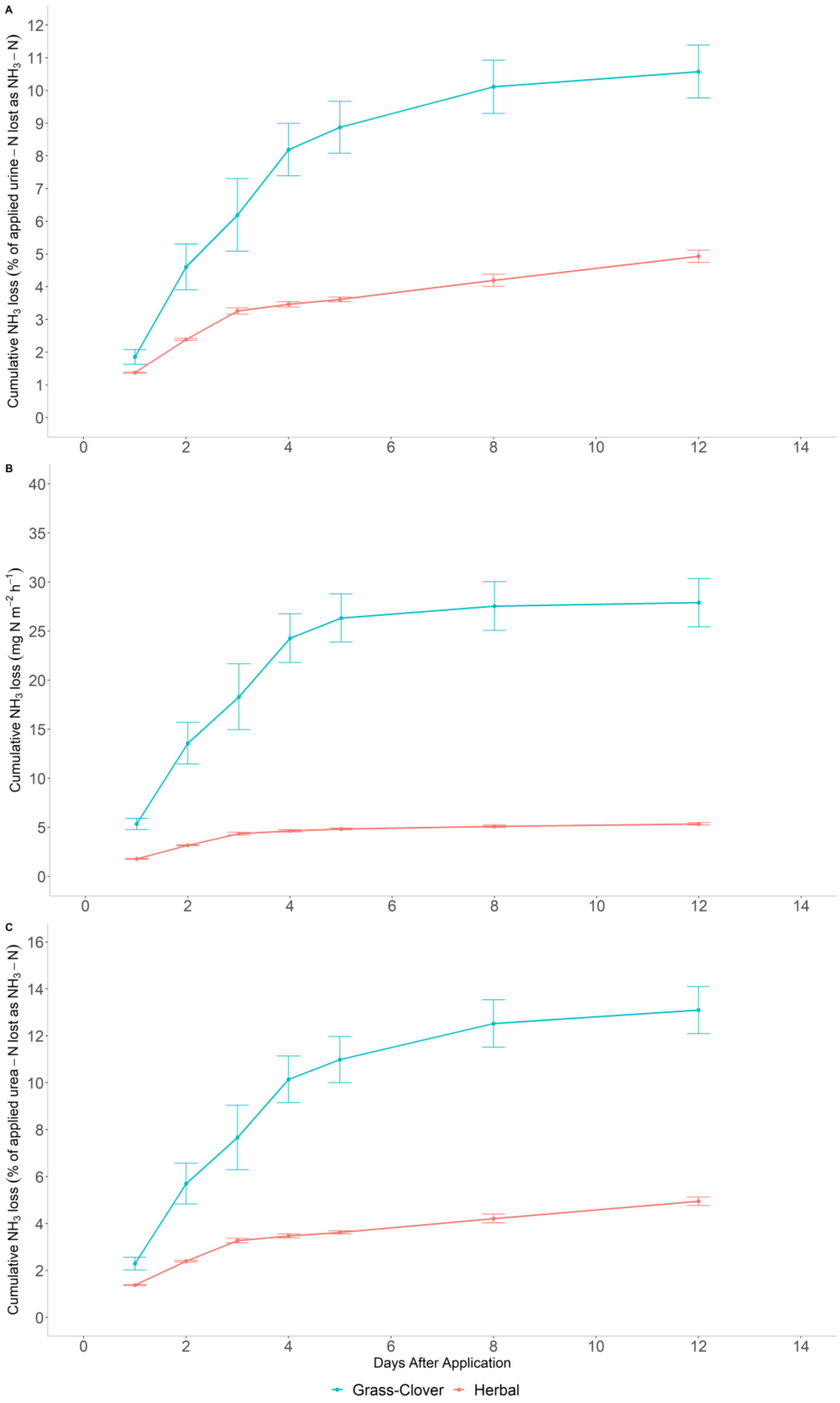


Figure 4.6. *Cumulative ammonia (NH_3) emissions from sward-specific lamb urine applied to intact soil cores from either a herbal (n = 3, 0-12 cm depth) or grass-clover ley (n = 3, 0-12 cm depth) measured over 12 days. Results are expressed as a percentage of applied total urine-*

N lost as NH_3-N (panel A), on an area basis (mg N m^{-2} h^{-1}) (panel B), and as a percentage of the total urine urea-N applied emitted as a NH_3-N (panel C). Data represents mean ± SEM.

4.4. Discussion

4.4.1. Changes in Lamb Excreta Composition and Urine-patch N Loading Rate

Excreta N concentration was not affected in lambs grazing the herbal ley in either experimental season under the field conditions and management methods explored in this study, therefore the first hypothesis is rejected. Our lamb-based study conflicts with previous studies reporting a reduction in urine-N concentration and an increase in faecal-N concentration in cattle grazing low-complexity herbal mixtures (e.g., 3-6 species) compared to conventional pastures (Cheng et al., 2017; Minneé et al., 2017; Totty et al., 2013; Wilson et al., 2020). High concentrations of plant secondary metabolites in key herb and legume species frequently sown in herbal leys can reduce rumen protein degradation, reducing blood urea content and subsequently urinary-N excretion (Jordon et al., 2022). However, to date, there are currently no studies investigating ruminant excreta composition when grazing a high-complexity herbal ley mixture (e.g., 6-18 species) promoted via agri-environment schemes.

Despite no observed dietary effect, urine-N concentration obtained from lambs in both autumn and spring (4.8-6.6 g N l^{-1}) were lower than averages reported in published meta-analyses (8.7 g N l^{-1}; Selbie et al., 2015) and from previous studies of sheep grazing grass-clover swards (7.0 ± 0.2 g N l^{-1}; Marsden et al. (2020); 7.9 g N l^{-1}; Hoogendoorn et al. (2010)). No difference was found in the concentration of urine urea or purine derivatives allantoin, hippuric acid, creatinine, uric acid, and benzoic acid between sward types in either season (Table 4.4). However, urea-N provided a slightly greater proportion of total urine-N in the herbal ley in both autumn (90.8 ± 4.7 % herbal *vs*. 80.3 ± 3.6 % grass-clover) and spring (77.3

± 8.3 % herbal *vs*. 71.6 ± 3.1 % grass-clover) but this was mostly within the range of previous published sheep urine studies, e.g., 78-85 % (Marsden et al., 2020) and 75-93 % (Bristow et al., 1992). Hippuric acid excreted in spring lamb urine was considerably lower than urine from autumn lambs, however, due to numerous variations in experimental conditions between seasons (e.g., lamb sex and age, sward maturity and botanical composition) it is difficult to know the exact cause of this, thus further investigation is warranted.

The lack of reduction in urine-N concentration in the herbal ley grazed lambs in this study was likely due to dilution of beneficial plant secondary metabolites (e.g., verbascoside, tannins) in the sward, as the proportion of herbs (13.8 %) sown was lower than grasses (46 %) and legumes (39.9 %). While this represented a commercial herbal ley mixture, the combination of low herb proportion, poor establishment of plant secondary metabolite rich legumes such as *Onobrychis* and *Medicago sativa*, and seasonal variation in herb presence in the sward may have contributed to the insignificant impact on excreta N manipulation in grazing lambs in this study. Of the total herb proportion sown, key species such as *Plantago lanceolata* and *Cichorium intybus* only comprised 1.5 % and 4.6 % of the sward composition, respectively (Table 4.1). However, a sward botanical survey in July 2021 revealed that of the 18 species sown in the herbal ley, only 11 species persisted after two grazing seasons (Supplementary Figure 2), with key herbs *Plantago lanceolata* and *Cichorium intybus* comprising 4.9 ± 1.2 % and 21.1 ± 1.3 % of the sward composition, respectively. Assuming this proportion of *Plantago lanceolata* was ingested, it was likely too small to have a significant effect on urine-N concentration.

Previous studies have reported that a 20 % reduction in cattle urine-N concentration only occurred when the herb proportion consisted of 20-40 % of the sward composition (Box et al., 2023), with a similar effect on dairy cattle only observed when herb proportion exceeded 50 % of the biomass (500 g kg^{-1} DM) (Cheng et al., 2017). However, it is unclear if this is the proportion needed to ensure consumption by the grazing ruminant and reduce opportunities for selective grazing, or the proportion needed to deliver a sufficient concentration of plant secondary metabolites to induce a reduction in urinary-N excretion. Bryant et al. (2018) found no difference in dairy cattle urine-N excretion when a low proportion of herbs *Plantago lanceolata* and *Cichorium intybus,* comprising of ca. 5 % of the sown composition respectively, was introduced into a ryegrass sward. Our study utilised a similar percentage composition of *Cichorium intybus*, but a much lower percentage of *Plantago lanceolata*. However, as only one sward botanical composition measurement was conducted after establishment in July 2021, it is difficult to know which species persisted under grazing, therefore assumptions can only be made from the sown composition.

Higher proportions of *Cichorium intybus* and *Plantago lanceolata* (e.g., > 20 %) in the pasture are likely required to ensure ingestion, and therefore greater delivery of key plant secondary metabolites, as it reduces potential livestock avoidance of these herbs due to their astringent taste (Jaramillo et al., 2021; Somasiri et al., 2020). Similarly, increasing grazing pressure through a higher stocking density or utilising rotational grazing may encourage consumption of these species compared to a set stocked pasture (Jaramillo et al., 2021). Future studies investigating herbal leys would benefit from monitoring preferential grazing of species to better understand livestock grazing behaviours and the proportion of key bioactive species that are ingested. In addition to recording seasonal differences in botanical composition, further work is needed to determine seasonal changes in the total sward secondary metabolite content, as previous studies often only measure plant secondary metabolite concentration of key species in isolation (e.g., Hamacher et al. (2021)) at one time point.

Other aspects of sward nutritional quality such as the dry matter, crude protein, and macronutrient content can also manipulate N excretion and urination behaviours in livestock (Dijkstra et al., 2013). Despite their greater digestibility and lower rumen retention time, *Plantago lanceolata* and *Cichorium intybus* have a lower dry matter content than grasses which can inadvertently suppress livestock dry matter intake and increase water ingestion, resulting in increased urination frequency and volume (Minneé et al., 2017). Suppressing dry matter intake can reduce crude protein intake, leading to a lower urine-N excretion and greater urination volume, creating a more dilute urine (Cheng et al., 2017). Previous studies of cattle consuming *Plantago lanceolata* and *Cichorium intybus* have reported a 1.6-2.4-fold increase in urination volume (Cheng et al., 2017; Mangwe et al., 2019; Wilson et al., 2020). In sheep, daily urination volume increased by up to 0.5 L when fed *Plantago lanceolata* (O'Connell et al., 2016). This was attributed to the increased water intake and potential consumption of aucubin, a diuretic plant secondary metabolite found in high concentrations in *Plantago lanceolata* (Gardiner et al., 2018; O'Connell et al., 2016; Wilson et al., 2020).

This study found surprisingly inconsistent effects of sward type on urination volume, with average urine event volume increasing by 77 % in lambs grazing the herbal ley compared to the grass-clover ley in autumn, but not in spring, where urine volume declined by 18 % (Table 4.4). Average urination event volume in both autumn and spring (114-180 ml) were lower than the average reported in previous studies of sheep grazing grass-clover swards (364 ± 32 ml; Marsden et al. (2020)). In autumn, the higher urination volume and subsequent urine-patch surface area led to a higher urine N loading rate in the herbal ley (1020 ± 46 kg N ha^{-1}) than the grass-clover sward (555 ± 33 kg N ha^{-1}). This was greater than the suggested 500 kg N ha^{-1} loading rate for sheep urine reported in Williams and Haynes (1994) but was within the 203-2283 kg N ha^{-1} range reported for upland and lowland sheep grazing in Marsden et al. (2020). No difference was observed in urine N loading rate in spring due to a similar urine patch size and N content. While sward type had no effect, daily urination frequency (6.9 ± 0.9 *vs*. 7.7 ± 1.1 urine events $sheep^{-1}$ day^{-1}, herbal *vs*. grass-clover) and volume (0.77 ± 0.10 *vs*. 1.15 ± 0.10 L $sheep^{-1}$ day^{-1}, herbal *vs*. grass-clover) was lower than those reported in previous studies utilising the same breed of sheep, e.g., 9.7 ± 0.7 urine events sheep^{-1} d^{-1} and 2.77 ± 0.15 L sheep^{-1} d^{-1}; Marsden et al. (2020). However, the daily rates generated from the excreta collection pens should be interpreted with caution, as they do not fully capture the daily and diurnal variation in urine composition and urination behaviour. During the collection period, animal movement inside the pen and natural grazing behaviour is restricted, thus the data may not be representative of typical behaviour when under normal grazing conditions. For a more accurate estimate of daily urinary excretion, urine sensors (e.g., accelerometers for detecting urination), urine collection bags (attached via catheter) or metabolism crates (e.g., Liu and Zhou, 2014) should be used instead.

A multitude of factors may be responsible for the inconsistent effects on urination volume and N loading rate, including differences in lamb physiology (e.g., sex, age, health) affecting feed utilisation and efficiency, grazing behaviours (e.g., selective grazing of herbs), seasonal variations in sward botanical composition, nutritional quality (e.g., dry matter content) and plant secondary metabolite concentration (e.g., aucubin) (Box and Judson, 2018). Despite no difference in sward dry matter and crude protein content between the two ley types, seasonal differences were observed with a lower dry matter content recorded in both swards in autumn (ca. 13 % DM) than spring (ca. 26-29 % DM) which may have contributed towards the greater urine volume in the herbal ley in autumn (Table 4.3). Higher sward sodium content in the herbal ley in both experimental seasons may have led to greater sodium ingestion, inadvertently impacting urination behaviour in grazing lambs.

Previous studies have reported a positive linear relationship between dietary sodium intake and urine volume, where dairy cattle produced an additional 0.136 kg of urine per additional gram of sodium ingested (Spek et al., 2012). Due to their deep-rooting nature, *Plantago lanceolata* and *Cichorium intybus* are known to accumulate greater concentrations of macronutrients, such as sodium, than grass or legume species (Barry, 1998; Scales et al., 1995).

While the chemical composition of individual species was not measured in this study, overall sward measurements showed that the herbal ley produced higher sodium concentrations in autumn (6.9 ± 0.8 g kg^{-1} herbal *vs*. 2.1 ± 0.4 g kg^{-1} grass-clover) and spring (0.9 ± 0.1 g kg^{-1} herbal *vs*. 0.4 ± 0.1 g kg^{-1} grass-clover), subsequently increasing urine sodium excretion by 521 % and 727 % in each respective season. Urine sodium concentrations measured in autumn (439 ± 62 mg Na l^{-1} herbal *vs*. 71 ± 26 mg Na l^{-1} grass-clover) and spring (389 ± 150 mg Na l^{-1} herbal *vs*. 47 ± 18 mg Na l^{-1} grass-clover) greatly exceeded previous studies of lowland sheep grazing grass-clover swards (28 ± 7 mg Na l^{-1}; Marsden et al. (2020)). However, it is difficult to draw comparisons to previous studies of sheep grazing under field conditions, as the excreta sodium content is seldom reported.

In an indoor feeding experiment, sheep fed a high-salt diet containing 6 g NaCl kg^{-1} DM excreted 2.4 g Na l^{-1} in their urine (Liu and Zhou, 2014). Despite a similar sward sodium concentration of 7 g Na kg^{-1} in the herbal ley in autumn 2020, urinary sodium excretion from ram lambs was considerably lower (< 0.5 g Na l^{-1}). When expressed on a urine-patch scale, accounting for seasonal variations in urination volume, the sodium loading rate from herbal grazed lambs amounted to 9.3 ± 1.3 g Na m^{-2} and 7.7 ± 3.0 g Na m^{-2} in autumn and spring, exceeding the loading rate from grass-clover grazed lambs by 1229 % and 601 %, respectively. However, as the excreta sodium content is frequently overlooked in grazing studies, and subsequently urine-patch sodium loading rates not calculated, comparisons to published literature cannot be made. Future studies would benefit from measuring urine and dung chemical composition in greater detail, reporting non-urea nitrogenous compounds (e.g., hippuric acid) and cation content (e.g., K, Ca, and Na) that may affect soil biogeochemistry in grazing pastures. The elevated urinary sodium excretion found in herbal grazed lambs in this study warrants further investigation to assess repeatability under differing soil types and locations, as currently the impact on urine-patch nutrient cycling, NH_3 volatilisation and N_2O emissions are unknown.

4.4.2. Soil Nutrient Cycling and N_2O Emissions

The herbal ley did not reduce N_2O emissions from lamb urine and dung in either experimental season, nor did it reduce emissions following the application of 50 kg N ha^{-1} ammonium nitrate in spring; as a result, the N_2O emission factors for urine and fertiliser were unaffected and thus the second hypothesis rejected. This conflicts with previous studies reporting a reduction in yield-scaled N_2O emissions following fertiliser application to a low diversity (e.g., 6-species) herbal ley mixture compared to grass-clover leys or fertilised ryegrass monocultures (Bracken et al., 2022; Cummins et al., 2021). Increasing plant species richness in grazing systems by utilising herbal leys is proposed to reduce N losses by altering the root architecture, increasing root density, and subsequently manipulating soil physical (e.g., porosity) and chemical (e.g., moisture content) properties (Niklaus et al., 2016). Similarly, BNI compounds, such as aucubin and verbascoside, released in *Plantago lanceolata* root exudates can reduce the transformation of NH_4^+ to NO_3^-, therefore increasing the stability of the soil mineral N pool and increasing plant N uptake (Cummins et al., 2021; Luo et al., 2018).

When included in high proportions of the sward mixture, *Plantago lanceolata* has been shown to reduce N_2O emissions from dairy cattle urine additions to pasture, with this effect largely linked to its BNI capabilities and dilution of the urine from grazing ruminants (Gardiner et al., 2018; Luo et al., 2018; Simon et al., 2019). Similar findings were reported from sheep grazing pure cultivars of *Plantago lanceolata,* where bioactive compounds excreted in urine reduced soil NO_3^- concentrations by inhibiting the nitrification enzyme ammonia monooxygenase (Peterson et al., 2022). However, when included in low proportions, e.g., 5 %

of the total sward composition, *Plantago lanceolata* had no effect on dairy cattle urine-patch N_2O emissions or subsequent emission factors (Nyameasem et al., 2021). In this study, the low proportion of *Plantago lanceolata* established in the herbal ley (ca. 1.5 % of the sown sward composition) did not reduce soil NO_3^- concentrations nor N_2O emissions, suggesting this proportion was insufficient to provide any BNI benefit.

As the magnitude of N_2O emissions emitted throughout each experimental season was extremely small, peaking at 53.6 ± 3.4 µg N_2O-N m^{-2} h^{-1} and 46.5 ± 5.3 µg N_2O-N m^{-2} h^{-1} in autumn and spring respectively (Figure 4.5), it is difficult to draw accurate conclusions about the effect of sward type on N_2O emissions. The fluxes emitted in this study were considerably lower than previous studies reporting N_2O emissions from low-diversity herbal leys following fertiliser (e.g., 44.6 g N_2O-N ha^{-1} d^{-1}, Bracken et al. (2020)) or dairy cattle urine additions (e.g., 24.2 mg N_2O-N m^{-2} d^{-1}, Nyameasem et al. (2021)). This was likely due to low precipitation during the autumn (October 2020 – January 2021) and spring (May – July 2021) experimental seasons, producing a low soil WFPS of ca. 45-52 % that was not sufficient to induce denitrification in any treatment (Supplementary Figure 4.3). Similarly, the dry conditions may have enabled rapid infiltration of urine to the subsoil and poor integration of dung on the soil surface, which, along with rapid plant N uptake, may be responsible for the low concentrations of soil NH_4^+, NO_3^-, TOC and TN measured in the 0-10 cm soil layer (Figure 4.3 and 4.4), contributing to subsequent low N_2O emissions (Bowatte et al., 2018). Likewise, as the optimal soil WFPS for denitrification (60-80 %) was not reached during either measurement period, it is highly unlikely that the negative N_2O fluxes measured in each season were due complete denitrification reducing N_2O to N_2 (Butterbach-Bahl et al., 2013; Chapuis-lardy et al., 2007; Davidson, 1993). Negative N_2O fluxes have been reported in previous studies following sheep urine addition to lowland grasslands (e.g., Mancia et al. (2022)), however, these are typically produced from anaerobic, low soil NO_3^- environments, unlike the conditions measured in our study. Instead, it is more likely that the negative N_2O fluxes in our study are due to the very low concentrations of headspace N_2O and the limit of detection of the N_2O analysis (± 18 µg N_2O-N m^{-2} h^{-1}), or N_2O consumption that has been previously reported for this soil (e.g., Button et al., 2023).

Consequently, the low and negative N_2O fluxes generated negative cumulative emissions for the herbal ley dung and control treatment in autumn and for both swards and all treatments in spring (Table 4.6), producing negligible N_2O emission factors. Sward-specific lamb urine emission factors determined in autumn and spring were much lower than the disaggregated urine-only 2019 IPCC Tier 1 EF_{3PRP} of 0.004 % (IPCC, 2019) and the 2022 UK Tier 2 EF_{3PRP} of 0.315 % (Churchill et al., 2022) used in current inventory calculations. Similarly, the EF_{3PRP}s generated in this study were also lower than previous studies reporting emission factors from sheep urine applied to a grass sward in autumn (0.04 ± 0.06 %) and spring (-0.01 ± 0.04 %) (Mancia et al., 2022), or a grass-clover sward in summer (0.07 %) (Hoogendoorn et al., 2016). As this study is the first to develop an emission factor for urine deposited to an herbal ley it is not yet possible to draw comparisons with other similar studies. However, while our emission factors support the further refinement of national GHG inventories, they do not currently demonstrate a need for disaggregated sward-specific EF_{3PRP}s. Instead, further research is needed over multiple years and locations, fully capturing national variations in weather, soil type and sward botanical composition, as the atypically dry conditions experienced in this study may have hidden any potential sward benefits.

4.4.3. NH_3 Volatilisation

In grazing systems, sheep urine-patches represent a major source of NH_3 emissions, with up to 20 % of applied N emitted as NH_3 (Sherlock and Goh, 1984), leading to indirect

N_2O emissions, soil acidification, habitat loss, and negative implications for human health (Laubach et al., 2013). However, due to increasing demand, NH_3 emissions from sheep production are increasing by 0.5 % annually, and are responsible for an average of 6.4 % of global livestock NH_3 emissions between 1961-2018 (Yang et al., 2023). Thus, to counter this, more effective on-farm NH_3 abatement strategies need to be identified. In pastures, NH_3 volatilisation is largely controlled by soil (e.g., pH, temperature), climatic (e.g., windspeed), and excreta composition (e.g., urine urea concentration, urine patch-size) factors (Laubach et al., 2013; Webb et al., 2005). As it is impractical to reduce emissions directly from urine-patches (via urease inhibitors) due to their heterogenous spatial and temporal distribution, current abatement strategies, e.g., the 2019 UK Clean Air Strategy or the 2015 UNECE Framework Code for Good Agricultural Practice, focus on improving animal husbandry and diet manipulation, recommending that ruminant dietary protein requirements are accurately met to reduce excreta N losses (DEFRA, 2019; United Nations Economic Commission for Europe, 2015).

By reducing fertiliser N applications, this can reduce excess N concentrations in grazed pastures, subsequently leading to lower N excretion and NH_3 volatilisation (Webb et al., 2005). However, low N input grasslands such as herbal leys offer a conflicting solution to reducing NH_3 emissions, as despite containing high proportions of legumes to reduce N fertiliser requirements, this can lead to excessive protein ingestion and thus greater N excretion in livestock. In commercial herbal ley mixtures, the proportion of plant secondary metabolite-rich herb and legume species is often inadequate to counter this and reduce urinary-N excretion, as observed in this study (Table 4.5). Instead, herbal leys may drive lower NH_3 emissions through increasing urination volume via greater sodium ingestion, creating a more dilute urine (Liu and Zhou, 2014). However, currently there are no field-based studies reporting NH_3 volatilisation from urine applied to a herbal ley.

In the bench-scale experiment, cumulative NH_3 emissions following the addition of sward-specific urine were 140 % lower in the herbal than the grass-clover ley (Figure 4.6, panel A). Sward-specific NH_3 emission factors generated over the 12-day measurement period were lower from the herbal (3 %) than the grass-clover ley (7 %), with the herbal ley emission factor also lower than the 2022 UK inventory estimate for sheep urine deposited to pasture, where it is assumed that 6 % of applied N is lost as NH_3 (Brown et al., 2022). The emission factors produced from our study were within the range of previous studies, with Whitehead and Raistrick (1992) reporting a 23 % loss as NH_3 from a ryegrass sward following the addition of synthetic livestock urine, and Sherlock and Goh (1984) reporting NH_3 losses between 7.5 – 37 % from sheep urine applied to a ryegrass sward, depending on season and number of applications. While our study supports the refinement of NH_3 inventory calculations, it is unclear what mechanisms are responsible for the reduction in NH_3 volatilisation from the herbal ley.

As urine N, urea and non-urea nitrogenous compound concentrations did not differ between the two swards, this reduction may be caused by differences in applied urine volume or urinary sodium concentration. The urine application rate in this experiment was reflective of seasonal differences in urination volume obtained from autumn ram lambs, with herbal ley cores receiving less urine (0.5 ml cm^{-2}) than grass-clover cores (0.9 ml cm^{-2}) to replicate differences in urine-patch dynamics where urine is spread over a greater area in the herbal ley. As urine-patch size is a key driver of NH_3 emissions, the greater urine volume received by the grass-clover cores likely produced a higher soil WFPS, subsequently leading to higher NH_3 emissions due to deeper infiltration of urine into the core (Moring et al., 2016). This is supported by a process-based model assessing NH_3 emissions from urine patches which found that increasing soil moisture field capacity by 10 % increased NH_3 volatilisation by 9.12 % (Moring et al., 2016). However, these results may be confounded by the closed system design of the bench-scale ammonia volatilisation system restricting the urine-patch diffusional area (both laterally and vertically) within the soil cores. This limited urine drainage through the soil profile and may overestimate emissions compared to *in-situ* studies.

Despite the higher urinary sodium content applied in the herbal ley (14.0 mg Na l^{-1}) compared to the grass-clover ley (4.1 mg Na l^{-1}), it is unlikely that this was sufficient to induce osmotic stress in soil microbes or an inhibitory effect on urease activity (Haj-Amor et al., 2022). Conversely, in high salinity soils, NH_3 emissions are often increased due to the inhibitory effect of salinity on nitrification increasing the accumulation of NH_4^+ and preventing uptake by plants (Haj-Amor et al., 2022). Instead, reductions in NH_3 emissions following urine deposition from sheep fed a high-salt diet (6 g NaCl kg^{-1} DM) were linked to an increase in urination volume combined with lower urine-N concentration, rather than the urinary sodium content (Liu and Zhou, 2014). Our findings were similar to those reported by Liu and Zhou (2014), who reported lower urinary NH_3-N emissions from sheep fed a high-salt diet (3.72 mg N m^{-2} h^{-1}) than the control diet (7.21 mg N m^{-2} h^{-1}). Although NH_3-N emissions from the herbal ley (5.35 ± 0.12 mg N m^{-2} h^{-1}) were close to this range, emissions from the grass-clover ley (27.90 ± 2.47 mg N m^{-2} h^{-1}) greatly exceed this. However, as there is no difference in urine N concentration between sward types, despite an increase in autumn 2020 urination volume in the herbal ley, it is not clear if this is driving the lower NH_3-N emissions measured in this study.

Although the findings from this experiment are promising and support further refinements of national NH_3 inventories, it is unclear what mechanisms underpinned the reduction in NH_3 volatilisation from the herbal ley. Further investigation is warranted both in laboratory and field-scale experiments to better assess the effect of sward-type on urine-patch NH_3 volatilisation. Finally, as N uptake in plant matter, N leaching, or N_2O emissions as an indicator of potential ‘pollution swapping’ was not quantified in this experiment, future studies would benefit from developing a N mass balance to better understand the fate of urine-N in herbal leys.

4.5. Conclusion

Understanding how sward composition affects ruminant excreta composition and subsequent N_2O emissions from grazing is vital for identifying pathways to achieving sustainable agricultural intensification. This study has shown that grazing lambs on a commercial herbal ley mixture does not alter the excreta N concentration nor reduce urine N loading rates when compared to a conventional grass-clover ley. The lack of differences in excreta composition may be due to seasonal variations in sward composition and plant secondary metabolite concentration within key herb and legume species, however, as this was not measured in our study this requires further investigation. Increased urinary sodium excretion in herbal ley grazed lambs was unexpected and has not been reported in the literature before. This was likely due to the high sward sodium content within the herbal ley, potentially caused by greater sodium accumulation in plant species such as *Cichorium intybus*. However, as the soil, sward, and excreta sodium content are frequently overlooked in grazing studies, future research would benefit from evaluating this as the implications of persisted increased sodium excretion in urine patches on soil nutrient cycling is largely unknown.

Contrary to previous research on low-diversity herbal mixtures, our study showed no effect of sward-type on N_2O emissions from urine, dung, or 50 kg N ha^{-1} ammonium nitrate applications. The atypical dry conditions in each measurement season created suboptimal conditions for denitrification and potentially contributed to urine infiltration to depth and poor dung integration into the soil, generating low N_2O fluxes that may have obscured any potential sward benefits. Subsequently, the resulting sward-specific EF_{3PRP}s produced in autumn and spring were negligible compared to the disaggregated 2019 IPCC Tier 1 EF_{3PRP} and the 2022 UK specific Tier 2 EF_{3PRP}. However, while these emission factors support future refinements of national GHG inventories, they do not currently demonstrate a need for sward-specific disaggregated EF_{3PRP}.

Furthermore, the reduction in NH_3 volatilisation following urine addition to intact herbal ley cores in the bench-scale experiment is promising for future field-scale applications, as this may reduce indirect N_2O emissions from grazing, subsequently reducing the overall environmental impact of sheep production. However, as this was only observed under laboratory conditions, future studies need to upscale and investigate the effect of herbal leys on NH_3 volatilisation under field conditions, fully capturing the national variability of soil, sward, and climatic factors, as the mechanisms underpinning the reduction in NH_3 emissions are currently unknown.

Overall, despite the promised benefits and promotion of herbal leys in agri-environment schemes, the herbal ley utilised in our study did not affect lamb excreta composition or reduce N_2O emissions associated with lamb grazing in either experimental season. Further environmental monitoring and refinements of commercial seed mixtures are needed to identify the optimum proportions of key plant species (e.g., *Cichorium intybus*, *Plantago lanceolata*) required in the sward composition to deliver environmental benefits prior to wide-scale on-farm adoption.

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Ethical Statement

Lamb excreta collection was approved by the Bangor University Ethics Committee (code COESE2019EC01A).

CRediT authorship statement

Funding acquisition, J.R.L and D.L.J; Conceptualisation, experimental design, sampling: E.C.C, D.R.C, D.L.J; Formal data analysis, E.C.C and K.A.M; Writing – first draft E.C.C, D.R.C, D.L.J; Writing – review and editing E.C.C, J.R.L., D.R.C, D.L.J; Supervision, D.L.J and D.R.C.

Declaration of interest

The authors declare they have no competing interests.

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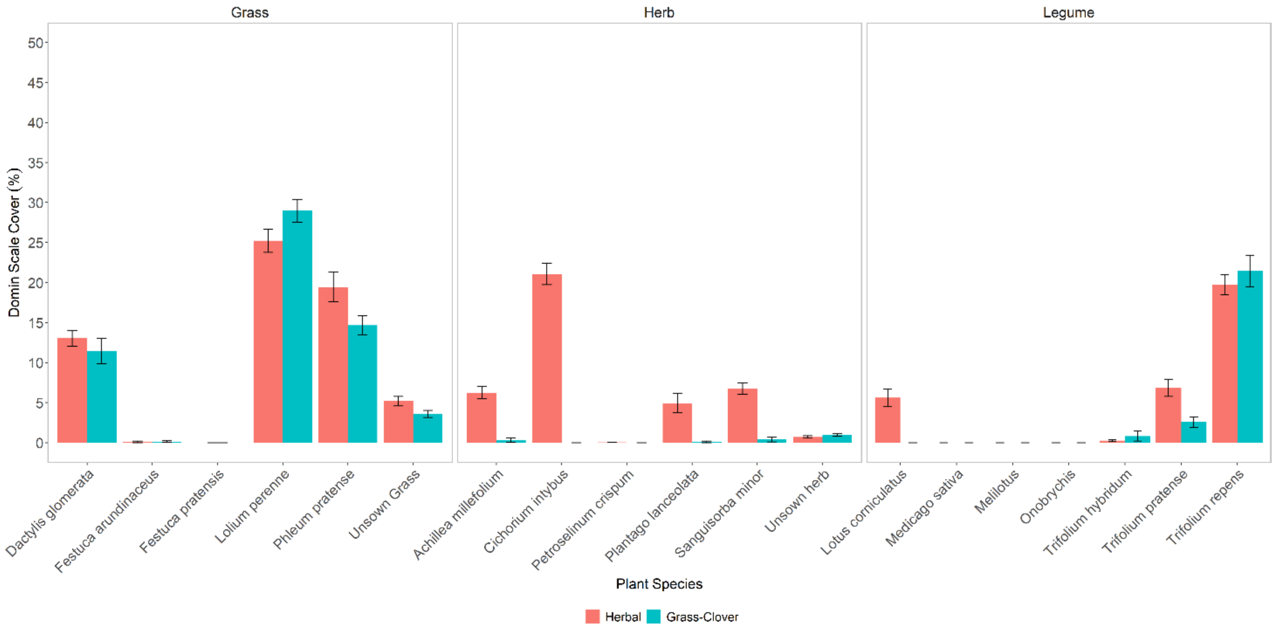
4.7. Supplementary Materials

ICP-OES methods:

Samples were analysed using an Agilent 5800 ICP-OES fitted with an Agilent SPS 4 autosampler using an axial torch configuration. Plasma flow was set at 15 l min^{-1} and nebuliser pressure set at 240 kPa, with a sample uptake delay of 40 s and a rinse time of 20 s using dry argon as a carrier gas. Data was analysed using Software ICP Expert Pro with all elements measured fully quantified against a 3-point calibration using standards from Sigma-Aldrich.

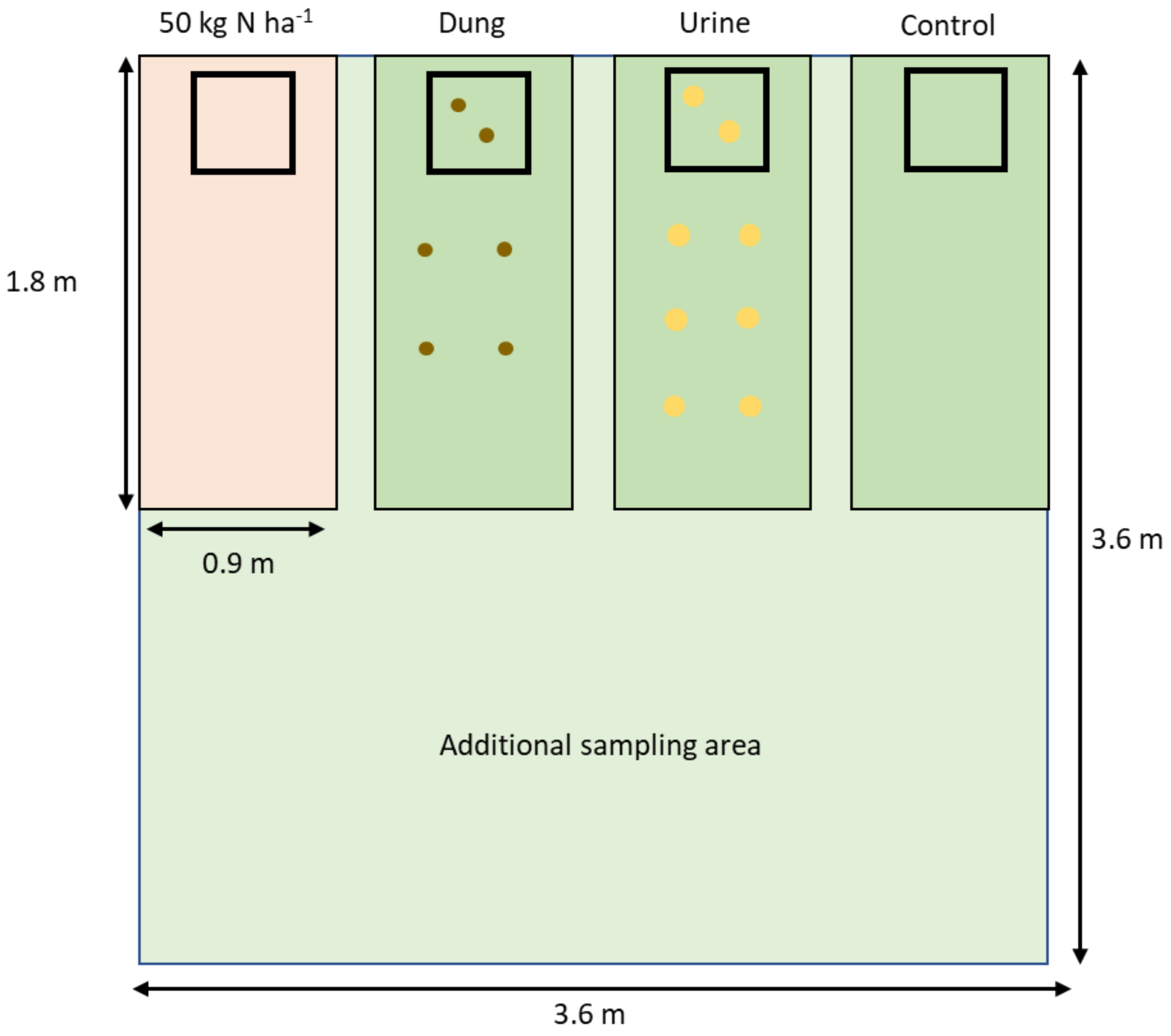
Field site:

Supplementary Figure 4.1: *Aerial drone photography of the field site. Grass-clover paddocks can be seen in the foreground, with the multispecies 'herbal' paddocks in the background. Exclusion plot location can be seen in each ca. 0.3 ha paddock. (Photography credit: Martine Graf, 2021).*

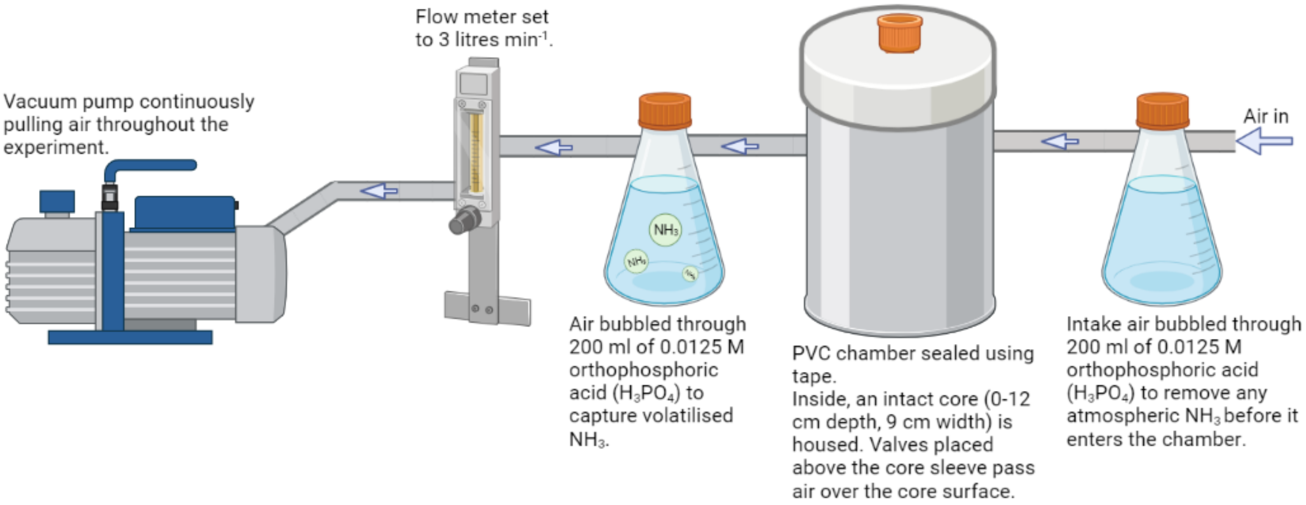


Supplementary Figure 4.2: *Domin score cover (%) of sown and unsown species in the herbal and the grass-clover ley assessed in July 2021. Data represents mean ± SEM, n = 3 per sward.*

Supplementary Figure 4.3: *Overhead photography of the herbal ley (left) and grass-clover ley (right) in summer 2021.*



Supplementary Figure 4.4. *Exclusion area on the field trial demonstrating subplot design. Headings denote treatments, however, note that the 50 kg N ha^{-1} ammonium nitrate treatment was only present in spring 2021. Black boxes indicate static greenhouse gas chambers. In autumn 2020, only one urine or dung patch was applied to each respective static greenhouse gas chamber, whereas in spring 2021 static gas chambers received two urine or dung patches. Plot design graphic is not to scale.*



Supplementary Figure 4.5. *Schematic of the bench-scale ammonia (NH_3) volatilisation system, described in Misselbrook et al. (2005). Captured NH_3 in the centre conical flask is used for analysis to determine NH_3 volatilisation from urine treated cores.*

***Supplementary Table 4.1**. Two-way ANOVA results of sward type, season, or the interaction between sward and season on measured sward characteristics. * indicates statistically significant values, significance level was determined as p < 0.05, degrees of freedom are presented within brackets.*

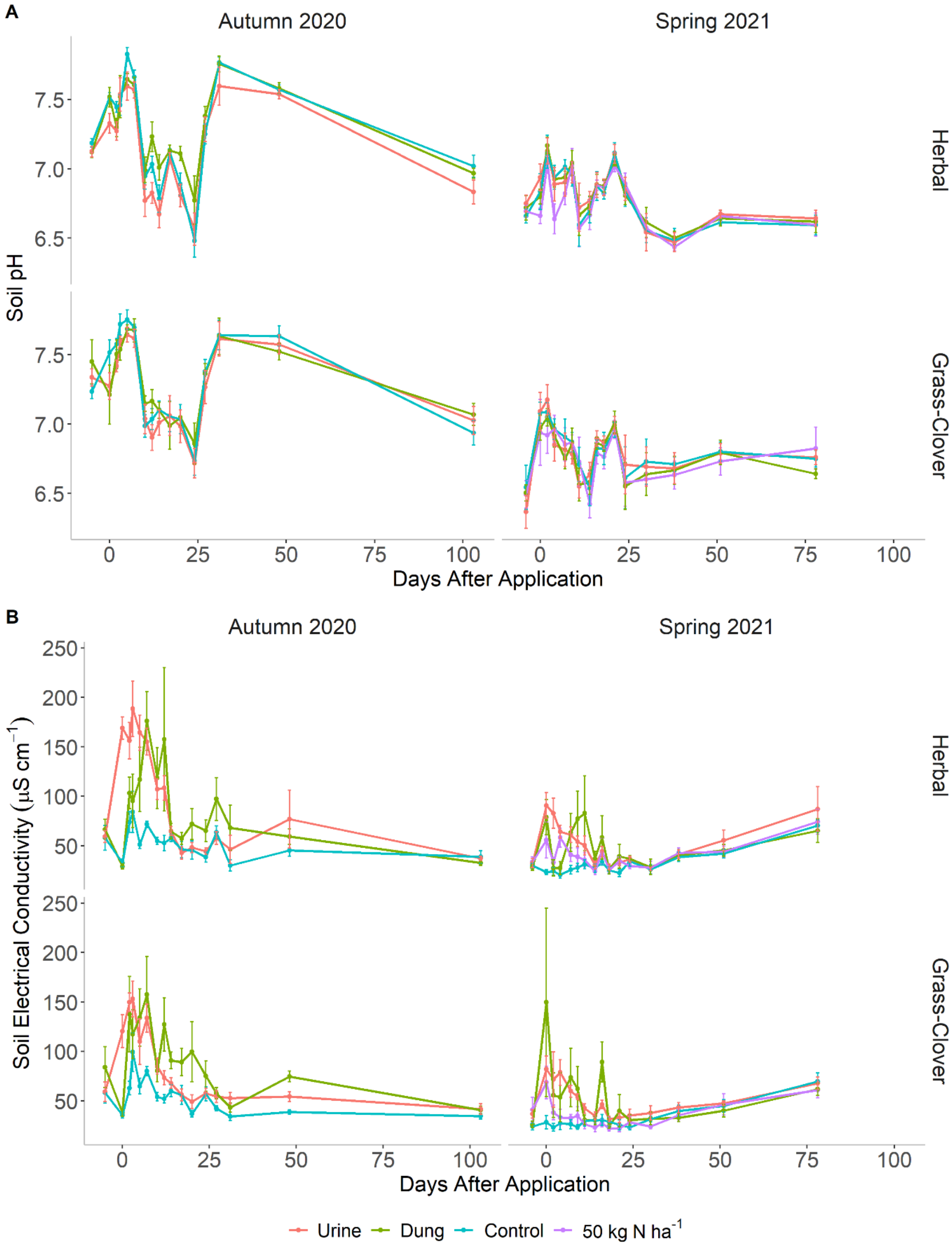
Sward Characteristic	Effect of sward type		Effect of season		Interaction between Sward × Season	
	$F_{(1,8)}$	*p*-value	$F_{(1,8)}$	*p*-value	$F_{(1,8)}$	*p*-value
Dry Matter	1.027	0.341	174.5	< 0.001*	1.534	0.251
Crude Protein	0.000	0.994	13.661	0.006*	4.465	0.068
Sugar	0.021	0.889	23.123	0.001*	1.436	0.265
Metabolisable energy	3.658	0.092	3.658	0.092	3.658	0.092
D-value	2.689	0.140	3.649	0.093	3.780	0.088
Sodium	27.780	< 0.001*	90.590	< 0.001*	26.730	< 0.001*

Supplementary Table 4.2. *Statistical results for the urine composition dataset following either a T-test or a Kruskal-Wallis test. Significant results are indicated by *. Significance level is set as $p < 0.05$, degrees of freedom are presented in brackets.*

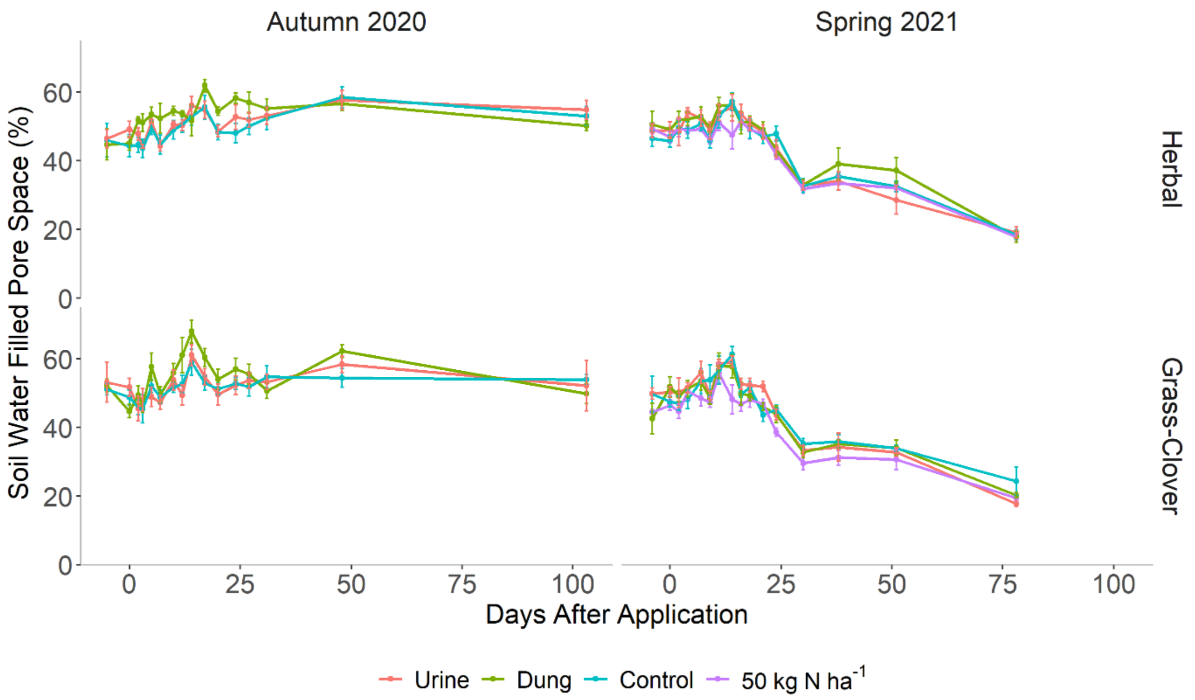
T-test				
Individual Urine Properties				
Urine properties	Autumn 2020		Spring 2021	
	T	*p*-value	T	*p*-value
pH	(173) 6.912	< 0.001*	-	-
Electrical conductivity	-	-	(129) 0.255	0.799
Urination event volume	(156) -3.718	< 0.001*	(132) 2.328	0.021*
Urine patch area	(149) 4.360	< 0.001*	(124) 0.630	0.530
Total N	(142) 1.151	0.252	(125) 0.333	0.740
Total organic C	(143) -0.006	0.996	(125) 0.426	0.671
N loading rate	(136) -8.433	< 0.001*	(125) 1.594	0.113
Ammonium	-	-	(121) -1.12	0.265
Kruskal-Wallis Test				
Urine properties	Autumn 2020		Spring 2021	
	Chi-squared	*p*-value	Chi-squared	*p*-value
pH	-	-	(1) 0.0002	0.987
Electrical conductivity	(1) 2.473	0.116	-	-
Nitrate	(1) 4.195	0.041*	(1) 0.035	0.852
Ammonium	(1) 7.186	0.007*	-	-
Bulked Urine Properties				
T-test				
Urine properties	Autumn 2020		Spring 2021	
	T	*p*-value	T	*p*-value
Urea	(11.0) -1.418	0.184	(6.64) -0.116	0.911
Sodium	(7.93) -5.459	< 0.001*	(9.99) -4.068	0.002*
Potassium	(8.76) 2.068	0.069	(9.82) -0.151	0.883
Calcium	-	-	(7.14) -0.745	0.480
Phosphorus	(10.4) 2.730	0.021*	(8.09) -0.818	0.437
Hippuric acid	(10.9) 0.625	0.545	-	-
Allantoin	(9.41) -0.977	0.353	(9.51) -0.593	0.567
Creatinine	(7.04) 0.778	0.462	(8.55) -0.536	0.606
Benzoic acid	(11.0) 0.557	0.589	(9.88) -0.729	0.483
Uric acid	(8.49) -0.403	0.697	(8.00) -0.887	0.401
Kruskal-Wallis Test				
Urine properties	Autumn 2020		Spring 2021	
	Chi-squared	*p*-value	Chi-squared	*p*-value
Calcium	(10) 12.000	0.285	-	-
Hippuric acid	-	-	(1) 0.936	0.333

Supplementary Table 4.3. *Statistical results for the dung composition dataset following either a T-test or a Kruskal-Wallis test within each grazing season. Significant results are indicated by *. Significance level is set as p < 0.05, degrees of freedom are given in brackets.*

T-test				
Dung Properties	Autumn 2020		Spring 2021	
	T-value	*p*-value	T-value	*p*-value
pH	(10.1) -0.814	0.434	(10.5) 0.956	0.361
Electrical conductivity	(5.67) 1.363	0.225	(8.80) -0.349	0.736
Moisture content	(9.38) 0.880	0.401	-	-
Dunging event weight	-	-	(102) 2.370	0.020*
Total N	(10.7) -1.861	0.090	(10.6) -0.240	0.815
Total dissolved C	(9.50) -1.867	0.093	(9.59) -0.122	0.905
Extractable nitrate	(7.80) -0.891	0.399	(9.76) 0.070	0.946
Extractable ammonium	(8.12) 0.649	0.534	(10.6) -0.815	0.433
Extractable phosphorus	n.d.	n.d.	(10.9) -0.519	0.614
Exchangeable sodium	n.d.	n.d.	(9.30) -0.580	0.576
Exchangeable calcium	n.d.	n.d.	(9.92) -1.096	0.299
Exchangeable potassium	n.d.	n.d.	(10.1) 1.039	0.323
Kruskal-Wallis Test				
Dung Properties	Autumn 2020		Spring 2021	
	Chi-squared	*p*-value	Chi-squared	*p*-value
Moisture content	-	-	(1.00) 0.000	1.000
Event weight	(1.00) 5.729	0.017*	-	-



Supplementary Figure 4.6: *Soil pH (panel A) and electrical conductivity (panel B) measured in autumn 2020 and spring 2021. Data represents mean ± SEM, n = 3 per treatment per sward, legend applies to both figures.*



Supplementary Figure 4.7. *Soil water filled pore space (%WFPS) measured at 0-10 cm depth over each experimental season. Data represents mean ± SEM.*

Chapter 5

Herbal leys have no effect on soil porosity, earthworm abundance, and microbial community composition compared to a grass-clover ley in a sheep grazed grassland after 2-years

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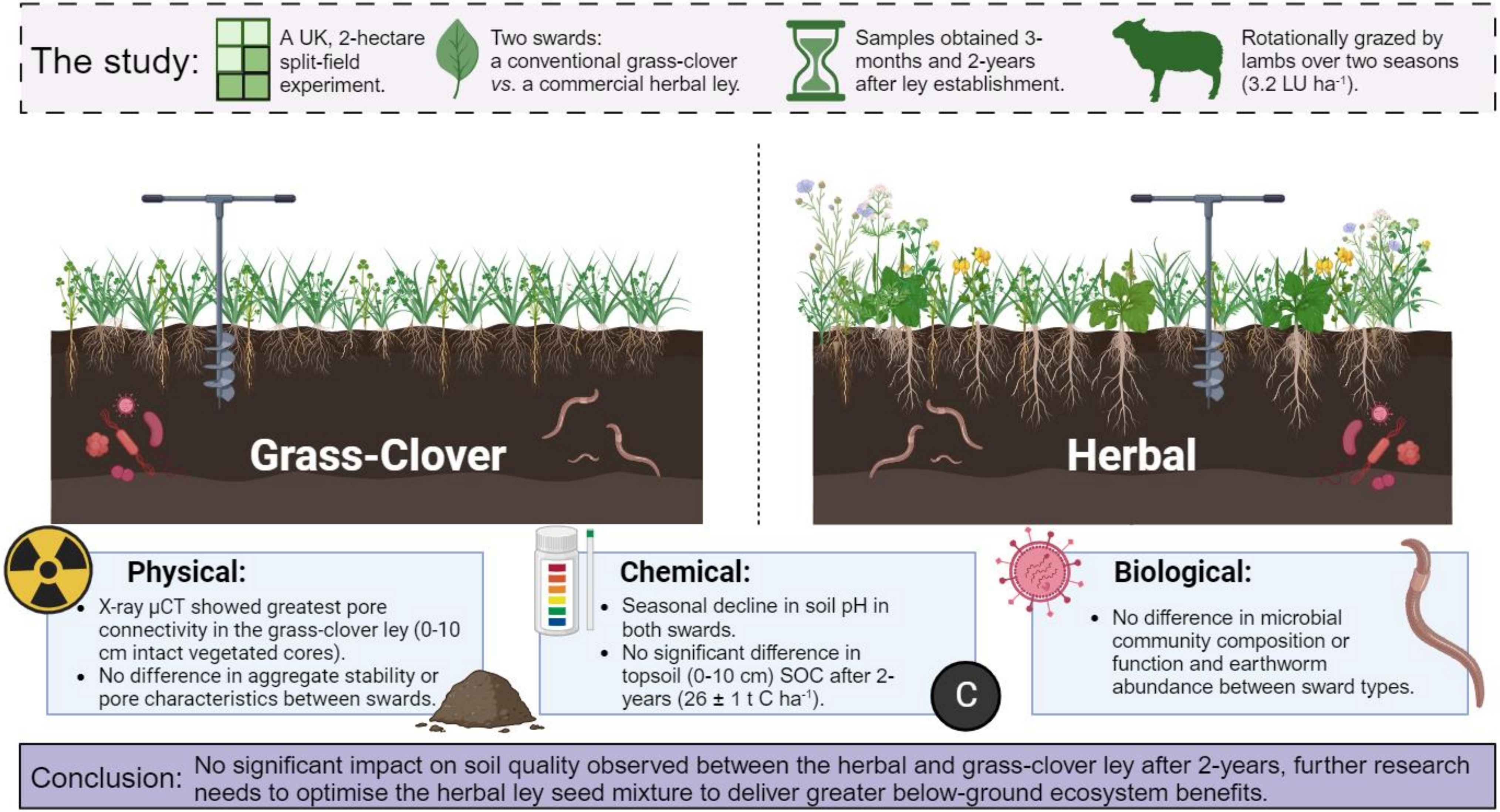

The study:

A UK, 2-hectare split-field experiment.

Two swards: a conventional grass-clover *vs.* a commercial herbal ley.

Samples obtained 3-months and 2-years after ley establishment.

Rotationally grazed by lambs over two seasons (3.2 LU ha^{-1}).

Physical:

- X-ray µCT showed greatest pore connectivity in the grass-clover ley (0-10 cm intact vegetated cores).
- No difference in aggregate stability or pore characteristics between swards.

Chemical:

- Seasonal decline in soil pH in both swards.
- No significant difference in topsoil (0-10 cm) SOC after 2-years (26 ± 1 t C ha^{-1}).

Biological:

- No difference in microbial community composition or function and earthworm abundance between sward types.

Conclusion: No significant impact on soil quality observed between the herbal and grass-clover ley after 2-years, further research needs to optimise the herbal ley seed mixture to deliver greater below-ground ecosystem benefits.

Figure 5.0. *Chapter 5 graphical abstract.*

Highlights

- Increasing grassland species richness can improve soil quality.
- X-ray µCT showed greatest pore connectivity in intact grass-clover ley cores.
- Sward type did not affect soil physical or chemical characteristics.
- Sward diversity did not affect earthworm abundance or microbial community composition.

Keywords

Multispecies sward, grass-clover ley, soil carbon, aggregate stability, X-ray µCT imaging

Abstract

Herbal leys (multispecies swards) can potentially deliver greater agronomic and environmental benefits than conventional grass-clover swards in grazed agroecosystems. However, despite their popularity in agri-environment schemes, little is known about the effect of herbal leys on soil physical (e.g., porosity), chemical (e.g., carbon), and biological (e.g., soil fauna) characteristics. In the UK, a 2-ha split-field experiment utilising a herbal or grass-clover ley (n = 3 per sward) aimed to investigate the effect of sward type on soil quality. Each sward was rotationally grazed by weaned lambs (3.2 LU ha^{-1}) over two grazing seasons, with soil physiochemical and biological characteristics assessed after 2-years using techniques such as X-ray micro Computed Tomography (µCT) and shallow shotgun sequencing. Soil chemical characteristics (e.g., pH, nitrate, ammonium) were unaffected by sward type, but varied seasonally. Similarly, topsoil (0-10 cm) organic carbon stocks measured after 2-years did not differ between the herbal (26.1 ± 1.1 t C ha^{-1}) and grass-clover ley (25.7 ± 1.1 t C ha^{-1}). X-ray µCT analysis revealed greater pore connectivity (Euler number) in grass-clover ley intact soil cores (0-10 cm depth, 7.5 cm diameter) than herbal ley cores dominated by *Plantago lanceolata* ($p = 0.008$). However, there was no sward-type difference in aggregate stability or general pore characteristics, determined using X-ray µCT, in air-dried 4 mm aggregates obtained from 0-5 or 5-10 cm depth, nor did sward type affect earthworm abundance, microbial community composition or functional genes. This study is the first to explore the effects of a commercial herbal ley on soil physical, chemical, or biological quality in rotationally grazed sheep pastures. While no improvements in soil quality were observed after 2-years, these findings have significant implications for agri-environment schemes aimed at promoting soil quality and sustainability, with further research needed to optimise the seed mixture and management regime to deliver greater long-term below-ground ecosystem service benefits.

5.1. Introduction

Permanent grasslands comprise 3.2 billion hectares (67 %) of global agricultural land (FAO, 2023) and provide numerous ecosystem services (e.g., climate regulation, carbon sequestration) (Bengtsson et al., 2019; Sollenberger et al., 2019) vital for supporting the delivery of climate change mitigation policies (e.g., ‘4 per mille’ initiative) (Brynes et al., 2018; Minasny et al., 2017). Globally, grasslands store approximately 525 to 634 Pg C (ca. 25-34 % of the terrestrial carbon stock) (Liu et al., 2023), with the potential to store an additional 0.28 Mg C yr^{-1} with improved grassland management (Conant et al., 2017). However, rapid agricultural intensification has contributed to the loss of grassland plant species diversity and reduced the abundance of legumes that symbiotically fix nitrogen, subsequently increasing reliance on agrochemicals (e.g., synthetic N inputs) to meet production demands (Allan et al., 2015). This has contributed to the widespread degradation of ca. 49 % of global grasslands (Bardgett et al., 2021) and the release of greenhouse gas emissions associated with fertiliser production and use (Dangal et al., 2019).

In grazed grasslands, soils are vulnerable to degradation via poor field (e.g., acidification) and livestock (e.g., overgrazing and excessive trampling) management practices, with erosion and compaction altering nutrient cycling and water infiltration rates at a local and catchment scale (Brynes et al., 2018). Further loss of grassland plant species richness has serious environmental and economic impacts through the decline of grassland ecosystem services (e.g., forage production, pollination) and multifunctionality (Bardgett et al., 2021; Cardinale et al., 2012; Schils et al., 2022). In 2007, the degradation of global grazed grasslands was estimated to cost 7.7 billion US dollars through the loss of meat and milk production, and has likely greatly increased since then (Nkonya et al., 2015). More recent disaggregated estimates of the total cost of soil degradation, attributed to the decline of ecosystem services, in improved grasslands across England and Wales identified soil compaction as a major driver of annual costs, accounting for losses of £145.9 million per year (Graves et al., 2015). This was followed by soil organic matter loss (£74.1 million yr^{-1}) and erosion (£14.8 million yr^{-1}) (Graves et al., 2015). As sward composition controls soil physical (e.g., porosity) (Gould et al., 2016), chemical (e.g., carbon) (Fornara and Tilman, 2008), and biological (e.g., soil fauna) (Zhang et al., 2022) characteristics that underpin ecosystem service delivery, it is a target for improved sustainability. As such, efforts to alleviate grassland degradation while improving productivity are often focussed on restoring plant species diversity through the reintroduction of legume and herb species into grass dominated systems (Schils et al., 2022).

Herbal leys (multispecies swards) consist of a highly diverse mixture of grasses, legumes, and herb species that can provide a multifaceted approach to improving ecosystem service delivery in grazed grasslands (Jordon et al., 2022). Deep-rooting herb and legume species frequently sown in herbal leys such as chicory (*Cichorium intybus*), ribwort plantain (*Plantago lanceolata*), and lucerne (*Medicago sativa*) can alter the release of root exudates and generate biopores that improve soil porosity (Uteau et al., 2013), aggregate stability (Pérès et al., 2013), and topsoil and subsoil carbon storage (Jobbágy and Jackson, 2000; McNally et al., 2015). This enhances water infiltration (Smettem and Collis-George, 1985), nutrient cycling (Kautz, 2015), earthworm abundance (Hyvönen et al., 2021), microbial activity (Lange et al., 2015), and forage production (Finn et al., 2013; Grange et al., 2021). However, as many previous studies utilise low-diversity experimental mixtures (e.g., 3-6 species) to explore the effect of sward composition on belowground ecosystem services, there is a lack of understanding of how commercial herbal leys (e.g., 9-18 species) that are widely marketed to improve soil quality affect soil quality in grazed grasslands. Consequently, as herbal leys are growing in popularity due to their promotion in agri-environment schemes (e.g., the UK Sustainable Farming Incentive Scheme, DEFRA (2023)), this represents a significant knowledge gap.

This study aimed to investigate if a commercial herbal ley can improve soil ecosystem service delivery in a grazed grassland compared to a conventional grass-clover ley. We hypothesised that i) biopores generated by deep and thick rooted plant species (e.g., *Cichorium intybus* and *Plantago lanceolata*) will increase soil porosity and improve overall soil structure beneath the herbal ley; ii) higher plant species richness within the herbal ley will increase topsoil (0-10 cm) soil organic matter content and soil organic carbon stocks; and iii) the highly diverse rhizosphere community generated by the high diversity of plant roots and litter in the herbal ley will generate a distinct microbial community composition and functional gene profile.

5.2. Materials and Methods

5.2.1. Site Description and Experimental Design

In July 2020, a 2-ha lowland permanent grass-clover pasture located at Bangor University’s Henfaes Research Centre, Abergwygregyn, North Wales, UK (10 m a.s.l., 53.240329 N, -4.014574 W) was ploughed and reseeded with either a commercially available 18-species (19 cultivar) herb- and legume-rich multispecies ley (herbal) or a conventional 5-species (9 cultivar) grass-clover ley (grass-clover) (Table 5.1) supplied by Cotswold Seeds (Cotswold Seeds Ltd., Moreton-in-Marsh, UK). Each sward covered 1 ha, split into three permanently fenced 0.33 ha paddocks (n = 3 per sward) to allow weaned Welsh mountain lambs (*Ovis aries*) to rotationally graze at a stocking density of 3.2 LU ha^{-1} (approximately n = 40 lambs per sward). The breed and sex of lamb grazed each experimental season represented seasonal differences in the local grazing system, with male ram lambs approximately 6-7 months old (average starting liveweight 22.1 ± 0.4 kg) grazing in autumn, and female ewe lambs approximately 10-11 months old (average starting liveweight 23.0 ± 0.3 kg) grazing in spring.

Lambs were grazed over two measurement seasons: autumn-winter 2020 (18/09/20 to 28/10/20) and spring-summer 2021 (12/04/21 to 28/06/21) (see Chapter 3 for more details). The field was left empty when sheep were not present. One fertiliser application of 50 kg N ha^{-1} ammonium nitrate, 20 kg P ha^{-1} and 60 kg K ha^{-1} was applied in March 2021 prior to the spring grazing season to promote forage growth, following the RB209 nutrient recommendations (AHDB, 2020).

Within each paddock, fenced exclusion areas (ca. 13.4 m^2) were established two months prior to grazing to provide an area protected from livestock access for soil sampling. The soil type at the field site was classified as a sandy clay loam, crumb structured, Eutric Cambisol, with an average rainfall and temperature of 1060 mm and 10.8 °C, respectively. Meteorological data was recorded every 30 min from an automated on-site weather station located approximately 200 m from the field site (Campbell Scientific Ltd., Leicestershire, UK) (see Supplementary Figure 5.1).

The botanical composition of each sward was assessed in July 2021 at the end of the spring-summer 2021 grazing experiment. Five 4 m2 quadrats were evaluated in each paddock, accounting for spatial variability, with the resulting Domin scores then converted to percentage cover using the Domin 2.6 transformation described in Currall (1987). Sward botanical composition and overhead photographs of the sward can be seen in Supplementary Figures 5.2 and 5.3, respectively.

Table 5.1. *Species composition and seeding rate of the herbal ley and grass-clover ley sown in July 2020.*

Plant type	**Herbal ley**			**Grass-clover ley**		
	Species and Cultivar	Proportion (%)	Seeding rate (kg ha^{-1})	Species and Cultivar	Proportion (%)	Seeding rate (kg ha^{-1})
Grass	Cocksfoot (*Dactylis glomerata*) cv. ‘Amba’	11.5	3.75	Perennial ryegrass (*Lolium perenne*) cv. ‘Glenstal’	30.8	10.0
	Festulolium cv. ‘Lofa’	11.5	3.75	Perennial ryegrass (*Lolium perenne*) cv. ‘AberMagic’	16.9	5.50
	Perennial ryegrass (*Lolium perenne*) cv. ‘Oakpark’	7.7	2.50	Timothy (*Phleum pratense*) cv. ‘Winnetou’	15.4	5.00
	Perennial ryegrass (*Lolium perenne*) cv. ‘Glenstal’	3.8	1.25	Cocksfoot (*Dactylis glomerata*) cv. ‘Amba’	15.4	5.00
	Timothy (*Phleum pratense*) cv. ‘Winnetou’	4.6	1.50	Hybrid ryegrass (*Lolium perenne*) cv. ‘Tetragraze’	11.5	3.75
	Tall fescue (*Festuca arundinacea*) cv. ‘Kora’	3.8	1.25			
	Meadow fescue (*Festuca pratensis*) cv. ‘Pardus’	3.1	1.00			
Legume	Sainfoin (*Onobrychis*)	19.2	6.25	Red clover (*Trifolium pratense*) cv. ‘AberClaret’	3.9	1.25
	Sweet clover (*Melilotus*)	6.2	2.00	White clover (*Trifolium repens*) cv. ‘AberDai’	3.1	1.00
	Red clover (*Trifolium pratense*) cv. ‘Milvus’	5.4	1.75	White clover (*Trifolium repens*) cv. ‘AberHerald’	2.3	0.75
	White clover (*Trifolium repens*) cv. ‘AberHerald’	3.8	1.25	Wild white clover (*Trifolium repens*) cv. ‘AberAce’	0.8	0.25
	Lucerne (*Medicago sativa*) cv. ‘Luzelle’	2.3	0.75			
	Alsike clover (*Trifolium hybridum*) cv. ‘Aurora’	1.5	0.50			
	Birdsfoot trefoil (*Lotus corniculatus*) cv. ‘Bull’	1.5	0.50			
Herb	Burnet (*Sanguisorba minor*)	5.4	1.75			
	Chicory (*Cichorium intybus*) cv. ‘Puna II’	4.6	1.50			
	Ribwort Plantain (*Plantago lanceolata*) cv. ‘Endurance’	1.5	0.50			
	Sheep’s Parsley (*Petroselenium crispum*)	1.5	0.50			
	Yarrow (*Achillea millefolium*)	0.8	0.25			

5.2.2. Soil Characteristics

Soil samples (0-10 cm, *n* = 3 per sward) were taken in August 2020 and November 2022 to identify differences in soil characteristics 1-month and 28-months, respectively, after ley establishment (Table 5.2). To account for any spatial variability across the field, 10 (0-10 cm depth) soil samples were randomly sampled across a 'W' transect within each paddock and homogenised to produce one replicate per paddock.

Briefly, soil pH and electrical conductivity (EC) were determined on fresh soil following a 1:2.5 w/v (soil:solution) DI H_2O extraction using standard electrodes. Gravimetric soil moisture content was determined by drying soil at 105 °C for 24 h. Following drying, soil organic matter was determined by loss-on-ignition (450 °C; 16 h; Ball, 1964). Initial soil bulk density (0.97 ± 0.06 g cm^{-3} herbal; 0.94 ± 0.04 g cm^{-3} grass-clover) was determined by inserting stainless steel rings (100 cm^{3}) into the soil at a depth of 0-5 cm and removing intact cores. Cores were then oven dried at 105 °C and sieved to < 2 mm to remove stones and large roots, then corrected for stone weight and volume to determine bulk density. Soil ammonium (NH_4^+) and nitrate (NO_3^-) were determined colorimetrically from 0.5 M K_2SO_4 extracts (1:5 w/v) using the methods described in Mulvaney (1996) and Miranda et al. (2001), respectively. Total extractable nitrogen and total extractable organic carbon were analysed using a Multi N/C 2100S analyser (AnalytikJena, Jena, Germany). Available P was determined colorimetrically from soil extracted in 0.5 M acetic acid (1:5 w/v) using the molybdate blue method of Murphy and Riley (1962). Exchangeable cations (Ca, Na, and K) were analysed using an Agilent 5800 ICP-OES (Agilent, USA) (see supplementary for more details). Soil microbial biomass C and N was determined by the chloroform fumigation-extraction method described in Voroney et al. (2008), with a k_{EC} and k_{EN} correction factor of 0.45 and 0.54 applied, respectively. Soil nitrogen mineralisation rate was determined through measuring soil NH_4^+ concentration before and after an anaerobic incubation at 40°C for 7 days then extracting soils in 1 M KCl (1:1 w/v) (Keeney, 1982).

5.2.3. Soil Physical and Chemical Properties

5.2.3.1. X-ray µCT Imaging for Soil Structure

X-ray micro Computed Tomography (µCT) imaging of intact vegetated soil cores (0-10 cm depth, 7.5 cm diam., 441.8 cm^3) and air-dried soil aggregates (ca. 4 mm) sampled from each sward type towards the end of the study (in March 2022) was conducted at the Hounsfield Facility, University of Nottingham, UK.

Intact vegetated soil cores (n = 9 per sward type, i.e., 3 per paddock) were collected and stored at 4 °C prior to scanning with a Phoenix v|tome|x m 240 kV X-ray tomography system (Waygate Technologies (a Baker Hughes company), Wunstorf, Germany) within 48 h of sampling to minimise disturbance from soil mesofauna. While cores from the grass-clover ley were sampled at random, key plants in the herbal ley such as *Cichorium intybus* and *Plantago lanceolata* were targeted to examine the effect of these taproots on the soil alongside a grass-clover mixture in the same sward (n = 3 per plant type was collected for the herbal ley). Each core was imaged using a voltage of 170 kV, current of 200 µA, voxel size resolution of 45 µm, and a scan time of 24 min. A total of 2879 images were collected per core and reconstructed using Datos|Rec software version 2.2.2 (Waygate Technologies (a Baker Hughes company) Wunstorf, Germany). To avoid edge effects caused by sampling, a 700 pixel × 700 pixel × 1651 image slice cuboid (31.5 mm × 31.5 mm × 74.3 mm) was selected for image analysis. Roots for illustration purposes were segmented from the solid matter using the 3D region growing tool in VGStudioMax version 3.4.3 (Volume Graphics GmbH, Heidelberg, Germany) (Supplementary Figure 5.4). The erode/dilate function with an opening/closing radius of 3 was applied to roots selected via the region grower tool within the intact cores to reduce the interference from the internal structure of tap roots, i.e., in *Cichorium intybus* or *Plantago lanceolata* dominated cores, on soil porosity measurements. Detected earthworms were also selected in the ROI and removed from the final exported solid ROI that was used for analysis.

Following imaging, intact soil cores were split into 0-5 cm and 5-10 cm fractions and sieved to ca. 4 mm then ca. 2 mm to remove plant and stone material. Sieved fractions were then air-dried at 20 °C for 24 h prior to selecting a 4 mm aggregate at random from each depth then scanning using a Nanotom® CT system (Waygate Technologies (a Baker Hughes company) Wunstorf, Germany). Each aggregate (*n* = 9 per sward type per depth) was scanned using a voltage of 80 kV, current of 100 µA, voxel size resolution of 3.5 µm, for 30 min per sample. A total of 2400 images were obtained per aggregate and reconstructed as described previously. As with the intact soil cores, a 400 pixel × 400 pixel × 301 image slice cuboid (1.4 mm × 1.4 mm × 1.1 mm) within each aggregate was selected for image analysis to avoid edge effects.

All reconstructed images were filtered by Adaptive Gauss (smoothing of 1, edge threshold of 0.08) and filtered with a median of 1.5 pixels. Reconstructed images were then analysed using ImageJ (version 2.9.0, FIJI 64-bit) to determine pore characteristics such as area, size and perimeter, pore size distribution and coefficient of uniformity (a ratio of the pore size distribution expressed by D60:D10). The BoneJ (Domander et al., 2021) plugin was used to determine pore connectivity (Euler number) and pore thickness.

5.2.3.2. Aggregate Stability

Soil aggregate stability was determined using the Kemper and Rosenau (1986) method. Briefly, 2 g of air-dried aggregates obtained from cores used for the X-ray µCT imaging ca. 2

mm in size were added to 250 µm soil sieves then immersed in DI H_2O and gently vertically shaken for 30 mins using a wet sieving apparatus (Royal Eijkelkamp, Giesbeek, the Netherlands). Remaining aggregates were then dried at 30 °C overnight and reweighed before immersing in 2 M NaOH and gently vertically shaken for a further 30 mins. Aggregates remaining after this step were dried overnight at 30 °C and the final weight recorded.

5.2.3.3. Bulk Density (0-10 cm), Soil Organic Matter and Soil Organic Carbon Stock

As it was not possible to determine bulk density from the intact vegetated cores used for the X-ray µCT imaging dataset, further cores were taken to determine soil bulk density by inserting plastic rings (441.8 cm^3) into the soil at a depth of 0-10 cm and removing intact cores from each grazing exclusion plot (n = 3 analytical replicates per plot per paddock, resulting in n = 3 per sward) after two seasons of sheep grazing (April 2023, 33-months after establishment). Collected cores were then oven-dried at 105 °C, ground and sieved to < 2 mm to remove stones and large roots, then corrected for stone weight and volume to determine fine earth bulk density. Soil organic matter was then determined from dry soil using the loss-on-ignition method described previously. Soil organic carbon (SOC) stock was determined using a regression equation relating weight loss on ignition to SOC previously validated for soils within the region (Ball, 1964). Briefly, the equation is as follows:

$$\text{SOC (\%)} = 0.458 \times \text{SOM (\%)} - 0.4$$

$$\text{SOC stock (t C ha}^{-1}\text{)} = \text{SOC (\%)} \times \text{Bulk density (g cm}^{-3}\text{)} \times 10$$

5.2.3.4. VESS and VSA Scoring

In November 2022, intact soil blocks (15 ×15 × 20 cm) were extracted from within each exclusion area (n = 3 per sward) to visually assess soil structure, with each soil horizon assessed using the visual evaluation of soil structure (VESS) guide and visual scoring assessment (VSA) outlined in Shepherd (2000).

5.2.4. Soil Biological Properties

5.2.4.1. Earthworm Abundance and Biomass

Earthworm sampling was conducted in November 2022, when each sward was approximately 28 months old. Sampling was conducted in November as it was not possible to sample sooner as the field experiment underwent drought conditions in spring and summer of the same year. Briefly, soil blocks (15 × 15 × 20 cm) used for VESS and VSA scoring described previously were obtained from each exclusion area (*n* = 3 per sward) and destructively sampled to obtain earthworms, based on the method described in Fusaro et al. (2018). Once collected, earthworms were washed to remove soil, then dried with paper towel before hand-sorting into type (epigeic, endogeic or anecic) prior to counting and weighing. Results were then upscaled to provide earthworm abundance (number) and biomass (weight) per square meter.

5.2.4.2. Microbial Community Composition

Soil microbial community composition was determined using the shallow shotgun sequencing service provided by Microbiome Insights (British Columbia, Canada). Briefly, freeze-dried soil samples (0-10 cm; 250 mg soil) collected from each of the herbal or a grass-clover ley paddocks (*n* = 9 analytical replicates per sward) in August 2022 (25-months after establishment) were extracted with the Qiagen MagAttract PowerSoil DNA DF kit (Qiagen, Hilden, Germany) using a KingFisher robot to obtain soil DNA. Extracted DNA quality was then evaluated visually via gel electrophoresis and quantified using a Qubit 3.0 fluorometer

(Thermo-Fischer, Waltham, MA, USA). Libraries were prepared using an Illumina Nextera library preparation kit using an in-house protocol (Illumina, San Diego, CA, USA).

Paired-end sequencing (150 bp x 2) was conducted on a NextSeq 500 (Illumina, San Diego, CA, USA) with shotgun metagenomic sequence reads processed using the Sunbeam Pipeline. Initial quality evaluation was done using the FastQC software (version 0.11.5; Bioinformatics Group at the Babraham Institute, 2022). Processing took place in four steps: adapter removal, read trimming, low-complexity-reads removal, and host-sequence removals. Adapter removal was completed using cutadapt software (version 2.6; Martin, 2015). Read trimming was done using the Trimmomatic software (version 0.36; Bolger and Lohse, 2014) set with custom parameters (LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:36). Low-complexity sequences were detected using Komplexity software (version 0.3.6; Clarke et al., 2019). High-quality reads were mapped to the human genome (Genome Reference Consortium Human Reference 37) and those reads mapped to it were removed from the analysis. Remaining reads were taxonomically classified using Kraken2 with the PlusPF database from May 17th, 2011 (Wood et al., 2019) (Supplementary Table 5.1). For functional profiling, high-quality filtered reads were aligned against the SEED database via translated homology search and annotate to their subsequent subsystems, or functional levels, 1-3 using the Super-Focus software (Silva et al., 2016).

5.2.5. Statistical Analysis

Data was analysed in R studio (version 4.2.1) with graphical images produced using the ‘ggplot2’ package (version 3.3.6., Wickham (2016)). Prior to analysis, all data was tested for normality using the Shapiro Wilks test (R core stats package) and homogeneity of variance using the Levene’s test (‘car’ package, version 3.1.1.). If assumptions were not met following log10-transformation, then data were analysed using a non-parametric test (e.g., Kruskal-Wallis test) where appropriate.

Data that met the assumptions for parametric testing were then analysed as follows. Independent sample t-tests were used for within season soil chemical characteristics (microbial biomass nitrogen, N mineralisation rate, total extractable nitrogen, exchangeable Na, Ca, and K), final soil bulk density and soil organic matter measurements, soil organic carbon stock estimates, and VESS and VSA scores. A one-way ANOVA was used for intact soil core pore characteristics. A two-way ANOVA was used to assess the interaction between the fixed factors of season and soil pH, electrical conductivity, moisture content, microbial biomass carbon, total dissolved carbon, extractable NH_4^+, NO_3^-, available P, aggregate pore characteristics, aggregate stability, earthworm abundance and biomass. A permutational multivariate analysis of variance (PERMANOVA) was conducted to assess the effect of sward type on the Shannon diversity index, taxonomic and functional gene diversity data using the ‘adonis2’ function in the ‘vegan’ package (version 2.6.4., Oksanen et al. (2020)). Significance level was set at $p < 0.05$. Values presented in the text represent mean ± SEM unless otherwise stated.

5.3. Results

5.3.1. Soil Chemical Properties

Sward type did not affect soil chemical characteristics measured 1-month (August 2020) and 28-months after sward establishment (November 2022) ($p > 0.05$; Table 5.2 and Supplementary Table 5.2). Season, however, did have a significant effect on soil organic matter content ($F_{(1,8)} = 7.416$, $p = 0.026$), nitrate concentration ($F_{(1,8)} = 143.35$, $p < 0.001$), pH ($F_{(1,8)} = 17.447$, $p = 0.003$) and microbial biomass C ($F_{(1,8)} = 9.423$, $p = 0.015$). A Tukey post-hoc test showed a small but significant decline in soil microbial biomass C of 0.65 g C kg^{-1} in the grass-clover ley ($p = 0.025$) and a decrease in soil pH in the herbal ($p = 0.004$) and grass-clover ($p = 0.011$) ley 28-months after establishment. Similarly, an increase in soil nitrate was observed in the herbal ($p = 0.005$) and grass-clover ($p = 0.007$) ley after 28-months.

5.3.2. Soil Bulk Density, Soil Organic Matter and SOC Stock

Soil bulk density did not differ between the herbal (0.86 ± 0.02 g cm^{-3}) and grass-clover ley (0.81 ± 0.03 g cm^{-3}) sampled from the grazing exclusion areas 33-months after establishment ($T_{(12.8)} = 1.200$, $p = 0.252$). Similarly, no difference was found between sward types in the topsoil (0-10 cm) soil organic matter content (75 ± 3.4 g kg^{-1} herbal *vs*. 89 ± 11 g kg^{-1} grass-clover; $K_{(1)} = 0.926$, $p = 0.336$). Subsequently, soil organic carbon stock in the 0-10 cm soil layer was unaffected by sward type (26.1 ± 1.1 t C ha^{-1} herbal *vs*. 25.7 ± 1.1 t C ha^{-1} grass-clover; $T_{(14.0)} = 0.240$, $p = 0.814$).

Table 5.2. *Soil chemical properties (0-10 cm) of a grass-clover and herbal ley measured 1-month and 28-months after establishment in August 2020 and November 2022, respectively. Results are expressed on a dry weight basis and as mean ± SEM, n = 3 per treatment. n.d. = not determined. l.o.d = limit of detection. Lowercase and uppercase letters indicate statistical differences within and between seasons respectively, significance level is determined as* $p < 0.05$.

Soil chemical properties	August 2020		November 2022	
	Grass-Clover	Herbal	Grass-Clover	Herbal
pH	6.99 ± 0.06^{aA}	6.90 ± 0.08^{aC}	6.51 ± 0.12^{aB}	6.65 ± 0.07^{aD}
Electrical conductivity (μS cm^{-1})	38 ± 5^{aA}	51 ± 14^{aA}	44 ± 3^{aA}	55 ± 10^{aA}
Moisture content (g g^{-1})	0.31 ± 0.01^{aA}	0.33 ± 0.02^{aA}	0.31 ± 0.01^{aA}	0.32 ± 0.02^{aA}
Organic matter (g kg^{-1})	56.4 ± 3.7^{aA}	54.5 ± 3.1^{aC}	63.6 ± 1.5^{aB}	65.3 ± 4.3^{aD}
Microbial biomass carbon (g C kg^{-1})	1.67 ± 0.02^{aA}	2.02 ± 0.44^{aA}	1.02 ± 0.10^{aB}	1.15 ± 0.21^{aA}
Microbial biomass nitrogen (mg N kg^{-1})	124.0 ± 17.7^{a}	115.4 ± 14.5^{a}	l.o.d.	l.o.d.
N mineralisation rate (mg N kg^{-1} day^{-1})	5.87 ± 2.45^{a}	6.68 ± 0.93^{a}	n.d.	n.d.
Total extractable nitrogen (mg N kg^{-1})	29.75 ± 9.68^{a}	20.35 ± 1.69^{a}	l.o.d.	l.o.d.
Total dissolved carbon (mg C kg^{-1})	74.3 ± 2.5^{aA}	71.7 ± 3.2^{aA}	22.3 ± 9.1^{aA}	73.7 ± 29.3^{aA}
Extractable ammonium (mg NH_4^+-N kg^{-1})	13.97 ± 11.85^{aA}	5.58 ± 0.77^{aA}	3.75 ± 0.36^{aA}	3.05 ± 0.34^{aA}
Extractable nitrate (mg NO_3^--N kg^{-1})	0.91 ± 0.27^{aA}	0.94 ± 0.04^{aC}	2.86 ± 0.12^{aB}	3.00 ± 0.15^{aD}
Extractable phosphorus (mg P kg^{-1})	11.17 ± 2.96^{aA}	13.35 ± 4.96^{aA}	7.26 ± 0.86^{aA}	4.68 ± 0.60^{aA}
Exchangeable sodium (mg Na kg^{-1})	0.55 ± 0.01^{a}	0.65 ± 0.12^{a}	n.d.	n.d.
Exchangeable calcium (mg Ca kg^{-1})	105.1 ± 18.9^{a}	85.2 ± 4.2^{a}	n.d.	n.d.
Exchangeable potassium (mg K kg^{-1})	2.74 ± 1.40^{a}	1.83 ± 0.39^{a}	n.d.	n.d.

5.3.3. Soil Pore Characteristics

5.3.3.1. Intact Cores

X-ray µCT imaging did not show any major differences in soil pore characteristics measured from intact soil cores obtained from either a grass-clover ley or an herbal ley dominated by *Cichorium intybus*, *Plantago lanceolata*, or a grass mixture (Table 5.3). Example 2D and 3D images demonstrating pore structure from each core type are shown in Figure 5.1. Biopores generated by earthworm activity were observed in all samples.

In general, total porosity was greatest in the herbal ley cores dominated by *Cichorium intybus* (7.45 ± 0.32 %), with the lowest porosity values observed in the grass-clover cores (4.99 ± 0.56 %), although no statistical significance between core type was found ($F_{(3)} = 2.269$, $p = 0.125$). Surprisingly, however, sward type had a significant effect on pore connectivity (indicated by Euler number, with higher numbers indicating poorer connectivity) ($F_{(3)} = 6.003$, $p = 0.008$). A Tukey post-hoc test showed greater pore connectivity in grass-clover cores than herbal ley cores dominated by *Plantago lanceolata* ($p = 0.004$), however, no statistical differences were observed between the other cores ($p > 0.05$).

Table 5.3. *Average pore characteristics per image slice measured from the intact soil cores ROI. Data represents mean ± SEM, n = 9 for grass-clover cores, n = 3 per herbal ley cores dominated with either Cichorium intybus, Plantago lanceolata, or a grass mixture. Superscripted letters indicated statistical differences; significance level is determined as p < 0.05.*

Pore Characteristic	Herbal – *Cichorium intybus*	Herbal – *Plantago lanceolata*	Herbal – Grass Mix	Grass-Clover
Total porosity (%)	7.45 ± 0.32^{a}	5.16 ± 0.70^{a}	6.14 ± 0.86^{a}	4.99 ± 0.56^{a}
Total pore area (mm^2)	73.9 ± 3.2^{a}	51.2 ± 6.9^{a}	60.9 ± 8.5^{a}	49.5 ± 5.6^{a}
Perimeter (mm)	2.21 ± 0.30^{a}	1.54 ± 0.11^{a}	2.01 ± 0.17^{a}	2.08 ± 0.14^{a}
Thickness (mm)	1.09 ± 0.18^{a}	0.70 ± 0.08^{a}	0.83 ± 0.14^{a}	1.15 ± 0.19^{a}
Euler number	12843 ± 4941^{a}	27873 ± 4394^{ab}	13532 ± 3413^{a}	10021 ± 1791^{ac}
$PSD_{D60/D10}$	72.5 ± 10.5^{a}	53.6 ± 8.1^{a}	50.7 ± 4.8^{a}	54.4 ± 7.0^{a}

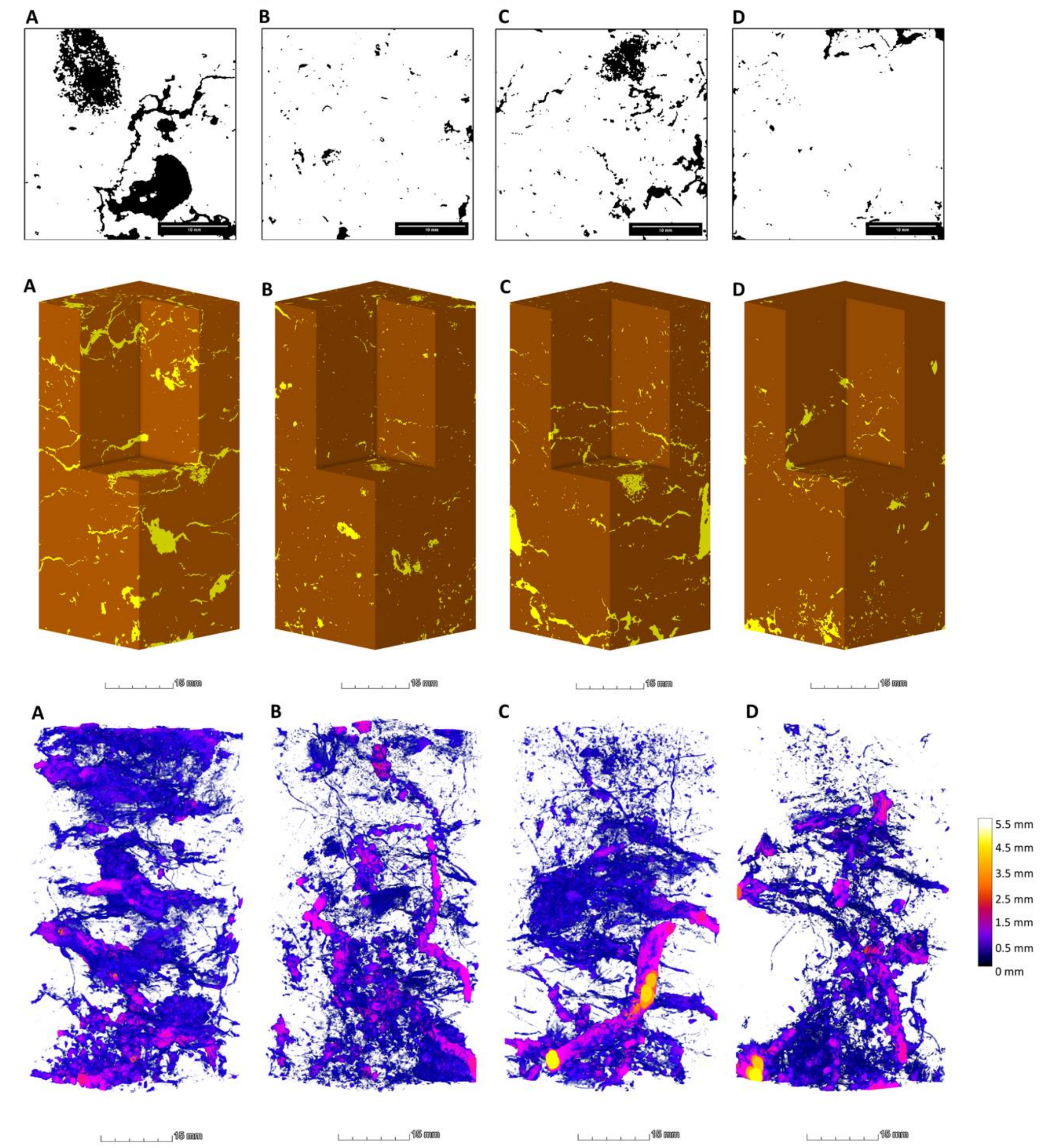

Figure 5.1. *Example X-ray µCT images of the ROI measured from intact soil cores; A = herbal - Cichorium intybus, B = herbal – Plantago lanceolata, C = herbal – grass-mix, D = grass-clover. Top row shows 2D binary slices of the middle of the ROI, black indicates pore space and white soil; scale bar displays 10 mm. Middle row shows a 3D reconstruction of the ROI, pore space is indicated in yellow and soil in brown; scale bar displays 15 mm. Bottom row shows a 3D heatmap of pore thickness with the soil removed; the heatmap scale bar showing pore width (0 mm to 5.5 mm) and bottom scale bar displays 15 mm.*

5.3.3.2. 4 mm Aggregate Size Fractions

At the aggregate scale, pore characteristics were not affected by dominant plant type (grass-clover *vs*. herbal *Cichorium intybus*, *Plantago lanceolata*, or grass-mix) or soil depth (0-5 cm or 5-10 cm) ($p > 0.05$) (Supplementary Table 5.5 and 5.7). Aggregates obtained from the 0-5 cm soil layer from the herbal ley cores dominated by *Plantago lanceolata* tended to have a greater total porosity, however, the interaction between dominant plant species and sampling depth was not significant ($F_{(3,16)} = 1.353$, $p = 0.293$).

5.3.3.3. Pore Size Distribution of Intact Cores and 4 mm Aggregates

Pore size distribution was unaffected by dominant plant type in intact soil cores ($F_{(3,172)} = 0.670$, $p = 0.571$) (Figure 5.2) and in aggregates from the 0-5 cm and 5-10 cm soil layers ($F_{(1,208)} = 1.637$, $p = 0.182$) (Supplementary Figure 5.5).

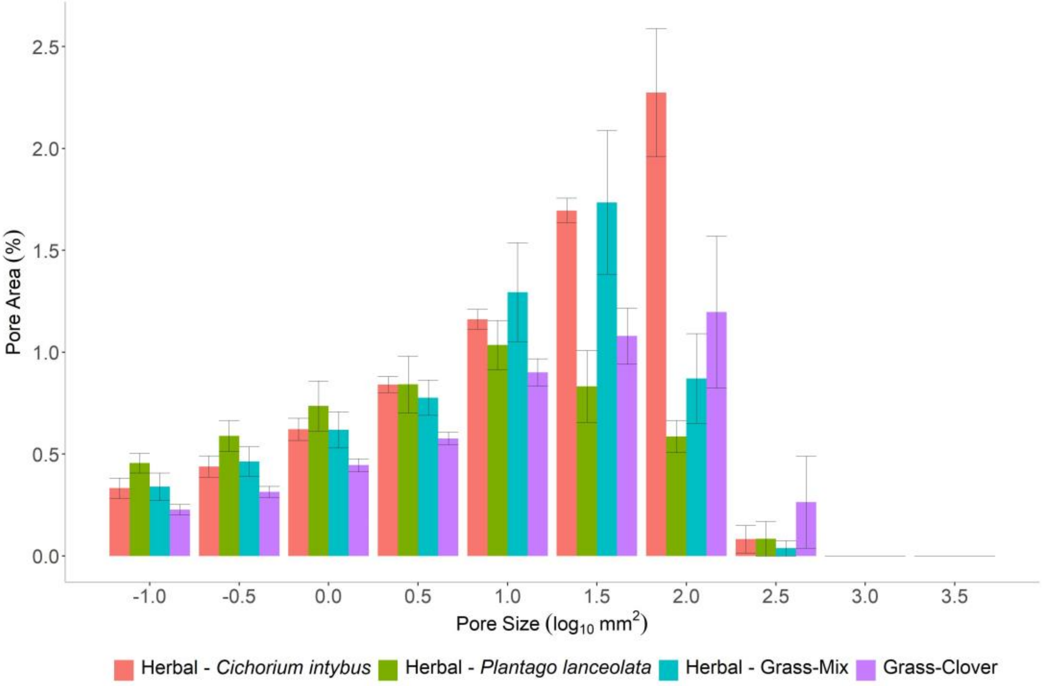

Figure 5.2. *Pore size distribution for the ROI from intact soil cores obtained from a grass-clover ley (n = 9) or a herbal ley dominated by Cichorium intybus, Plantago lanceolata or a grass mixture (n = 3 per plant type). Data represents mean ± SEM.*

5.3.4. Aggregate Stability

Aggregate stability was not influenced by sward type or soil depth ($F_{(5,24)} = 0.282$, $p = 0.918$). There were no differences in average stability of the ca. 2 mm aggregates in the 0-5 cm depths between cores obtained from the herbal ley that were predominately *Cichorium intybus* (65.2 ± 0.2 %), *Plantago lanceolata* (68.2 ± 1.5 %) or grass-mixture (67.7 ± 3.2 %), and the grass-clover ley (65.9 ± 1.0 %). Aggregates obtained from the 5-10 cm depth had consistently, but not significantly, lower aggregate stability of 64.6 ± 2.1 % in the grass-clover ley, with this ranging in the herbal ley from 59.2 ± 1.2 % with *Cichorium intybus*, 63.2 ± 5.0 % with *Plantago lanceolata* and 64.3 ± 6.9 % with the grass-mixture.

5.3.5. VESS and VSA Scoring

Sward type did not influence soil structure as assessed using the VESS ($K_{(1)} = 2.064$, $p = 0.151$) or VSA ($K_{(1)} = 0.083$, $p = 0.773$) scoring system. After 2-years, soils were generally deemed to be in good condition based on the overall scores. VESS scores were similar between the herbal (2.8 ± 0.2) and grass-clover (2.4 ± 0.2) ley, with minor differences in VSA scores between the two swards (26.0 ± 1.0 herbal *vs*. 26.5 ± 0.6 grass-clover) driven by slight variations in earthworm abundance.

5.3.6. Soil Biological Properties

5.3.6.1. Earthworm Abundance and Biomass

Following 33-months of ley establishment there was no significant difference in total earthworm abundance (903.7 ± 92.8 worms m^{-2} herbal *vs*. 908.6 ± 100.2 worms m^{-2} grass-clover) ($T_{(4)} = -0.036$, $p = 0.973$) or total earthworm biomass (363.7 ± 15.2 g m^{-2} herbal *vs*. 287.8 ± 68.9 g m^{-2} grass-clover) ($T_{(4)} = 1.077$, $p = 0.342$) between the two sward types. There was no significant interaction between earthworm ecotype and sward type on earthworm abundance ($F_{(2,36)}$ = 1.057, p = 0.358) and earthworm biomass ($F_{(2,36)}$ = 0.149, p = 0.862). Epigeic earthworms were dominant in both swards, followed by endogeic then anecic ecotypes (Figure 5.3).

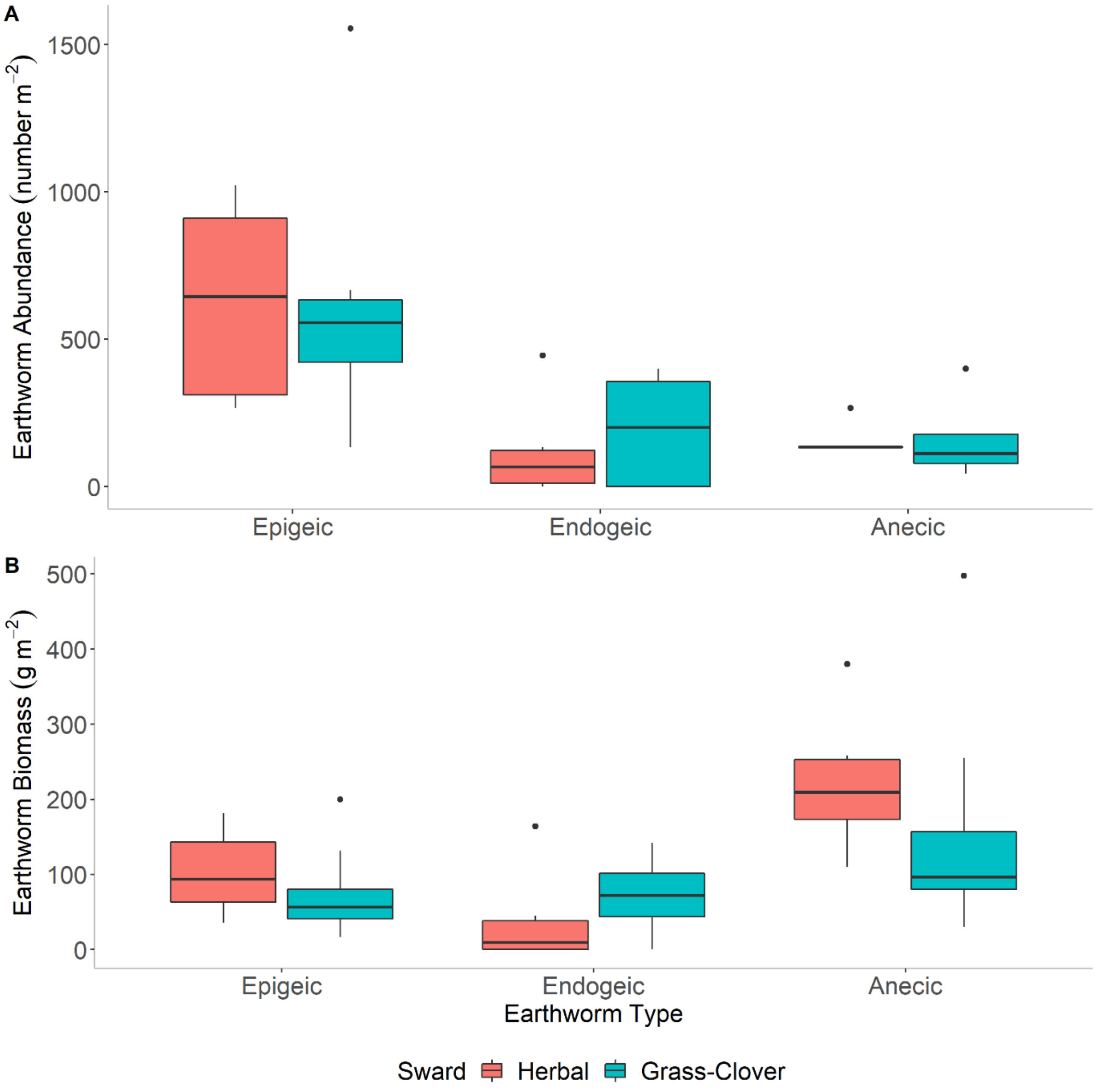

Figure 5.3. *Earthworm abundance (panel A) and earthworm biomass (panel B) measured in November 2022, 2-years after establishing the herbal and grass-clover ley. Legend applies to both panels, n = 6 and n = 8 measurements per earthworm type in the herbal and grass-clover ley, respectively. Boxplots display the median and interquartile range, with whiskers showing minimum and maximum values in the data, and dots indicating potential outliers.*

5.3.6.2. Microbial Community Composition

Sward type did not influence soil taxonomic profiles ($R^2 = 0.08$, $p = 0.131$) or functional profiles ($R^2 = 0.07$, $p = 0.117$) 2-years after ley establishment. Shallow shotgun sequencing obtained 179,168 ± 9945 classified reads per sample, with bacteria dominating the communities followed by archaea, eukaryotes, and viruses accounting for 99.26 %, 0.17 %, 0.57 %, and 0.002 % of the reads, respectively (Supplementary Table 5.1). Bacteria in soil from both swards were dominated by Proteobacteria (50.5 %), Actinobacteria (41.6 %), and Planctomycetes (2.3 %) (Supplementary Figure 5.6). At a species level, alpha diversity in taxonomic profiles using Shannon's diversity index (H') did not differ between the herbal and grass-clover ley ($K_{(1)} = 0.75$, $p = 0.387$) (Figure 5.4). Similarly, functional genes did not differ between sward types ($R^2 = 0.074$, $p = 0.117$) (Supplementary Figure 5.7).

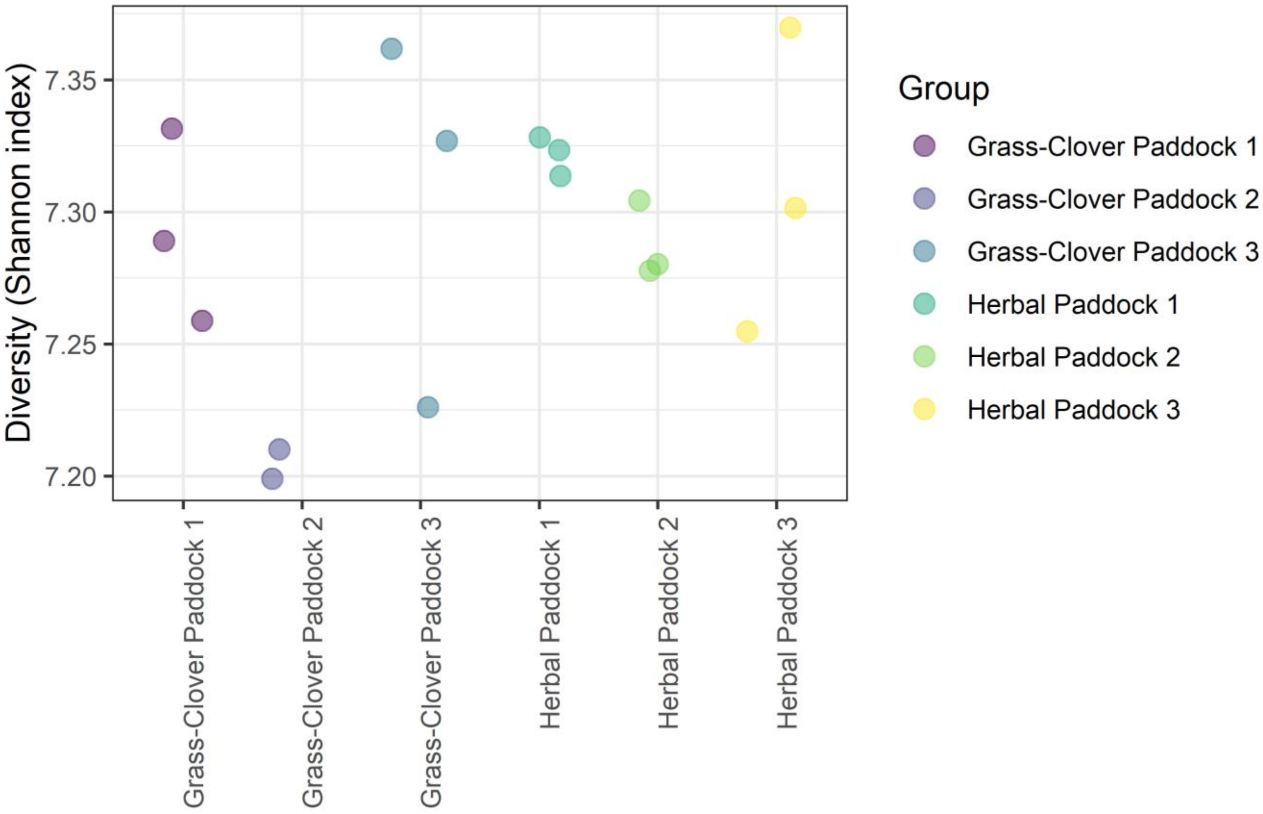

Figure 5.4. *Shannon Diversity index (H') at a species-level of the herbal and grass-clover ley, sampled 2-years after establishment, n = 8 grass-clover, n = 9 herbal ley.*

5.4. Discussion

5.4.1. Soil Structure, Aggregate Stability, and Earthworm Abundance

Previous studies have reported an improvement in soil porosity following the inclusion of dicotyledonous (tap-rooted) legume and herb species such as *Cichorium intybus*, *Medicago sativa* and *Plantago lanceolata* in leys within a crop rotation (Han et al., 2015; McCallum et al., 2004; Pagenkemper et al., 2015; Pulido-Moncada et al., 2020; Uteau et al., 2013). Taproot species can generate continuous biopores (> 2 mm in diameter) within the soil profile via the binding of soil particles through the secretion of extra cellular polysaccharides (Pierret et al., 2007), shrink-swelling processes (Uteau et al., 2013), and root channel generation once the original root material has decayed (Kautz, 2015).

Biopores can potentially improve the overall hydrological functioning of the soil by increasing subsoil infiltration, subsequently reducing the local flood risk (Smettem and Collis-George, 1985). The new channels created by biopores can allow growing monocotyledonous (fibrous-rooted) species access to subsoil nutrients and water (Kautz, 2015), consequently altering nutrient cycling across the soil profile (Pierret et al., 2007). However, beneficial effects for soil structure are highly site (e.g., field management, grazing *vs*. mowing), soil (e.g., parent material), depth (e.g., topsoil *vs*. subsoil), plant (e.g., root architecture), and time dependent, with mixed effects observed when taproot species are utilised in monocultures (e.g., Burr-Hersey et al., 2020) versus low-diversity mixtures (up to 9 species) at the mesocosm or field scale (e.g., Gould et al., 2016; Pulido-Moncada et al., 2020). Surprisingly, to date, there are currently no studies investigating the effect of taproot species on soil structure when included in a commercial herbal ley mixture, particularly under grazing. Our study has shown that after 2-years of establishment, the herbal ley did not significantly improve soil total porosity, bulk density, or aggregate stability compared to a conventional grass-clover ley, thus our first hypothesis is rejected.

In this study, a slightly greater total porosity was observed in herbal ley cores dominated by *Cichorium intybus* (7.5 %) than *Plantago lanceolata* (5.2 %) or comprised of a grass mixture within the herbal ley (6.1 %), however, it was surprising that these did not significantly differ from the grass-clover ley (5.0 %) despite the presence of taproot species and greater overall plant species richness. These findings conflict with previous research reporting improvements in porosity across the soil profile, particularly within the subsoil. For example, in an arable-ley experiment, Han et al. (2015) reported a greater biopore presence across a 0-75 cm depth following a year of *Cichorium intybus* (2.3 %) than tall fescue (*Festuca arundinacea*) (1.5 %), with the following wheat (*Triticum aestivum*) crop utilising the biopore network created by *Cichorium intybus* to produce a greater root length (24 m m^{-2}) than *Triticum aestivum* grown after *Festuca arundinacea* (6 m m^{-2}). In a similar experiment, greater macroporosity (> 4 mm diameter) was observed below 45 cm depth 2-years after the establishment of *Cichorium intybus* (2.2 %) compared to fescue (*Festuca*) (1.5 %) and *Medicago sativa* (1.1 %), with *Cichorium intybus* producing a 1.5-1.8 times greater biopore network than other measured species (Pagenkemper et al., 2015). Uteau et al. (2013) also reported greater subsoil (below 75 cm) macroporosity following *Medicago sativa* (13.6 %) than *Cichorium intybus* (2.5 %) or *Festuca* (3.4 %), however, this was mainly attributed to the high-water requirements of *Medicago sativa* generating biopores via shrink-swelling processes and the subsequent cracking of clay. While the topsoil (0-10 cm) layer sampled in our study is highly important for nutrient cycling and hydrological functioning in grazed pastures due to interaction with the grazing livestock (Brynes et al., 2018; Greenwood and McKenzie, 2001; Newell-Price et al., 2013), this shallow sampling depth missed changes in subsoil pore characteristics, where it is likely differences between swards might be greater.

The lack of differences in soil porosity, bulk density and aggregate stability measured between the swards in this study may be due to several factors. First, within the 2-year timeframe of the experiment, it is possible that due to slow root turnover the primary taproot of species such as *Cichorium intybus, Medicago sativa* and *Plantago lanceolata* present in the herbal ley may not have decayed and thus generated biopores that could have been quantified by X-ray µCT imaging. Instead, it is likely that rapid root turnover of fine roots (< 0.5 mm diameter) occurred and produced micropores that contributed to similar total porosity between cores (Houde et al., 2020; Stewart and Frank, 2008). Despite the importance of root turnover for soil carbon regulation and nutrient cycling, this is generally understudied in common pasture species, with median root longevity in temperate climates estimated to be between 14 and 131 days (Reid et al., 2015). Limited data are available for herb and legume species, with no estimates available for key species such as *Plantago lanceolata*. Typically, a faster turnover rate is observed in conventional grass-clover species such as perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*), with 1.3 % and 0.97 % of the root system replaced per day respectively, equivalent to the total root system replaced 3.5-3.8 times a year (Reid et al., 2015). In taproot species this is much slower, with 0.84 % and 1.0 % of the root system replaced per day in *Cichorium intybus* and *Medicago sativa*, respectively (Reid et al., 2015). Climatic (e.g., soil temperature, moisture) and field management factors (e.g., grazing *vs*. mowing) can alter the rate of root turnover (Wang et al., 2019), with a greater turnover rate observed under simulated grazing (Reid et al., 2015) and in the topsoil due to the greater nutrient availability enabling rapid exploration by fine roots (Houde et al., 2020).

Secondly, the dense root network created by the high proportion of sown grasses (46 % herbal *vs*. 90 % grass-clover) and legumes (39.9 % herbal *vs*. 10 % grass-clover) in both swards likely enabled the greater enmeshing of soil particles by fine roots and fungal hyphae, physically reducing soil porosity through the binding of soil aggregates (Haynes and Beare, 1997; Pérès et al., 2013; Pohl et al., 2012; Reinhart and Vermeire, 2016). A sward botanical composition survey conducted in July 2021 revealed that of the 18 species sown in the herbal ley, only 11 species persisted after two grazing seasons (Supplementary Figure 5.2), with key taproot herbs *Plantago lanceolata* and *Cichorium intybus* comprising 4.9 ± 1.2 % and 21.1 ± 1.3 % of the sward composition, respectively. These proportions were likely too small to improve total porosity and bulk density at the field scale. Likewise, root-generated biopores can form at the expense of smaller micropores (< 0.3 mm in diameter), leading to no overall change in total porosity and bulk density (McCallum et al., 2004). In grasslands, a positive relationship between higher plant species richness and aggregate stability is generally assumed due to the increased presence of fungal hyphae and exudation of binding agents from the elevated rhizosphere microbial biomass (Haynes and Beare, 1997; Pérès et al., 2013; Pohl et al., 2012; Zangerlé et al., 2011). Aggregate stability in grazed pastures is often used as an indicator of soil susceptibility to erosion and runoff, with surface aggregate formation linked to soil sealing and reduced infiltration (Pohl et al., 2012). In our study, we observed no difference in 2 mm aggregate stability between the herbal (ca. 64 %) and grass-clover ley (ca. 65 %) after 2-years. This finding conflicts with previous research by Pohl et al. (2012), who reported a linear increase in aggregate stability with plant species richness up to a maximum of 8-species in a loamy soil. However, this was measured 16 to 44 years after the initial disturbance (Pohl et al. 2012), unlike after 2-years as in our study. Similarly, it is important to note that most studies compare aggregate stability in arable-ley systems (e.g., Guest et al. (2022)) with differing soil structure, soil texture, and initial soil organic matter content, therefore there is limited information available for improved grasslands. Although it is difficult to compare our findings to previous research, it does support our theory that the fine root network produced by high proportions of grasses and legumes in both swards likely enmeshed soil aggregates, resulting in no difference in aggregate stability between the grass-clover and herbal ley.

Finally, the high earthworm abundance recorded in the herbal (ca. 904 worms m^{-2}) and grass-clover (ca. 909 worms m^{-2}) ley after 2-years potentially biased pore characteristics determined via X-ray µCT. During the imaging process, earthworms and earthworm-generated biopores were present in the majority of sampled intact cores, with the subsequent 3D image reconstruction illustrating their presence in the pore thickness images (Figure 5.1, panel C and D, bottom row). Earthworm activity likely contributed to the greater pore connectivity (lower Euler number) observed in the grass-clover cores than the herbal ley cores dominated by *Plantago lanceolata* despite clear differences in root architecture that would be expected to have an opposing effect (Table 5.3 and Supplementary Figure 5.4). It is possible that future mesocosm experiments established in the absence of earthworms may demonstrate greater improvements in pore characteristics due to the differences in root architecture, however, due to the field-scale nature of this experiment it was not possible to prevent earthworm interactions in this study. Compared to arable soils where the underlying root architecture drives changes in soil structure due to tillage practices reducing earthworm populations (Briones and Schmidt, 2017), in grasslands, earthworms have a greater influence on soil structure than roots, and thus should be included in soil quality analyses.

In grasslands, earthworms are used as a biological indicator of soil quality to evaluate the sustainability of soil management practices (Fusaro et al., 2018; Paoletti, 1999). Despite their importance for aggregate stability, nutrient cycling, and litter decomposition, little is known about the effect of increasing grassland plant diversity on earthworm abundance and community composition (Birkhofer et al., 2011; Hyvönen et al., 2021). While previous studies have reported shifts in earthworm diversity with greater plant species richness (e.g., Zhang et al. (2022)) it is unclear if these effects are driven by higher plant diversity itself, or by the abundance of legume species (e.g., *Trifolium repens*) within the mixture generating a more palatable plant litter with a low C:N ratio preferred by earthworms (Eisenhauer et al., 2009;

Hyvönen et al., 2021; Piotrowska et al., 2013). Bioactive herb and legume species (e.g., *Cichorium intybus* and *Plantago lanceolata*) frequently included in herbal ley mixtures can also produce plant litter rich in secondary metabolites such as phenolics, saponins and tannins that can reduce the rate of litter decomposition in soils (Veen et al., 2019). However, it is unclear if plant litter rich in secondary metabolites will have a beneficial or detrimental effect on soil fauna, as this is largely understudied in grasslands (Veen et al., 2019). Although litter composition was not determined in this study, no shift in earthworm community composition was observed between the herbal and grass-clover ley, supporting previous research by Birkhofer et al. (2011) and Gastine et al. (2003) who reported no positive effect of plant species richness on earthworm abundance and diversity. As with soil aggregate stability, this is likely driven by the high proportion of legumes included in each sward. Similarly, as earthworm abundance did not differ between the two swards, it is likely that this contributed to the lack of improvement in aggregate stability as earthworms can drive the formation of micro- and macroaggregates through the excretion of mucilage (Pérès et al., 2013; Six and Paustian, 2014). However, these results may be confounded by the single sampling point that occurred in November 2022, where the cooler soil temperatures and higher soil moisture content may have affected earthworm activity.

Due to the complex nature of herbal leys, it is difficult to identify the mechanisms underpinning changes, or the lack thereof, in soil physical characteristics. Potential improvements in soil porosity, bulk density and aggregate stability on commercial farms following an herbal ley will largely be dependent on the parent soil material (e.g., texture, mineralogy), seed mixture (e.g., proportions of plant functional groups), the establishment and subsequent abundance of the different species, which will change dynamically with grazing and seasonality, and field management regime (e.g., previous arable crops or livestock stocking density). Future research would benefit from assessing changes in topsoil and subsoil physical characteristics across a greater variety of temporal and spatial scales. This will capture variations in sward composition and subsequently root architecture, providing a better understanding of the bioengineering potential of these swards and their effect on soil fauna across seasons, ultimately building on our understanding of the role of herbal leys for nutrient cycling.

5.4.2. Soil Organic Matter and Carbon Stocks

The herbal ley did not significantly increase topsoil (0-10 cm) soil organic matter content or SOC stocks compared to the grass-clover ley over a 2-year period, thus our second hypothesis is rejected. This conflicts with previous studies reporting an increase in SOC in species rich grasslands compared to conventional swards (e.g., Dijkstra et al., 2006; Fornara and Tilman, 2008; Lange et al., 2023; Mellado-Vázquez et al., 2016). The inclusion of herbs and legumes in grasslands is proposed to increase SOC accumulation in the soil profile through the greater root biomass (McNally et al., 2015) leading to the enhanced physical protection of soil organic matter within soil aggregates (Schmidt et al., 2011), with deep-rooting plant species such as *Plantago lanceolata* contributing to the greater vertical deposition of SOC (Jobbágy and Jackson, 2000), enhanced symbiosis with mycorrhizal fungi (Pol et al., 2021) and higher fine root turnover (Kagiya et al., 2019; Reid et al., 2015). Similarly, combining plant functional groups can alter aboveground plant litter chemistry (Dijkstra et al., 2006) and rhizodeposition (Shahzad et al., 2015), particularly the diversity and amount of compounds released in root exudates (e.g., amino acids, sugars, secondary metabolites) (Badri and Vivanco, 2009), subsequently increasing SOC accumulation via enhanced microbial activity and necromass formation (Lange et al., 2015).

Rhizodeposition of carbon in grasslands involves a broad range of processes such as gaseous production and losses, root exudation, carbon flow via mycorrhizae, death and lysis of root cells, and polymer (e.g., mucilage) secretion by living root cells (Jones et al., 2009), and is largely controlled by biotic (e.g., plant age, mesofauna) and abiotic (e.g., soil pH, temperature) factors (Jones et al., 2004). As such, rhizodeposition rates vary between plant species and functional groups (Steinauer et al., 2016), with a higher carbon rhizodeposition observed in grass and legume species than taproot herbs (Hafner and Kuzyakov, 2016). This is often attributed to the faster fine root turnover and more evenly distributed root biomass across the soil profile, with previous studies reporting an 8-times greater topsoil rhizodeposition in legumes such as *Medicago sativa* (3.1 t C ha^{-1} yr^{-1}) than herb species such as *Cichorium intybus* (0.6 t C ha^{-1} yr^{-1}) (Hafner and Kuzyakov, 2016). However, as rhizodeposition rates can vary both temporally and spatially within the sward, it is notoriously difficult to estimate the contribution this makes to SOC accumulation beneath diverse species mixtures, such as herbal leys. Instead, previous studies reporting a higher SOC content beneath a 16-species mixture (0.7 ± 0.1 t C ha^{-1} yr^{-1}) than monocultures of the same species (0.1 ± 0.1 t C ha^{-1} yr^{-1}) associated this increase with the greater root biomass and root turnover within species rich mixtures (Fornara and Tilman, 2008). Similar findings were reported in McNally et al. (2015), who estimated that the higher root mass beneath a moderately diverse pasture (5.3-9.4 t ha^{-1}) containing *Cichorium intybus* and *Medicago sativa* increased topsoil (0-30 cm) carbon inputs by 1.2 t C ha^{-1} compared to the root mass of a conventional grass-clover ley (3.8-5.7 t ha^{-1}).

Although no measurement of root biomass or root turnover was made in this study, the similar topsoil SOC content within the herbal (26.1 ± 1.1 t C ha^{-1}) and grass-clover (25.7 ± 1.1 t C ha^{-1}) ley 2-years after establishment may be due to the high proportion of grasses and legumes in each sward creating a similar root architecture and thus root turnover rate. At the field-scale, the herbal ley contained a low proportion of taproot herb species, with *Cichorium intybus* and *Plantago lanceolata* only comprising 4.6 % and 1.5 % of the sown composition, respectively. As a result, dominating grass and legume species in each sward likely created a fine root network responsible for the similar topsoil (0-5 cm and 5-10 cm) aggregate stability discussed previously. This likely provided an equal level of physical protection for soil particulate organic matter (POM) within soil aggregates in the herbal and grass-clover ley. However, while there is limited data available for SOC sequestration in herbal leys, particularly when introduced in grasslands, our findings corroborate with previous studies reporting no correlation between plant species diversity and SOC accumulation when legume species with a strong effect on ecosystem function, such as red clover (*Trifolium pratense*), are included in the sward mixture (De Deyn et al., 2010).

It is important to note that as rates of SOC accumulation in mineral soils are typically slow and can reach an upper 'saturation' limit within the stable mineral-associated organic matter (MAOM) fraction of ca. 50 g C kg^{-1}, any additional topsoil SOC accrual produced via altering the sward composition likely occurs within the POM fraction and thus is vulnerable to losses (Guillaume et al., 2022; Paustian et al., 2019; Schmidt et al., 2011; Six et al., 2002). Although approximately 80 % of soils are below the MAOM fraction carbon saturation limit and have potential for further SOC accumulation (Cotrufo et al., 2019), permanent grasslands are likely saturated due to long-term management practices (e.g., lack of tillage) and, if grazed, carbon inputs via livestock excreta deposition (Abdalla et al., 2018; Guillaume et al., 2022). Therefore, it is likely that the field experiment in this study was already near the MAOM carbon saturation limit, as it was previously a sheep grazed permanent grassland prior to ploughing and reseeding with the herbal or grass-clover ley in July 2020, thus reducing the potential for SOC accrual beneath each respective sward. While a slight temporal increase in soil organic matter was observed in both swards between August 2020 and November 2022 (Table 5.2), it is not clear if this was in the unprotected POM fraction or the stable MAOM fraction, therefore further investigation is needed. However, we can assume that during the 2-year measurement period in this study, the increase in soil organic matter was likely in the former POM fraction.

Subsequently, as herbal leys require reseeding every 3-4 years to maintain the higher plant species diversity necessary to qualify for agri-environment schemes, conventional reseeding techniques (e.g., ploughing) will likely result in losses of newly acquired SOC within the POM fraction due to the faster microbial turnover rate releasing carbon dioxide (CO_2) (Paustian et al., 2019).

Conventional short-term ploughing and reseeding cycles to maintain the species composition of herbal leys in both grazed grasslands and crop rotations may lead to a slow increase in MAOM. However, to determine this, long-term experiments (e.g., 5-25 years) are required to capture net changes in topsoil and subsoil SOC dynamics, as changes in SOC accumulation are not always consistent (Hopkins et al., 2009) and the repeated 'refreshing' of herbal leys may lead to an overall loss of newly acquired SOC (Paustian et al., 2019), although some alternative techniques (e.g., min-tillage, overseeding) can be used to reduce this loss. While our study provides an insight into topsoil SOC accrual beneath a herbal and grass-clover ley, it is limited by the shallow sampling depth and short-term measurement period (ca. 2-years). Therefore, future studies would benefit from assessing how species-rich herbal leys affect SOC dynamics across various temporal and spatial scales, utilising national research networks to capture changes in SOC with varying field management and underlying soil mineralogy.

5.4.3. Soil Microbial Community Composition

Given that soil physiochemical characteristics were unaffected by sward type in this study, it is unsurprising that the topsoil (0-10 cm) microbial community composition also did not differ between the herbal and the grass-clover ley, thus our third hypothesis is rejected. While our findings conflict with previous studies reporting a higher microbial biomass (e.g., Chen et al., 2019; Tilman et al., 2001; Wardle and Nicholson, 1996) and altered microbial community composition (e.g., Fox et al., 2020; Lange et al., 2015; Leff et al., 2018) with increasing plant species richness, they do support previous research by Steinauer et al. (2016), who reported no effect of plant diversity on microbial richness, evenness, and Shannon Diversity index.

In grasslands, combining plant functional groups can affect the underlying soil microbial community composition and function (Berg and Smalla, 2009) by changing the soil structure (Gould et al., 2016), altering water and nutrient availability (Fornara and Tilman, 2008), above- and belowground plant litter chemistry (Dijkstra et al., 2006), and the release and chemical composition of root exudates (Badri and Vivanco, 2009). A recent meta-analysis has shown that, on average, increasing plant diversity can alter the ratio of soil fungi:bacteria, and can increase total microbial and fungal biomass by 12.5 % and 10.9 %, respectively, compared to monocultures of the same species (Chen et al., 2019). Introducing 'bioactive' plant species, such as *Plantago lanceolata*, into the sward can release high-levels of secondary metabolites (e.g., aucubin and catapol) into the soil through root exudates, protecting root tissue from pathogens and soil fauna herbivory (Wurst et al., 2010). As aucubin can inhibit the enzymatic activity of ammonia monooxygenase, it can therefore increase soil nitrogen availability for soil microbes and neighbouring plant species (Subbarao et al., 2007; Vi et al., 2023), contributing to increasing the overall soil microbial biomass. Similarly, sesquiterpene lactones and tannins released via the root exudates of *Cichorium intybus* have antimicrobial properties and can bind with soil organic matter to prevent degradation, subsequently reducing carbon mineralisation and increasing overall SOC accumulation (Kagiya et al., 2019). Consequently, this can induce shifts in the soil microbial community composition. However, although this has been well-documented in pot (e.g., Kagiya et al., 2019; Ristok et al., 2019; Wurst et al., 2010) and mesocosm scale (e.g., Fox et al. (2020) and Leff et al. (2018)) experiments that often explore the effect of single species and low-diversity (e.g., 3-9 species) mixtures, it has rarely been documented in field-scale experiments utilising complex commercial (e.g., 9-16 species) herbal mixtures.

Similarly, it is notoriously difficult to measure the rate and composition of root exudates in an ecological context *in situ* in the field (Oburger and Jones, 2018). Consequently, there is currently no information detailing how root exudation changes with increasing plant species richness in grasslands within a short-term (e.g., 1-4 years) or long-term (e.g., 5-25 years) context. Therefore, we can assume that although this study is limited by a single sampling point 2-years after sward establishment, the lack of changes in soil microbial community composition between swards is likely driven by the similar soil physiochemical characteristics and the low abundance of bioactive species within the herbal ley. The singular sampling point in this study likely missed any changes in community composition both in the short-term and long-term context, as it is possible that the pre-existing microbial community structure was stable as the field experiment was previously long-term permanent grass-clover pasture prior to reseeding for this study.

Given the rising popularity of herbal leys in both arable and grassland systems, future studies should aim to capture changes in microbial community composition and function (e.g., changes in N cycling genes) across the soil profile using isotopic tracing techniques (e.g., ^{15}N, ^{18}O or ^{14}C) throughout the sward lifecycle to fully understand how sward composition affects microbial activity (e.g., Jones et al., 2019), diversity (e.g., Schwartz et al., 2016), and rates of turnover (e.g., Caro et al., 2023).

5.5. Conclusions

Improving soil physical, chemical, and biological characteristics in grasslands can enhance overall ecosystem service delivery, vital for improving the sustainability of lowland agriculture. This is the first study to explore the effect of commercial herbal leys on below-ground ecosystem services, with the findings of our study revealing that a current commercial herbal ley mixture does not improve various aspects of soil quality compared to a conventional grass-clover ley on a sheep grazed pasture established on a sandy clay loam. Although this study is limited by the short sampling duration (i.e., 2-years after sward establishment) and shallow sampling depth (0-10 cm), it does provide a valuable insight into the effects of herbal leys on soil quality in a sheep grazed grassland. The lack of differences in soil ecosystem services delivered by the herbal ley in this study is likely due to the low proportion of taproot species and the dominance of grass and legume species generating a similar root architecture, and thus soil characteristics, to the grass-clover ley.

Therefore, further research is needed to explore the best practices to establish and maintain optimal functional diversity in herbal leys to deliver the promised ecosystem benefits given the growing popularity of herbal leys in agri-environment schemes. Long-term national-scale studies are needed to assess the impact of herbal leys compared to grass or grass-clover leys on soil quality, capturing variations in soil mineralogy, field and grazing management, sward composition and age. Overall, we can conclude that the additional costs to farmers of commercial herbal leys (with a typical seed cost of ca. £200-250 ha^{-1}) compared to grass-clover leys (ca. £150 ha^{-1}) is not rewarded in this case through the delivery of greater below-ground ecosystem services observed during this 2-year study. Instead, further refinement of herbal leys is needed prior to wide-scale adoption, as currently conventional grass-clover leys provide equal ecosystem benefits.

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Ethical Statement

No ethical approval required.

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Declaration of interest

The authors declare they have no competing interests.

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5.7. Supplementary Materials

ICP-OES methods:

Samples were analysed using an Agilent 5800 ICP-OES fitted with an Agilent SPS 4 autosampler using an axial torch configuration. Plasma flow was set at 15 l min^{-1} and nebuliser pressure set at 240 kPa, with a sample uptake delay of 40 s and a rinse time of 20 s using dry argon as a carrier gas. Data was analysed using Software ICP Expert Pro with all elements measured fully quantified against a 3-point calibration using standards from Sigma-Aldrich.

Supplementary Table 5.1. *Number of reads following shallow shotgun sequencing on soil samples (0-10 cm, n = 9 per sward) obtained from a 2-year-old herbal or grass-clover ley.*

Sample ID	**Number of raw reads**	**Classified reads**	**Chordate reads**	**Artificial reads**	**Unclassified reads**	**Microbial reads**	**Bacterial reads**	**Viral reads**	**Fungal reads**	**Protozoan reads**
1.1-H	886,815	230,369	282	0	656,446	230,036	227,226	149	333	72
1.2-H	880,741	222,173	254	0	658,568	221,879	218,964	122	366	74
1.3-H	837,973	219,191	211	0	618,782	218,920	216,146	119	357	64
2.1-H	635,568	163,112	180	0	472,456	162,896	160,931	73	243	39
2.2-H	497,702	131,586	138	0	366,116	131,420	129,789	80	221	53
2.3-H	661,251	179,896	166	0	481,355	179,687	177,531	102	275	48
3.1-H	586,473	145,438	153	0	441,035	145,258	143,548	76	228	54
3.2-H	1,094,392	290,711	346	0	803,681	290,301	286,565	154	456	94
3.3-H	801,276	193,823	224	0	607,453	193,558	191,018	106	338	76
1.1-GC	556,010	135,795	198	0	420,215	135,569	133,757	67	241	54
1.2-GC	654,105	165,799	177	0	488,306	165,584	163,525	78	224	40
1.3-GC	529,767	136,780	128	0	392,987	136,619	134,979	63	177	37
2.1-GC	487,788	136,110	144	0	351,678	135,941	134,447	80	225	31
2.2-GC	643,277	184,509	134	0	458,768	184,331	182,377	77	205	50
2.3-GC	649,258	179,109	134	0	470,149	178,928	177,069	65	191	33
3.1-GC	632,144	176,956	132	0	455,188	176,777	174,968	70	223	40
3.2-GC	558,838	136,601	165	0	422,237	136,405	134,672	69	215	37
3.3-GC	789,176	197,059	255	0	592,117	196,761	194,264	102	308	58

Supplementary Table 5.2. *Two-way ANOVA results of sward type and measurement season on soil characteristics. * indicates statistically significant values, significance level was determined as* $p < 0.05$.

Soil Characteristic	Effect of sward type		Effect of season		Interaction between Sward × Season	
	$F_{(1,8)}$	*p*-value	$F_{(1,8)}$	*p*-value	$F_{(1,8)}$	*p*-value
pH	1.764	0.221	17.447	0.003*	0.083	0.780
Electrical conductivity	0.022	0.885	0.382	0.553	1.748	0.223
Moisture content	0.683	0.433	0.708	0.425	1.092	0.327
Organic matter	0.003	0.961	7.416	0.026*	0.304	0.597
Microbial biomass carbon	0.932	0.363	9.423	0.015*	0.180	0.682
Total dissolved carbon	2.488	0.153	2.613	0.145	3.048	0.119
Extractable Ammonium	0.585	0.466	1.152	0.314	0.418	0.536
Extractable Nitrate	0.270	0.617	143.35	< 0.001*	0.097	0.763
Extractable Phosphorus	0.005	0.946	4.597	0.064	0.658	0.441

Supplementary Table 5.3. *Average pore characteristics per image slice of ca. 4 mm air-dried aggregates obtained from X-ray µCT imaging following the destruction of the intact soil cores. Data represents mean ± SEM, n = 9 per depth for aggregate obtained from grass-clover ley cores and n = 3 per depth per aggregate for herbal ley cores dominated with either Cichorium intybus, Plantago lanceolata, or a grass-mix. No significant differences were found between samples; thus no superscripted letters are presented (p > 0.05).*

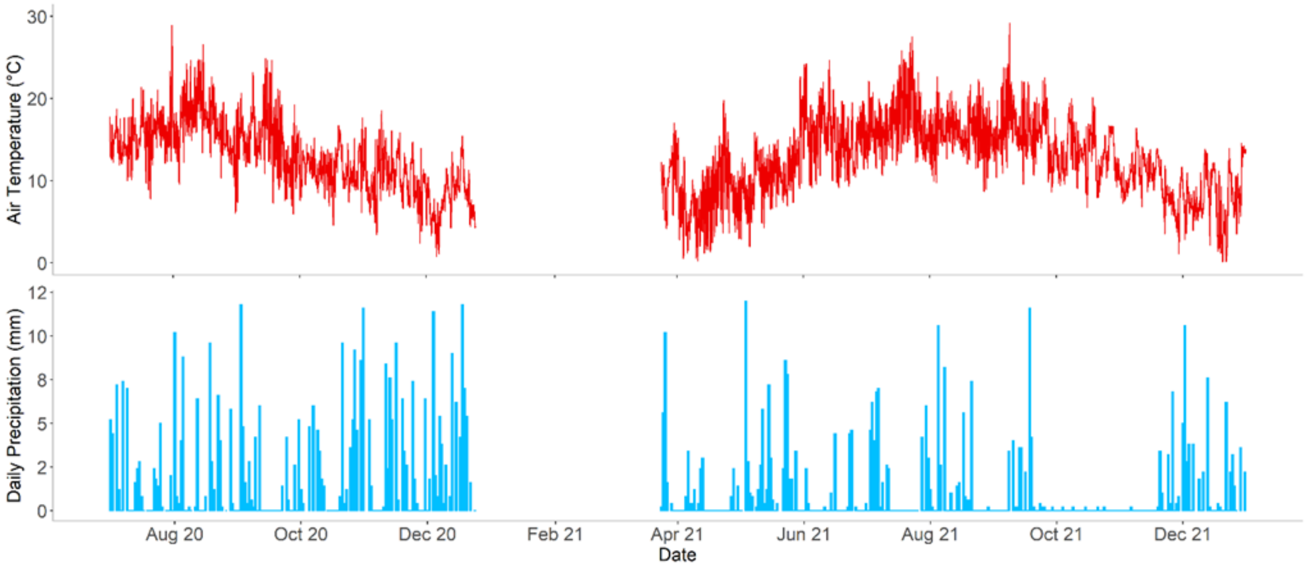
		0-5 cm depth		
Pore Characteristic	Herbal – *Cichorium intybus*	Herbal – *Plantago lanceolata*	Herbal – Grass Mix	Grass-Clover
Total porosity (%)	7.70 ± 2.51	15.65 ± 7.90	11.84 ± 2.47	7.08 ± 3.57
Total pore area (mm^2)	0.15 ± 0.05	0.31 ± 0.16	0.23 ± 0.05	0.14 ± 0.04
Perimeter (mm)	0.08 ± 0.004	0.08 ± 0.01	0.10 ± 0.01	0.06 ± 0.02
Euler number	20360 ± 7862	17889 ± 2048	13223 ± 2077	78811 ± 63329
$PSD_{D60/D10}$	56.36 ± 6.85	75.15 ± 13.33	46.93 ± 14.57	34.47 ± 12.18
		5-10 cm depth		
Pore Characteristic	Herbal – *Cichorium intybus*	Herbal – *Plantago lanceolata*	Herbal – Grass Mix	Grass-Clover
Total porosity (%)	8.64 ± 1.57	8.71 ± 2.16	6.42 ± 1.84	12.45 ± 3.37
Total pore area (mm^2)	0.17 ± 0.03	0.17 ± 0.04	0.13 ± 0.05	0.24 ± 0.07
Perimeter (mm)	0.09 ± 0.01	0.08 ± 0.003	0.08 ± 0.01	0.09 ± 0.003
Euler number	16567 ± 5542	16833 ± 5649	15888 ± 3337	15412 ± 2580
$PSD_{D60/D10}$	54.88 ± 8.54	52.93 ± 7.91	34.99 ± 2.07	71.68 ± 29.37

***Supplementary Table 5.4**. One-way ANOVA results of dominant plant type (grass-clover, herbal – grass mix, herbal – Cichorium intybus, or herbal – Plantago lanceolata) on measured pore characteristics obtained for intact cores imaged via X-ray µCT. * indicates statistically significant values, significance level was determined as $p < 0.05$.*

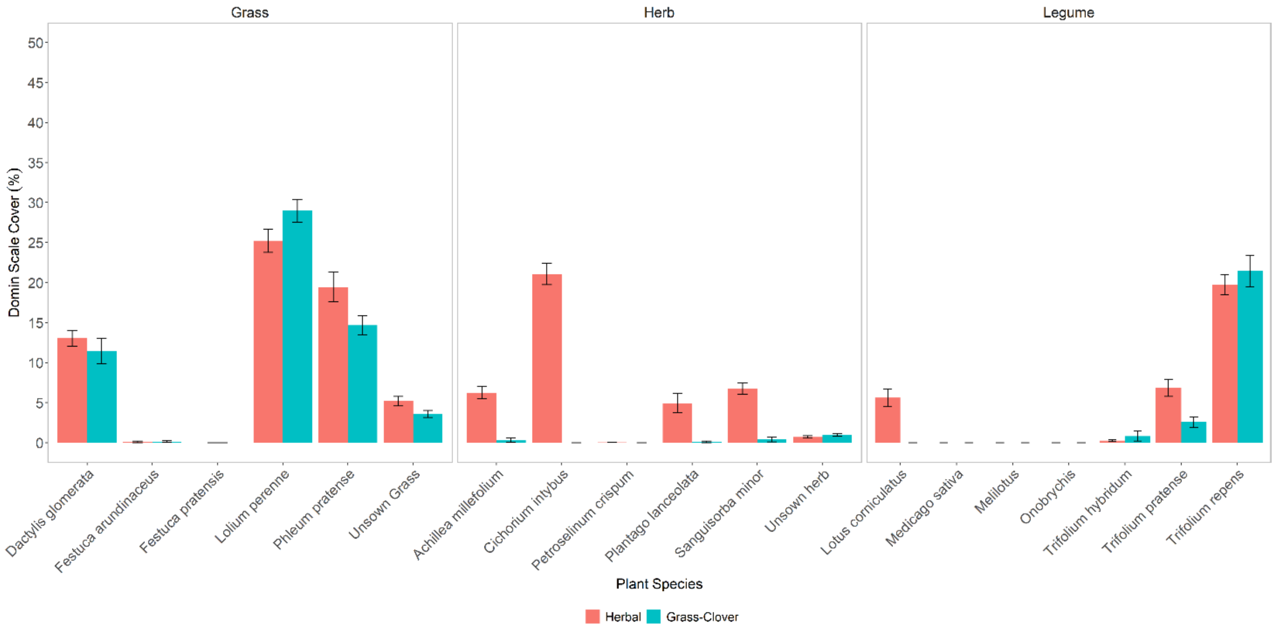
Pore Characteristic	Effect of Dominant Plant Type	
	$F_{(3,14)}$	*P*-value
Total porosity (%)	2.269	0.125
Total pore area (mm^2)	2.269	0.125
Perimeter (mm)	1.808	0.192
Thickness (mm)	1.053	0.402
Euler number	6.003	0.008*
$PSD_{D60/D10}$	0.922	0.456

***Supplementary Table 5.5**. Two-way ANOVA results of dominant plant type, depth (0-5 cm or 5-10 cm), or the interaction between dominant plant type and depth on measured pore characteristics obtained for 4 mm aggregates imaged via X-ray µCT. * indicates statistically significant values, significance level was determined as $p < 0.05$. † Degrees of freedom = 2,12.*

Characteristic	Effect of Dominant Plant Type		Effect of Soil Depth		Interaction between Plant Type × Depth	
	$F_{(3,16)}$	*P*-value	$F_{(1,16)}$	*P*-value	$F_{(3,16)}$	*P*-value
Total porosity (%)	0.190	0.902	0.048	0.830	1.353	0.293
Total pore area (mm^2)	0.190	0.902	0.048	0.830	1.353	0.293
Perimeter (mm)	0.410	0.7478	0.235	0.6345	2.510	0.6345
Euler number	0.823	0.462	1.124	0.310	†0.898	0.433
$PSD_{D60/D10}$	0.917	0.455	0.002	0.969	1.699	0.207

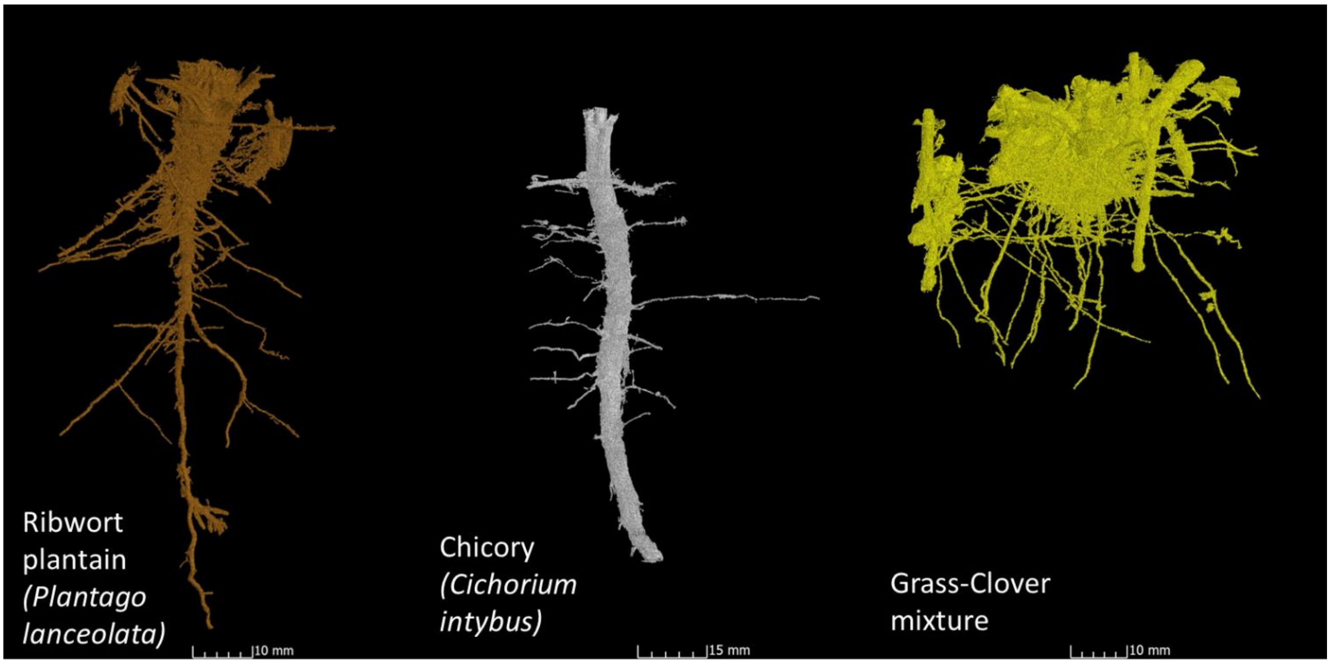


Supplementary Figure 5.1. *Daily air temperature (°C) and precipitation (mm) obtained 200 m from the field experiment at Henfaes Research Centre, Abergwyngregyn, (UK) between July 2020 and December 2021. Data are not reported between 24/12/20 and 24/03/21 due to a malfunction with the metrological apparatus.*

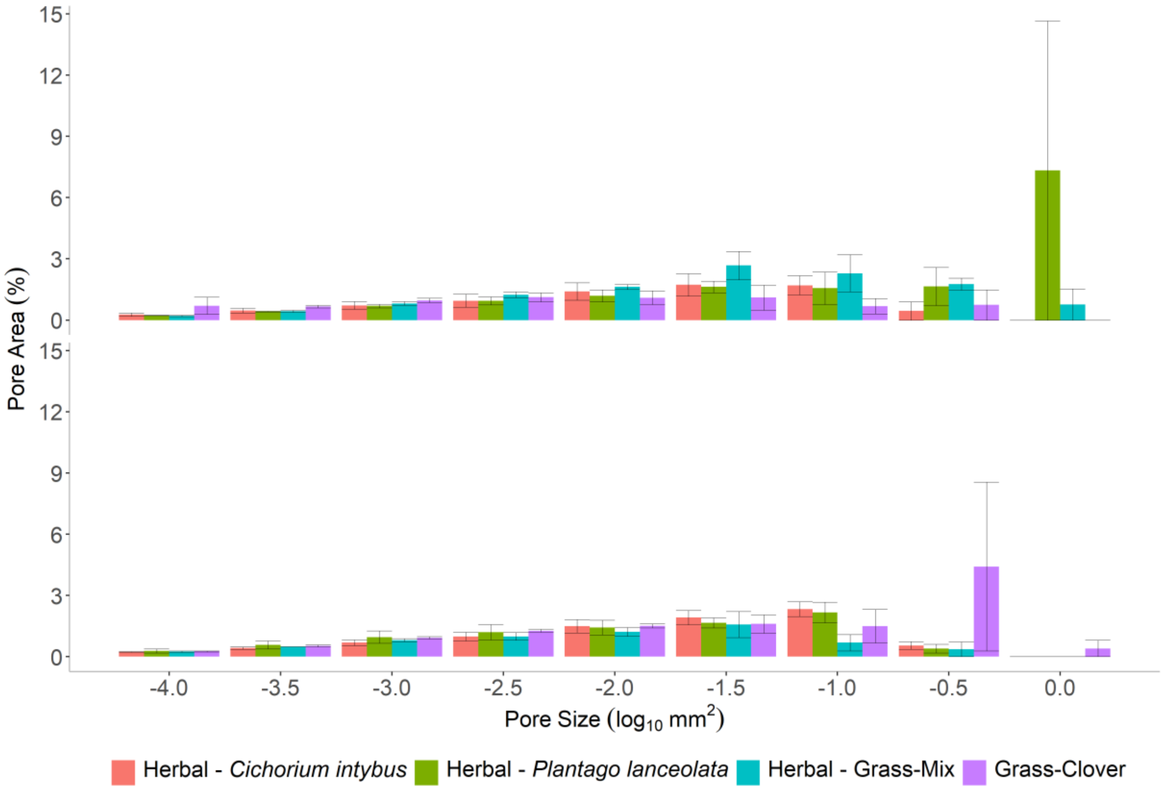


Supplementary Figure 5.2: *Domin score cover (%) of sown and unsown species in the herbal and the grass-clover ley assessed in July 2021. Data represents mean ± SEM, n = 3 per sward.*

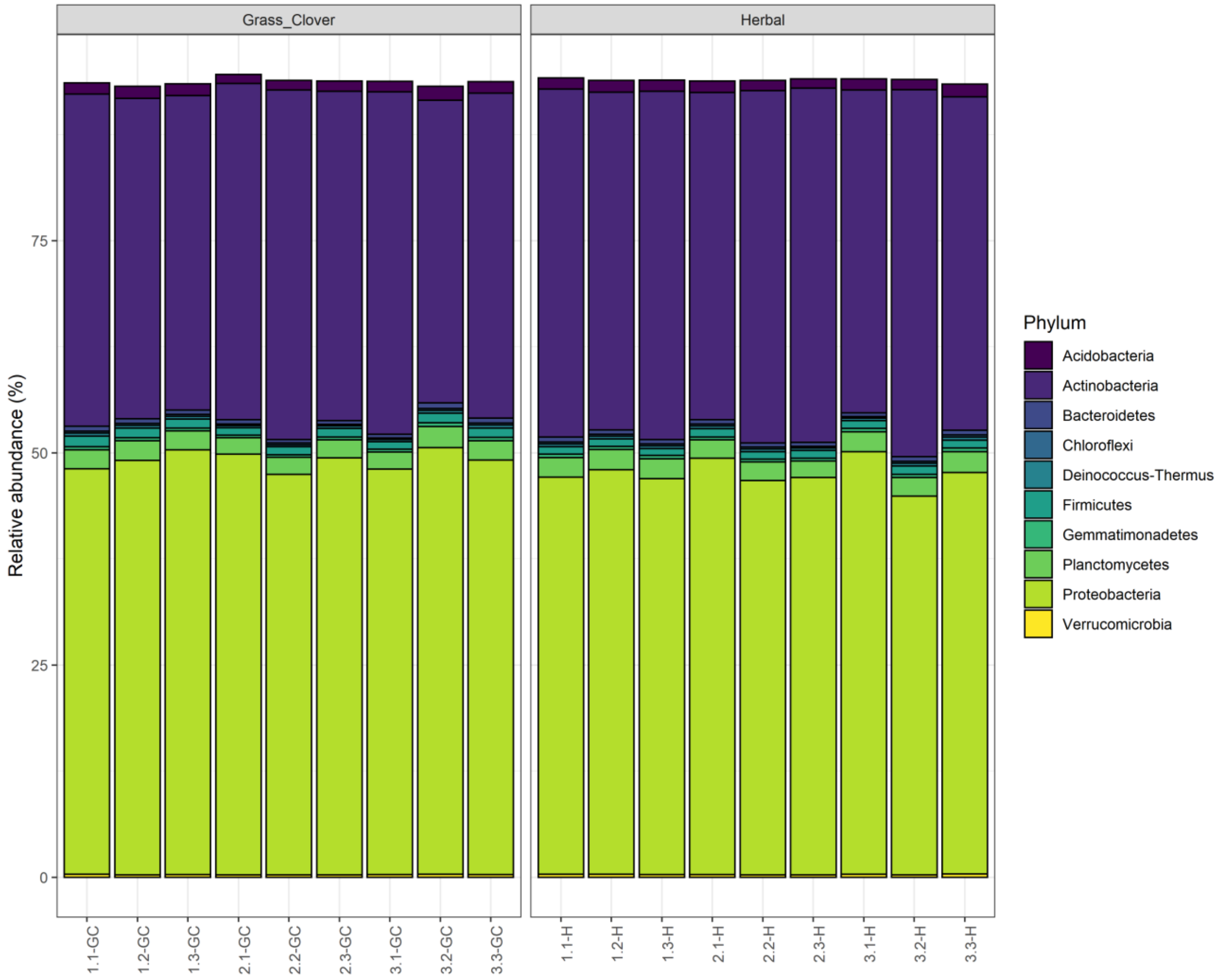
Supplementary Figure 5.3: *Overhead photography of the herbal ley (left) and grass-clover ley (right) in summer 2021.*



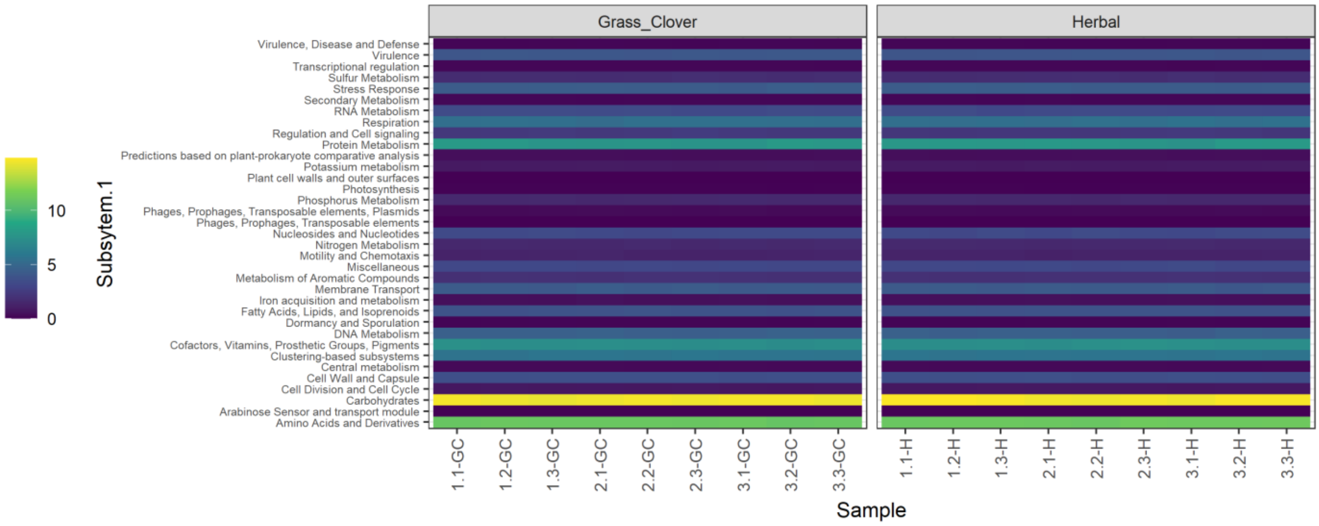
***Supplementary Figure 5.4**: X-ray µCT 3D reconstruction of root structures identified from intact vegetated cores (0-10 cm depth).*



Supplementary Figure 5.5*. Pore size distribution for the ROI from 4 mm soil aggregates obtained from two depths (0-5 cm or 5-10 cm) from a grass-clover ley or a herbal ley dominated by Cichorium intybus, Plantago lanceolata, or a grass mixture. n = 3 per plant type and depth). Data represents mean ± SEM.*



Supplementary Figure 5.6*. Bacteria composition at phylum level. Data shows relative abundance (%) of the ten most abundant bacteria phyla per sample. GC = grass-clover ley, H = herbal ley, n = 9 per sward.*



Supplementary Figure 5.7*. Functional genes organised into the SEED functional hierarchy. GC = grass-clover ley, H = herbal ley, n = 9 per sward.*

Chapter 6 - Discussion

6.1. Introduction

This section will synthesise the key results reported in experimental chapters 3-5 in relation to the initial thesis aims and objectives, before discussing the broader implications of these findings. Detailed discussions of results are provided in the individual experimental chapters and will not be repeated here. Instead, the focus will be on limitations of the research and the potential for future work, followed by some concluding remarks.

6.2. Synthesis of Key Findings

The overall aim of this thesis was to evaluate the agronomic and environmental benefits of a current commercial herbal ley, focussing on the effect of herbal leys on i) sward productivity, nutritional quality, and livestock productivity (Chapter 3); ii) livestock excreta composition and soil N cycling (Chapter 4); and iii) below-ground ecosystem services and soil physiochemical and biological characteristics (Chapter 5). A synthesis of key findings of each experimental chapter are provided below, with a schematic diagram summarising key results presented in Figure 6.1.

Firstly, the literature review (Chapter 2) revealed the widespread issues caused by global agricultural intensification and its subsequent impact on ecosystem service delivery (e.g., Bardgett et al., 2021; Kopittke et al., 2019; Lal, 2007; O’Mara, 2012), while exploring the potential of herbal leys to alleviate this (Cooledge et al., 2022; Schut et al., 2021). Although this literature review originally focussed on reintroducing herbal leys into arable rotations, much of the knowledge gaps identified are applicable to grassland systems, and subsequently formed the basis of the research questions explored throughout this thesis. The findings of this literature review supported further expert policy and research priority recommendations recently reported in Cooledge et al. (2022) and Buckingham et al. (2023). Research priorities arising from the literature review (Chapter 2) consequently contributed to the establishment of the 2-ha split-field experiment at Henfaes Research Centre (North Wales, UK) in July 2020 that aimed to address the thesis objectives presented in Chapter 1 and led to the research conducted in experimental Chapters 3-5.

Chapter 3 aimed to address the first thesis objective by exploring if herbal leys can improve livestock productivity, health, and sward productivity and nutritional quality compared to a conventional grass-clover ley. This chapter builds upon previous sheep grazing experiments conducted on low-diversity (e.g., 3-9 species) herbal ley mixtures in Ireland (Grace et al., 2019), Germany (Jerrentrup et al., 2020) and New Zealand (Golding et al., 2011; Somasiri et al., 2016), and directly addresses the lack of research on sheep performance when grazing a high-diversity (e.g., 9-18 species) commercial herbal ley. The results of this study were surprisingly highly seasonal and revealed that while there is no difference in nutritional quality (e.g., crude protein, sugar content) between sward types, deep-rooting taproot species (e.g., *Cichorium intybus* and *Plantago lanceolata*) in the herbal ley can consistently improve the sward macro- and micronutrient content across two experimental seasons (autumn-winter 2020 and spring-summer 2021). This contributed to a greater daily liveweight gain observed in lambs grazing the herbal ley (172 ± 7 g d^{-1}) than the grass-clover ley (144 ± 7 g d^{-1}) in spring 2021, with blood samples obtained after 11-weeks of grazing revealing higher concentrations of plasma cobalt and selenium in herbal ley grazed lambs, reducing their risk of micronutrient deficiencies. However, no difference in daily liveweight gain was observed in autumn 2020 (146 ± 15 g d^{-1} herbal *vs*. 119 ± 9 g d^{-1}, $p > 0.05$), with this potentially attributed to the high pre-existing gastrointestinal parasite burden (> 750 epg) that was not managed by the natural anthelminthic properties of the herbal ley.

Although our faecal egg count measurements conflict with previous studies reporting a reduction in gastrointestinal parasite burden when grazing livestock on ‘bioactive’ plant species such as *Cichorium intybus*, *Plantago lanceolata*, and *Medicago sativa* (Costes-Thiré et al., 2019; French, 2018; Hoste et al., 2006; Peña-Espinoza et al., 2018), it demonstrates that when combined in a highly diverse species mixture these anthelminthic benefits can be diluted, and therefore may not be a viable alternative to synthetic anthelminthic use in lowland sheep production as hoped. Instead, suppression of the gastrointestinal parasite burden in herbal ley grazed lambs in spring 2021 was likely driven by seasonal variations in plant secondary metabolite content (e.g., tannins), the older lamb age offering natural gastrointestinal parasite resistance, and the taller sward grazing height (ca. 8-13 cm) preventing parasitic larvae migration up the plant towards the grazing canopy (French, 2018; Marley et al., 2003). Despite expectations, the herbal ley and grass-clover ley had a similar sward standing biomass throughout the experiment, with significant differences only observed 66-days after the start of the spring grazing experiment, where the herbal ley had a greater biomass under drought conditions. Overall, the results of Chapter 3 have demonstrated that herbal leys can provide seasonal benefits for lowland sheep production, with some support for our initial hypotheses demonstrated through the greater sward macro- and micronutrient content, and seasonal improvements in lamb productivity and health.

Chapter 4 builds on the field experiment conducted in Chapter 3 to examine the environmental impact of grazing lambs on herbal leys. This chapter addresses the second thesis objective to determine if a commercial herbal ley mixture can reduce urine N excretion, soil N losses, ammonia (NH_3) volatilisation, and N_2O emissions associated with lowland sheep grazing. Previous studies have reported reductions in urine-N excretion and subsequent N_2O emissions in livestock consuming high proportions of *Plantago lanceolata* in low-diversity (e.g., 3-9 species) herbal ley mixtures (Box et al., 2023; Cheng et al., 2017; Totty et al., 2013), with no studies, to-date, exploring the effectiveness of these bioactive plant species when utilised in a highly diverse commercial herbal ley. As N_2O emissions from commercial herbal leys have not been reported before, this study aimed to produce the first sward-specific urine N_2O emission factor (EF_{3PRP}) to support future greenhouse gas inventory calculations. To assess this, sward-specific lamb excreta was collected at the start of each grazing season and reapplied to the soil of its respective pasture, with subsequent plot-scale N_2O emissions and soil (0-10 cm) chemical measurements conducted over 103 and 78 days in autumn 2020 and spring 2021, respectively.

Despite the greater plant species diversity, no reduction in urine-N excretion was observed in lambs consuming the herbal ley in either grazing season in this study. Instead, the diuretic properties of the herbal ley increased urination volume in autumn 2020, increasing the urine N loading rate of herbal ley grazed lambs by 84 % (1020 kg N ha^{-1} herbal *vs*. 555 kg N ha^{-1} grass-clover), with no difference observed in spring 2021 ($p > 0.05$). Similarly, urinary sodium excretion was consistently higher in herbal ley grazed lambs across both measurement seasons, and was attributed to the greater accumulation of macronutrients in deep-rooting species (e.g., *Cichorium intybus* and *Plantago lanceolata*) (Barry, 1998; Scales et al., 1995). The lack of reduction in urine-N concentration conflicted with our original hypothesis, and was attributed to the dilution of beneficial plant secondary metabolites (e.g., aucubin) when bioactive plant species are sown in low proportions in a commercial herbal ley (Bryant et al., 2018; Cheng et al., 2017). This probably also contributed to the lack of sward type effect on soil N cycling and N_2O emissions, with the proportion of these species likely too low to induce biological nitrification inhibition (Nyameasem et al., 2021). Due to dry sampling conditions (ca. 45-52 % WFPS), the magnitude of N_2O emissions emitted were extremely small, and consequently generated negligible sward-specific urine N_2O emission factors in autumn (0.008 ± 0.01 % EF_{3PRP} herbal *vs*. 0.008 ± 0.01 % EF_{3PRP} grass-clover, $p > 0.05$) and spring (-0.001 ± 0.001 %

EF_{3PRP} herbal *vs*. 0.001 ± 0.001 % EF_{3PRP} grass-clover, $p > 0.05$). These were much lower than the disaggregated urine-only 2019 IPCC Tier 1 EF_{3PRP} of 0.004 % (IPCC, 2019a) and the 2022 UK Tier 2 EF_{3PRP} of 0.315 % (Churchill et al., 2022). To explore why reductions in N_2O emissions were not observed in this study, a bench-scale ammonia volatilisation experiment was conducted to assess alternative pathways of N losses, with a 140 % reduction in NH_3 volatilisation observed following the application of sward-specific urine in the herbal ley than the grass-clover ley. This result was surprising as NH_3 emissions from a herbal ley have not been assessed previously, and was attributed to either differences in urine-patch size and urine infiltration rates (Moring et al., 2016), or osmotic stress generated by the high urinary sodium content (Haj-Amor et al., 2022). Overall, contrary to our hypotheses, the results presented in Chapter 4 have shown that current commercial herbal ley mixtures do not alter excreta N concentration, soil N cycling, or reduce N_2O emissions associated with lowland sheep grazing compared to a conventional grass-clover ley, although additional research is needed under conditions that are more favourable for N_2O production.

Finally, Chapter 5 addresses the third thesis objective to evaluate the delivery of below-ground ecosystem services by assessing the soil physical (e.g., porosity), chemical (e.g., soil organic carbon stocks), and biological (e.g., earthworm abundance) characteristics beneath the herbal and grass-clover ley after two seasons of sheep grazing. This chapter evaluates changes in soil properties 1-month and ca. 2-years after sward establishment utilising advanced techniques such as X-ray µCT imaging and shallow shotgun sequencing. Although we hypothesised that biopores generated by deep-rooting taproot herbs such as *Cichorium intybus* may improve the overall soil structure, with similar findings reported in previous studies (e.g., Han et al., 2015; Pagenkemper et al., 2015; Uteau et al., 2013), surprisingly no difference in pore characteristics was found in intact soil cores or ca. 2 mm air-dried soil aggregates obtained from 0-5 and 5-10 cm depths between the herbal and grass-clover ley. Instead, greater pore connectivity (Euler number) was observed in the grass-clover ley than herbal ley cores dominated by *Plantago lanceolata*, however, this was likely an artifact of high earthworm activity in the samples biasing biopore quantification determined via X-ray µCT imaging. Similarly, no differences in aggregate stability (using wet sieving techniques), bulk density, or VESS and VSA scores were observed between the sward types after 2-years. This was likely due to insufficient proportions of key 'bioengineering' plant species (e.g., *Cichorium intybus*, *Plantago lanceolata* and *Medicago sativa*) in the herbal ley and an overall decline in grassland plant species richness by the second year of the study. Consequently, as there were no significant changes in overall soil structure and root architecture, soil chemical (e.g., soil carbon stocks) and biological characteristics (e.g., microbial community composition and earthworm abundance) was unaffected by sward type, conflicting with previous studies of low-diversity herbal ley mixtures (Fox et al., 2020; Leff et al., 2018).

While the results presented in Chapters 3-5 sometimes did not support our initial hypotheses, they do provide a realistic and valuable insight into the potential of herbal leys in sheep production systems. The results of this thesis are the first, to our knowledge, to assess the ecosystem services and overall environmental impact of sheep grazing a commercial herbal ley currently promoted via agri-environment schemes (e.g., the UK Sustainable Farming Incentive Scheme, DEFRA (2023)). Further, these results highlight the importance of optimising the herbal ley seed mixture to deliver the promised ecosystem benefits, as commercial herbal leys are often expensive (ca. £200-250 ha^{-1}) compared to conventional grass-clover leys (ca. £150 ha^{-1}) with their potential for achieving sustainable agricultural intensification not yet realised.

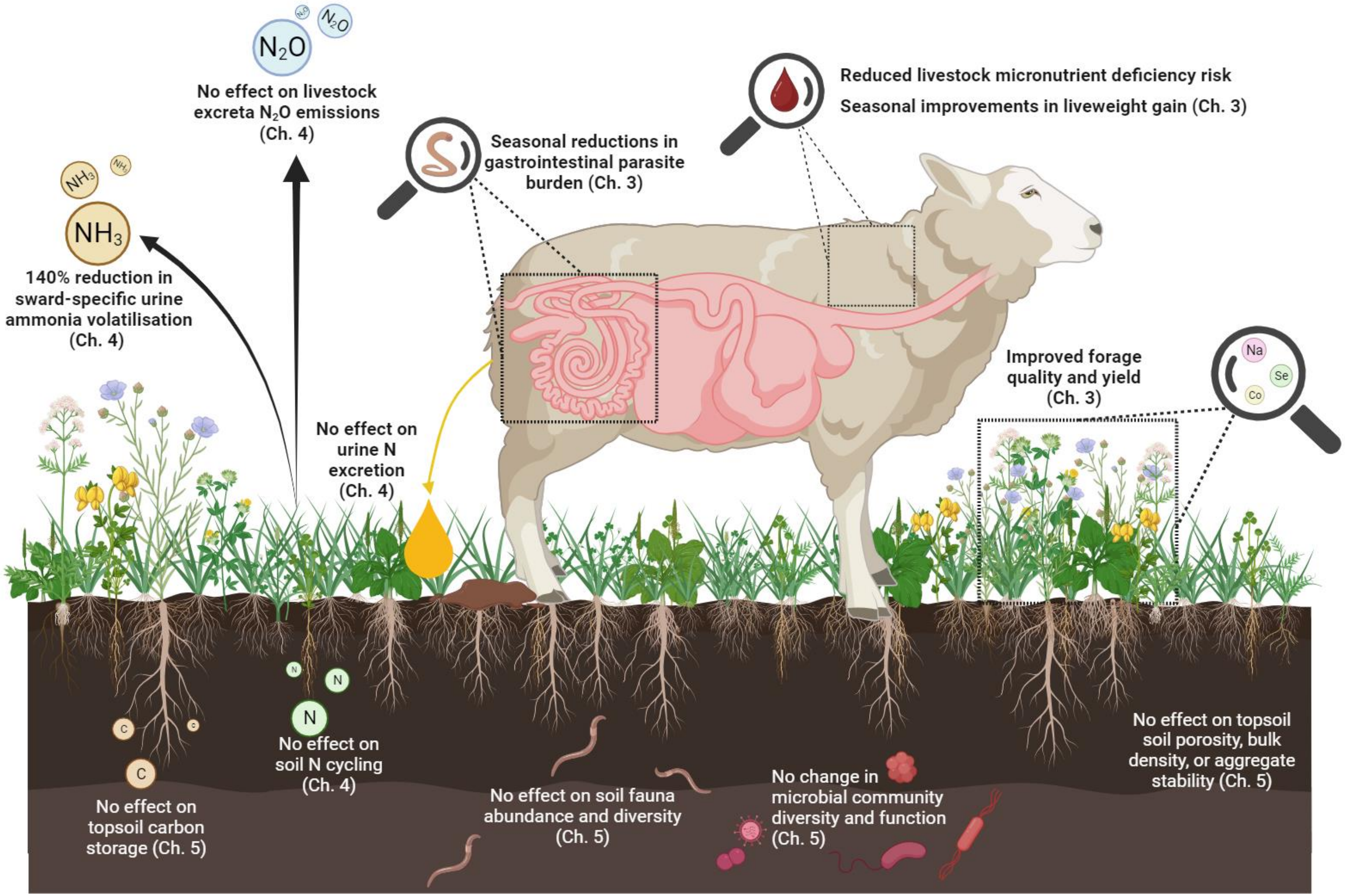


Figure 6.1. *Schematic diagram displaying a summary of key thesis results and their respective chapters.*

6.3. Research Limitations and Wider Implications

This thesis was initially aligned with the BBSRC-SARIC project (see foreword), however, complications arising from the COVID-19 pandemic limited access to pre-existing field experiments established on varying soil types across the UK. This subsequently limited the experimental work of this thesis to one geographical location (Henfaes Research Centre, North Wales, UK) and one soil type (Eutric Cambisol) under relatively wet and cool climatic conditions, with an average annual rainfall and temperature of 1060 mm and 10.8 °C, respectively. Consequently, this also removed the arable-ley component from this thesis as there was no arable comparison possible at Henfaes Research Centre, with the only available land for the field experiment previously long-term grazed grassland.

Throughout the field experiment, every effort was taken to control external factors (e.g., livestock illnesses) and ensure adequate replication to reduce potential limitations, however, some unavoidable challenges remained. Firstly, the statistical design of the field experiment was limited by resources (e.g., available land area, numbers of livestock, financial costs) and space requirements for conducting agronomic practices (e.g., manoeuvring of commercial farm machinery during mowing, fence establishment, sheep handling). As only 2 ha of land was available at Henfaes Research Centre when the experiment was established in 2020, this limited each replicate paddock size to ca. 0.33 ha^{-1} and only enabled the creation of three replicate paddocks per sward type. Consequently, as a randomised block experiment with smaller paddocks (e.g., 0.1 ha each) to capture greater spatial variability could not be created, this generated low statistical power (n = 3 per sward) for many of the soil and plant analyses conducted in this thesis. This increased the sensitivity of statistical tests to outliers and reduced the confidence to estimate experimental errors within treatment groups, subsequently increasing the probability of Type II errors (i.e., failure to reject a false null hypothesis). The lower degrees of freedom for the soil and plant analyses also limited the potential use of more complex statistical analyses (e.g., Principal Component Analysis).

The design of the field experiment affected Chapter 3, where the number of grazing lambs was limited by both land area and seasonal forage availability, with the field experiment only able to provide for a stocking density of 3.2 LU ha^{-1} without damaging the sward composition and soil structure through overgrazing. This prevented lambs from grazing in early spring (e.g., January-April) and late winter (e.g., November and December), inadvertently limiting the study from utilising younger lambs (e.g., < 9 months old) or ewes with lambs at foot. Instead, the experimental design only enabled the use of older lambs (e.g., > 9 months old) that required conditioning for breeding or slaughter, subsequently limiting the experimental duration for capturing potential growth rates. Similarly, ethical limitations arising from the lack of trained personnel with an appropriate Home Office licence prevented more detailed analyses (e.g., rumen microbiome sampling) that may have provided a greater understanding of the role and mechanisms of herbal leys on livestock productivity. This also limited the potential of the gastrointestinal parasite burden analyses, as direct from rectum faecal sampling was prohibited, thus reducing replication and introducing a risk of sample contamination as it was only possible to measure naturally excreted samples deposited onto the barn floor.

Consequently, a collaboration with the University of Nottingham's Veterinary Nutritional Analysis (NUVetNA) service was sought to conduct a blood sample of ewe lambs at the end of the spring 2021 grazing season utilising the secondary availability aspect of their Home Office licence. However, while this was conducted successfully in spring 2021, unexpected financial constraints prevented further sampling across multiple seasons and with greater numbers of sheep, thus limiting the replication of this dataset. Instead, the blood characteristics dataset presented in Chapter 3 should be acknowledged as an indicator of the potential of herbal leys to reduce the risk of micronutrient deficiencies, as it was not possible to determine temporal changes in mineral status between the start and end of the experiment. Financial constraints also restricted the replication of the forage quality sampling to once per grazing season, subsequently preventing this study from capturing temporal changes in seasonal forage composition and nutritional quality. Due to resource demands, personnel limitations, and external factors (e.g., livestock illnesses and deaths), annual replication of the grazing experiment was not possible thus this aspect of the thesis was limited to one year only.

The limitations discussed in Chapter 3 also had a major impact on the experimental work conducted in Chapter 4, with restrictions on grazing season and duration affecting the potential to capture seasonal changes in livestock excreta composition and provide annual replication of the soil N cycling and N_2O emission dataset. Subsequently, the N_2O measurements conducted in Chapter 4 can only provide an insight into emissions over two experimental seasons (e.g., autumn-winter 2020 and spring-summer 2021) as it was not possible to measure for the IPCC recommended one-year period for inventory calculations (IPCC, 2019b). However, the N_2O measurements conducted over 103 and 78 days in autumn 2020 and spring 2021, respectively, were within the minimum sampling period of 60-180 days reported in Vangeli et al. (2022) and were able to produce a sward-specific urine EF_{3PRP} that may be of use to future modelling studies (e.g., carbon footprinting) and meta-analyses. It should be noted that the atypically dry conditions experienced during the N_2O sampling periods in this study may limit the potential to scale these results up to a national or international level, as the magnitude of emissions was abnormally small due to the low WFPS (e.g., 45-52 %) and subsequently may underestimate N_2O emissions under wet conditions. The low WFPS likely affected the sward-specific urine NH_3 volatilisation measurements, with these results also confounded by the scale of the measurements (i.e., a bench-scale mesocosm study) as *in-situ* field measurements were not possible.

Finally, in Chapter 5, unavoidable limitations arose from the underlying soil structure and parent material as Henfaes Research Centre is located on ancient (10,000 Y.B.P) glacial outwash, and thus has a very shallow topsoil layer (ca. 0-30 cm) before boulder-rich glacial till is reached. This prevented subsoil (30-100 cm) sampling using conventional techniques (e.g., mechanical auger) to assess changes in soil physiochemical properties and root architecture, limiting our understanding of the effect of herbal leys on subsoil characteristics. In July 2022, an attempt was made to explore the soil profile to 100 cm depth using a mechanical excavator, however, due to the thickness of the glacial till and the instability of the subsequent soil pit, this was abandoned. As a result, much of the soil physical, chemical, and biological properties explored in Chapter 5 is limited to the topsoil (0-10 cm) layer. As with the previous chapters, financial constraints limited replication of detailed analyses, only enabling one measurement of soil physical and biological properties towards the end of the field experiment. Soil structure and pore characteristics determined using X-ray µCT were limited by competition funding and access to equipment at the University of Nottingham's Hounsfield Facility, preventing the analysis of larger column-scale (e.g., 100 cm) intact vegetated soil cores.

However, despite these limitations, the results of this thesis provide a valuable foundation for changing the social attitudes towards sustainable farming practices that may provide ecosystem service benefits (e.g., pollinator habitats) without compromising on productivity. As such, these results have wider implications for the agri-food industry and future agri-environment policies, and may contribute to increasing the adoption of herbal leys within the farming community. The soil physiochemical data obtained in Chapter 4 and 5 will contribute to building a 'farm-to-fork' life cycle assessment (LCA) and carbon footprint to assess the overall impact of herbal leys on lowland meat production, with the data collated in Chapter 3 contributing to our understanding of herbal leys on lamb productivity and health. This will subsequently be used to assess if herbal leys can reduce the need for additional inputs (e.g., boluses, synthetic anthelminthics) than lambs grazing conventional pastures, and may lead to the marketing of herbal ley grazed lamb as a healthier product than other intensively farmed meat products. Furthermore, as grazing livestock on herbal leys may reduce the risk of micronutrient deficiencies and subsequently improve immune system resilience (e.g., Sordillo (2016)), this may also reduce the need for veterinary intervention (e.g., antibiotic use), reducing the risk of on-farm antimicrobial resistance (AMR) spread.

6.4. Priorities for Future Research

Future research should prioritise the development of a national-scale project that utilises an ecosystem services approach to assess the potential agronomic and environmental benefits of herbal leys across a broad range of temporal and spatial scales. Although this thesis has laid the foundation for future research into the efficacy of commercial herbal leys, a multi-year, multi-site project will greatly advance our understanding by capturing differences in herbal ley composition and function with changes in the underlying soil parent material and texture (e.g., silt and clay content), field management (e.g., grazing intensity), and exposure to climate extremes (e.g., drought and flood events) that was beyond the scope of this study. As grazing studies on a commercial scale are often limited by land area, with this subsequently limiting the number of statistical replicates possible, we recommend that if on-farm field or paddock replication is not possible then multiple field sites are utilised to circumnavigate this issue.

As such, we provide recommendations for areas of future research, based on the five key research themes explored throughout this thesis:

<u>Forage quality</u>

- Regular, monthly forage measurements should be made over multiple years to capture seasonal changes in sward yield, composition (e.g., species abundance) and nutritional quality (e.g., macro- and micronutrient content). This will help to monitor changes in botanical composition and detect species loss within the aging sward, informing stakeholders (e.g., commercial seed merchants) of species persistence under varying conditions (e.g., drought) and the tipping point of when a herbal ley requires reseeding.

- Hay and silage yield and nutritional quality (e.g., metabolizable energy) measurements should also be conducted over multiple years to examine the potential of herbal leys as an additional feedstock once preserved as information on this is currently lacking.
- The concentration of plant secondary metabolites within the herbal ley mixture should be quantified to determine the concentration and bioavailability of key compounds (e.g., hydrolysable and condensed tannins) that have a secondary effect on grazing ruminants and other ecosystem services.

Livestock health and productivity

- Regular liveweight gain measurements from birth to slaughter using a variety of breeds across multiple years will capture changes in growth rates with sward seasonality at various production stages.
- Greater frequency faecal egg count measurements interlinked with other livestock productivity assessments (e.g., liveweight gain, body condition score) will capture seasonal variations in gastrointestinal parasite burden. This will enable researchers and farmers to record when synthetic anthelminthic intervention is required to identify the threshold where natural anthelminthic management using herbal leys is no longer possible.
- Although expensive, higher frequency blood sampling campaigns will enable researchers to determine the mineral status response in grazing ruminants to seasonal changes in sward macro- and micronutrient content. This will help to determine if

additional supplementation (e.g., mineral boluses) are required, subsequently contributing to lowering production costs.

- If possible, rumen microbiome samples coupled with *in-situ* enteric methane (CH_4) measurements of livestock grazing a fresh or processed (e.g., hay or silage) herbal ley will contribute to understanding the effect of plant secondary metabolite rich species on rumen protein degradation and microbial community composition. This will contribute to building carbon footprints and life cycle assessments (LCAs) of lowland meat production.

Livestock excreta composition

- While a current commercial herbal ley mixture was used in this study, further work is needed to identify the optimal proportions of key herb and legume species (e.g., *Plantago lanceolata* or *Cichorium intybus*) that can reduce urine N concentration to adapt and optimise new herbal ley mixtures.
- Quantification of non-urea nitrogenous compounds (e.g., hippuric acid), cations (e.g., Na, K and Ca) and plant secondary metabolites (e.g., aucubin) excreted in urine is also necessary to better understand the potential of herbal leys to alter urine chemistry and subsequently soil biogeochemical cycling.
- Future excreta collections should be conducted over a 24-hour period over multiple days (e.g., 1-week) to capture diurnal variations in excreta composition and excretion frequency. This will allow the calculation of daily N excretion from grazing livestock.
- Further investigating into sheep dung chemical characteristics will allow researchers to assess how herbal leys affect total dung N content and subsequently the dung N loading rate, as it was not possible to determine this in our study.

Nitrogen cycling

- Higher frequency N_2O measurements (e.g., by using automated greenhouse gas measurement systems) are needed following the application of sward-specific excreta or varying synthetic N fertiliser rates over multiple seasons and repeated experimental years. This will provide a more detailed dataset that can be used to develop a greater accuracy sward-specific N_2O emission factor (e.g., EF_{3PRP}) for use in national greenhouse gas inventory calculations.
- *In-situ* measurements of sward-specific urine NH_3 volatilisation are needed to assess the potential of herbal leys to reduce emissions under field conditions across multiple seasons, as it was only possible to use a bench-scale system in this thesis. Future studies should also aim to conduct an assay to identify any potential tipping point in NH_3 reductions with increasing urine N concentration and proportion of 'bioactive' plant species.
- Lysimeter, or in rare cases, hydrologically isolated field-scale experiments, should be conducted to assess the impact of sward type (i.e., herbal ley *vs*. a conventional pasture) and grazing management (e.g., set stocking *vs*. rotational grazing) on NO_3^- leaching across multiple seasons.
- Mesocosm-scale experiments should be conducted to determine the optimum proportion of *Plantago lanceolata* required in herbal leys to reduce nitrate leaching via biological nitrification inhibition.
- Future research utilising stable isotopes (e.g., ^{15}N) should aim to develop a nitrogen balance for herbal leys by determining N fixation rates by legumes and interplant N transfer into non-leguminous species. This will allow researchers to evaluate the symbiotic relationship between plant functional groups and determine the nitrogen use efficiency of the sward, subsequently identifying the nitrogen fertiliser replacement value (NFRV) for further economic analyses.

Soil quality

The following research recommendations for improving soil quality are split into soil physical, chemical, and biological characteristics:

- Physical:
    - Greater measurements of herbal ley soil structure (e.g., porosity, pore connectivity) should be conducted at various temporal (e.g., year 1 *vs*. year 4) and spatial (e.g., 0-100 cm cores) scales using X-ray µCT. This will allow researchers to assess the effect of deep-rooting taproot species within the herbal ley on gas exchange and water infiltration as a proxy for flood risk management.
- Chemical:
    - Further assessments into the carbon sequestration potential of herbal leys in the topsoil (0-20 cm) and subsoil (20-100 cm) layers are needed to determine their effect on carbon storage across relatively short (1-4 years) and longer (e.g., 2-25 years) timeframes. Multi-site field experiments are required for this as variations in underlying mineralogy, climate, and differing field management practices will alter carbon sequestration rates. Future analyses should assess the MAOM and POM fractions separately, as the MAOM fraction has the longest soil residence time and thus will capture long-term changes in carbon dynamics. As such, greater evaluation of soil carbon sequestration beneath herbal leys should better inform policy makers and stakeholders (e.g., farmers, carbon credit brokers) of their potential.
    - Similarly, where the underlying mineralogy and soil structure allows, subsoil chemical characteristics beneath a herbal ley should be assessed to determine how deep-rooting taproot species (e.g., *Cichorium intybus*) affect subsoil nutrient cycling. This will enable future researchers to assess the potential of

    - deep-rooting plant species to recover nutrients (e.g., phosphorus, water) lost at depth that are typically not recovered by grass species.
- Biological:
    - Seasonal measurements of above- (e.g., pollinator species) and belowground (e.g., soil mesofauna) biodiversity with varying management regimes will provide a greater understanding of how herbal leys drive ecosystem services (e.g., food production, pest management).
    - Detailed quarterly (e.g., every 3-months) microbial community measurements throughout the sward life cycle assessing bacterial abundance and functional genes, combined with microbial biomass, will illustrate how the microbial community composition varies with sward age and further inform how this affects soil quality restoration rates.

Finally, we can summarise that future studies should take a multidisciplinary, ecosystem services approach assessing the economic, environmental, and agronomic impact of herbal leys where possible to fully evaluate their effect on lowland livestock production compared to conventional pastures. This should be coupled with stakeholder engagement to advise farmers of the best management practices (e.g., inputs, grazing management) required to achieve the potential benefits of herbal leys.

6.5. Concluding Remarks

The experiments conducted in this thesis are the first, to our knowledge, to evaluate the agronomic and environmental benefits of a commercial herbal ley compared to a conventional grass-clover ley. During the COVID-19 pandemic, a 2-ha split-field experiment was established at Henfaes Research Centre that utilised a multidisciplinary approach to assess the effect of a high-diversity commercial herbal ley on lamb productivity and health, forage quality, nutrient losses, NH_3 and N_2O emissions, soil quality, and below-ground ecosystem service delivery.

The results of this thesis have shown that although current herbal ley mixtures only offer seasonal benefits for livestock productivity, they consistently provide greater concentrations of sward macro- and micronutrients, reducing the risk of micronutrient deficiencies in grazing livestock. However, as these commercial herbal ley mixtures often only utilise a low proportion (e.g., < 10 %) of bioactive and bioengineering plant species (e.g., *Cichorium intybus*) in the sward composition, this can dilute the beneficial effects of ingested (i.e., grazed) or excreted (e.g., urinary or root exudation) plant secondary metabolites (e.g., aucubin) on livestock excreta composition, soil nutrient losses, and greenhouse gas emissions. This also likely limited the potential of herbal leys to deliver the promised benefits for soil quality and belowground ecosystem service delivery, with no difference in soil physiochemical and biological characteristics observed between the herbal and grass-clover ley in this study.

As herbal leys are becoming increasingly popular due to their promotion in agri-environment schemes, this thesis offers a timely, pragmatic assessment of the agronomic and environmental effects of a current commercial herbal ley mixture. Although limited benefits were observed in this study, further work is needed to optimise the herbal ley mixture to deliver greater ecosystem services, reduce the environmental impact of lowland sheep production, and help the UK agri-food system achieve sustainable agricultural intensification.

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