



# 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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## 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 replicated-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 ( $3.2 \text{ LU ha}^{-1}$ ) over two grazing seasons, with soil physiochemical and biological characteristics assessed after 2-years using techniques such as X-ray micro-Computed Tomography ( $\mu$ CT) and microbial shallow shotgun sequencing. Soil chemical characteristics (e.g., pH) were unaffected by sward type. Similarly, topsoil (0–10 cm) organic carbon stocks measured after 2-years did not differ between the herbal ( $26.1 \pm 1.1 \text{ t C ha}^{-1}$ ) and grass-clover ley ( $25.7 \pm 1.1 \text{ t C ha}^{-1}$ ). X-ray  $\mu$ CT analysis revealed greater pore connectivity (Euler number) in grass-clover ley intact soil cores (0–10 cm depth, 7.5 cm width) 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  $\mu$ CT, 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 the functional gene profile. This study is the first to explore the effects of a commercial herbal ley on physical, chemical, and biological soil quality indicators in a rotationally grazed sheep pasture. While no improvements in soil quality indicators were observed after 2-years, these findings have significant implications for agri-environment schemes promoting herbal leys to achieve 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.

## 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 525–634 Pg C (ca. 25–34% of the terrestrial carbon stock) (Liu et al., 2023), with the potential to store an additional  $0.28 \text{ Mg C yr}^{-1}$  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.

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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<sup>-1</sup>) and erosion (£14.8 million yr<sup>-1</sup>) (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 (Pères 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.

## 2. Materials and methods

### 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, Abergwyngregyn, 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 herb- and legume-rich multispecies ley (herbal) or a conventional 6-species grass-clover ley (grass-clover) (Table 1). Each sward covered 1 ha and was subdivided 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<sup>-1</sup> (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 (18/09/20–28/10/20) and spring-summer 2021 (12/04/21–28/06/21) (see Cooledge et al. in review for more details). The field was left empty when sheep were not present. One fertiliser application of 50 kg N ha<sup>-1</sup> ammonium nitrate, 20 kg P ha<sup>-1</sup> and 60 kg K ha<sup>-1</sup> 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<sup>2</sup>) 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 were 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 1).

The botanical composition of each sward was assessed in July 2021 at the end of the spring-summer 2021 grazing experiment. Five 4 m<sup>2</sup> 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 2 and 3, respectively.

### 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 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<sub>2</sub>O 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<sup>-3</sup> herbal; 0.94 ± 0.04 g cm<sup>-3</sup> grass-clover) was determined by inserting stainless steel rings (100 cm<sup>3</sup>) 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<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) were determined colorimetrically from 0.5 M K<sub>2</sub>SO<sub>4</sub> 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 2100 S analyser (AnalytikJena, Jena,

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

**Table 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 <sup>aA</sup>	6.90 ± 0.08 <sup>aC</sup>	6.51 ± 0.12 <sup>aB</sup>	6.65 ± 0.07 <sup>aD</sup>
Electrical conductivity (µS cm <sup>-1</sup> )	38 ± 5 <sup>aA</sup>	51 ± 14 <sup>aA</sup>	44 ± 3 <sup>aA</sup>	55 ± 10 <sup>aA</sup>
Moisture content (g g <sup>-1</sup> )	0.31 ± 0.01 <sup>aA</sup>	0.33 ± 0.02 <sup>aA</sup>	0.31 ± 0.01 <sup>aA</sup>	0.32 ± 0.02 <sup>aA</sup>
Organic matter (g kg <sup>-1</sup> )	56.4 ± 3.7 <sup>aA</sup>	54.5 ± 3.1 <sup>aC</sup>	63.6 ± 1.5 <sup>aB</sup>	65.3 ± 4.3 <sup>aD</sup>
Microbial biomass carbon (g C kg <sup>-1</sup> )	1.67 ± 0.02 <sup>aA</sup>	2.02 ± 0.44 <sup>aA</sup>	1.02 ± 0.10 <sup>aB</sup>	1.15 ± 0.21 <sup>aA</sup>
Microbial biomass nitrogen (mg N kg <sup>-1</sup> )	124.0 ± 17.7 <sup>a</sup>	115.4 ± 14.5 <sup>a</sup>	l.o.d.	l.o.d.
N mineralisation rate (mg N kg <sup>-1</sup> day <sup>-1</sup> )	5.87 ± 2.45 <sup>a</sup>	6.68 ± 0.93 <sup>a</sup>	n.d.	n.d.
Total extractable nitrogen (mg N kg <sup>-1</sup> )	29.75 ± 9.68 <sup>a</sup>	20.35 ± 1.69 <sup>a</sup>	l.o.d.	l.o.d.
Total dissolved carbon (mg C kg <sup>-1</sup> )	74.3 ± 2.5 <sup>aA</sup>	71.7 ± 3.2 <sup>aA</sup>	22.3 ± 9.1 <sup>aA</sup>	73.7 ± 29.3 <sup>aA</sup>
Extractable ammonium (mg NH <sub>4</sub> <sup>+</sup> -N kg <sup>-1</sup> )	13.97 ± 11.85 <sup>aA</sup>	5.58 ± 0.77 <sup>aA</sup>	3.75 ± 0.36 <sup>aA</sup>	3.05 ± 0.34 <sup>aA</sup>
Extractable nitrate (mg NO <sub>3</sub> <sup>-</sup> -N kg <sup>-1</sup> )	0.91 ± 0.27 <sup>aA</sup>	0.94 ± 0.04 <sup>aC</sup>	2.86 ± 0.12 <sup>aB</sup>	3.00 ± 0.15 <sup>aD</sup>
Extractable phosphorus (mg P kg <sup>-1</sup> )	11.17 ± 2.96 <sup>aA</sup>	13.35 ± 4.96 <sup>aA</sup>	7.26 ± 0.86 <sup>aA</sup>	4.68 ± 0.60 <sup>aA</sup>
Exchangeable sodium (mg Na kg <sup>-1</sup> )	0.55 ± 0.01 <sup>a</sup>	0.65 ± 0.12 <sup>a</sup>	n.d.	n.d.
Exchangeable calcium (mg Ca kg <sup>-1</sup> )	105.1 ± 18.9 <sup>a</sup>	85.2 ± 4.2 <sup>a</sup>	n.d.	n.d.
Exchangeable potassium (mg K kg <sup>-1</sup> )	2.74 ± 1.40 <sup>a</sup>	1.83 ± 0.39 <sup>a</sup>	n.d.	n.d.

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<sub>4</sub><sup>+</sup> 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](#)).

### 2.3. Soil physical and chemical properties

#### 2.3.1. X-ray µCT imaging for soil structure

X-ray micro-Computed Tomography (µCT) imaging was conducted on intact vegetated soil cores (0–10 cm depth, 7.5 cm diam., 441.8 cm<sup>3</sup>) 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 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  $\mu$ A, voxel size resolution of 3.5  $\mu$ m, 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  $\times$  400 pixel  $\times$  301 image slice cuboid (1.4 mm  $\times$  1.4 mm  $\times$  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.

### 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  $\mu$ CT imaging ca. 2 mm in size were added to 250  $\mu$ m soil sieves then immersed in DI H<sub>2</sub>O 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.

### 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  $\mu$ CT imaging dataset, further cores were taken to determine soil bulk density by inserting plastic rings (441.8 cm<sup>3</sup>) 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:

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

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

### 2.3.4. VESS and VSA scoring

In November 2022, intact soil blocks (15  $\times$  15  $\times$  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).

## 2.4. Soil biological properties

### 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  $\times$  15  $\times$  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.

### 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  $\times$  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 17<sup>th</sup>, 2011 (Wood et al., 2019) (Supplementary Table 1). For functional profiling, high-quality filtered reads were aligned against the SEED database via translated homology search and annotate to their subsequent sub-systems, or functional levels, 1–3 using the Super-Focus software (Silva et al., 2016).

## 2.5. 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 were 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 log<sub>10</sub>-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 for soil pH, electrical conductivity, moisture content, microbial biomass carbon, total dissolved carbon, extractable  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , available P, aggregate pore characteristics, aggregate stability, and earthworm abundance and biomass. A permutational multivariate analysis of variance (PERMANOVA) was conducted for 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 are mean  $\pm$  SEM unless otherwise stated.

### 3. Results

#### 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 2 and Supplementary Table 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  $0.65 \text{ g C kg}^{-1}$  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.

#### 3.2. Soil bulk density, soil organic matter and SOC stock

Soil bulk density did not differ between the herbal ( $0.86 \pm 0.02 \text{ g cm}^{-3}$ ) and grass-clover ley ( $0.81 \pm 0.03 \text{ g cm}^{-3}$ ) 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 ( $75 \pm 3.4 \text{ g kg}^{-1}$  herbal vs.  $89 \pm 11 \text{ g kg}^{-1}$  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 ( $26.1 \pm 1.1 \text{ t C ha}^{-1}$  herbal vs.  $25.7 \pm 1.1 \text{ t C ha}^{-1}$  grass-clover;  $T_{(14,0)} = 0.240$ ,  $p = 0.814$ ).

#### 3.3. Soil pore characteristics

##### 3.3.1. Intact cores

X-ray  $\mu\text{CT}$  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 3). Example 2D and 3D images demonstrating pore structure from each core type are shown in Fig. 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* ( $7.45 \pm 0.32\%$ ), with the lowest porosity values observed in the grass-clover cores ( $4.99 \pm 0.56\%$ ), 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 ( $p > 0.05$ ).

##### 3.3.2. 4 mm Aggregate size fractions

At the aggregate scale, pore characteristics were not affected by

**Table 3**

Average pore characteristics per image slice measured from the intact soil cores ROI. Data represents mean  $\pm$  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 – <i>Cichorium intybus</i>	Herbal – <i>Plantago lanceolata</i>	Herbal – Grass Mix	Grass-Clover
Total Porosity (%)	7.45 $\pm$ 0.32 <sup>a</sup>	5.16 $\pm$ 0.70 <sup>a</sup>	6.14 $\pm$ 0.86 <sup>a</sup>	4.99 $\pm$ 0.56 <sup>a</sup>
Total Pore Area (mm <sup>2</sup> )	73.9 $\pm$ 3.2 <sup>a</sup>	51.2 $\pm$ 6.9 <sup>a</sup>	60.9 $\pm$ 8.5 <sup>a</sup>	49.5 $\pm$ 5.6 <sup>a</sup>
Perimeter (mm)	2.21 $\pm$ 0.30 <sup>a</sup>	1.54 $\pm$ 0.11 <sup>a</sup>	2.01 $\pm$ 0.17 <sup>a</sup>	2.08 $\pm$ 0.14 <sup>a</sup>
Thickness (mm)	1.09 $\pm$ 0.18 <sup>a</sup>	0.70 $\pm$ 0.08 <sup>a</sup>	0.83 $\pm$ 0.14 <sup>a</sup>	1.15 $\pm$ 0.19 <sup>a</sup>
Euler number	12843 $\pm$ 4941 <sup>a</sup>	27873 $\pm$ 4394 <sup>ab</sup>	13532 $\pm$ 3413 <sup>a</sup>	10021 $\pm$ 1791 <sup>ac</sup>
PSD <sub>D60/D10</sub>	72.5 $\pm$ 10.5 <sup>a</sup>	53.6 $\pm$ 8.1 <sup>a</sup>	50.7 $\pm$ 4.8 <sup>a</sup>	54.4 $\pm$ 7.0 <sup>a</sup>

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 3 and 5). 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 ( $F_{(3,16)} = 1.353$ ,  $p = 0.293$ ).

##### 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 ( $F_{(3,172)} = 0.670$ ,  $p = 0.571$ ) (Fig. 2) and in aggregates obtained from the 0–5 cm and 5–10 cm soil layers ( $F_{(1,208)} = 1.637$ ,  $p = 0.182$ ) (Supplementary Figure 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* ( $65.2 \pm 0.2\%$ ), *Plantago lanceolata* ( $68.2 \pm 1.5\%$ ) or a grass-mixture ( $67.7 \pm 3.2\%$ ), and the grass-clover ley ( $65.9 \pm 1.0\%$ ). Aggregates obtained from the 5–10 cm depth had consistently, but not significantly, lower aggregate stability of  $64.6 \pm 2.1\%$  in the grass-clover ley, with this ranging in the herbal ley from  $59.2 \pm 1.2\%$  with *Cichorium intybus*,  $63.2 \pm 5.0\%$  with *Plantago lanceolata* and  $64.3 \pm 6.9\%$  with the grass-mixture.

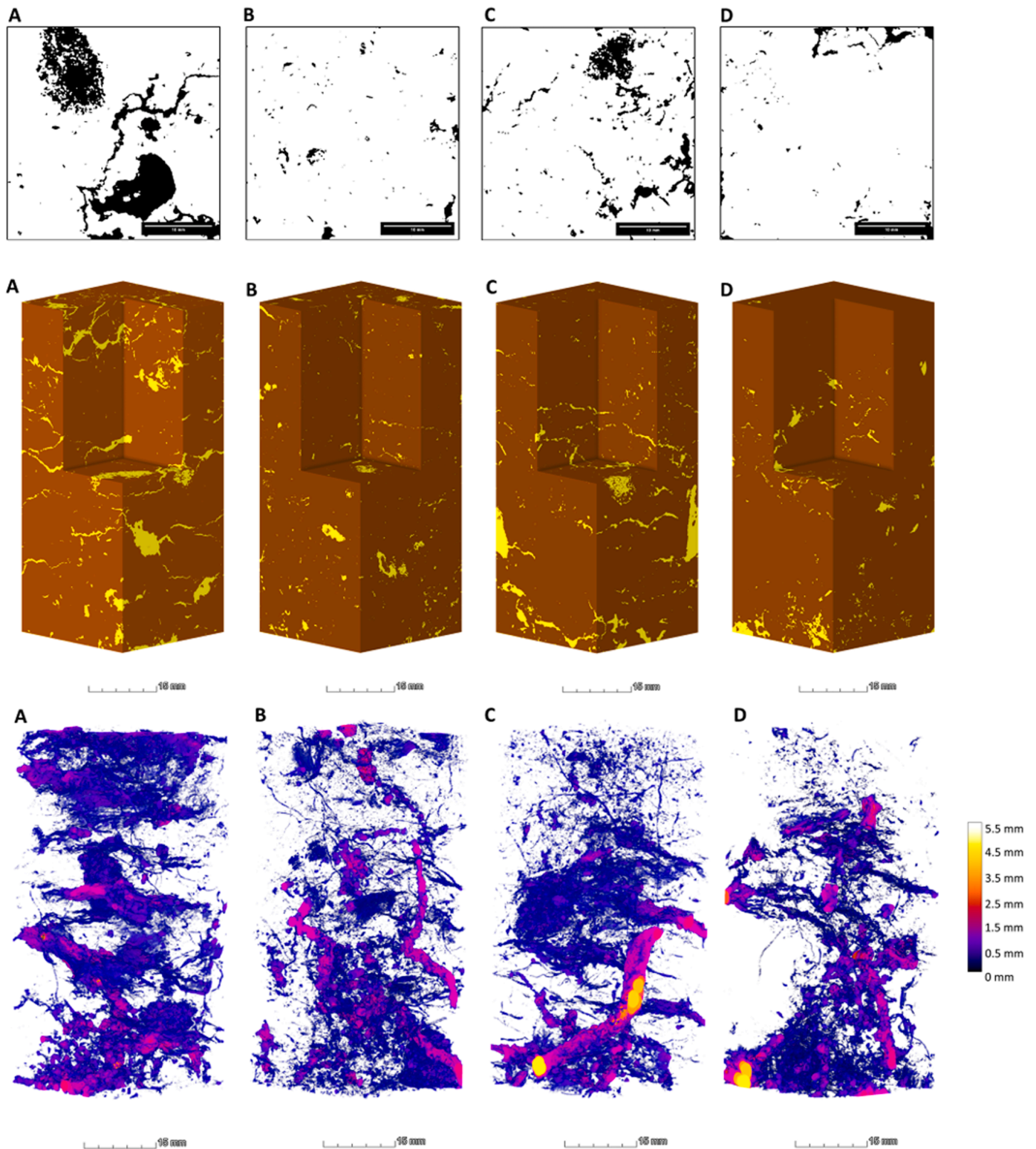
#### 3.5. VESS and VSA scoring

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 ( $2.8 \pm 0.2$ ) and grass-clover ( $2.4 \pm 0.2$ ) ley, with minor differences in VSA scores between the two swards ( $26.0 \pm 1.0$  herbal vs.  $26.5 \pm 0.6$  grass-clover) driven by slight variations in earthworm abundance.

#### 3.6. Soil biological properties

##### 3.6.1. Earthworm abundance and biomass

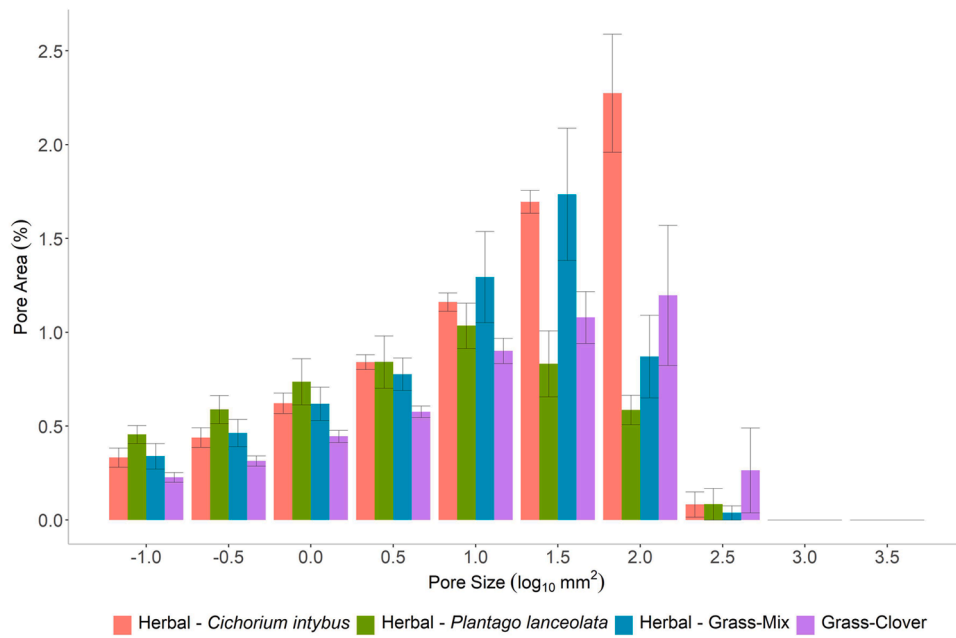
Following 33-months of ley establishment there was no significant difference in total earthworm abundance ( $301.2 \pm 75.0$  worms  $\text{m}^{-2}$  herbal vs.  $311.1 \pm 68.7$  worms  $\text{m}^{-2}$  grass-clover) ( $T_{(12)} = 0.1457$ ,  $p = 0.887$ ) or total earthworm biomass ( $121.25 \pm 24.6 \text{ g m}^{-2}$  herbal vs.



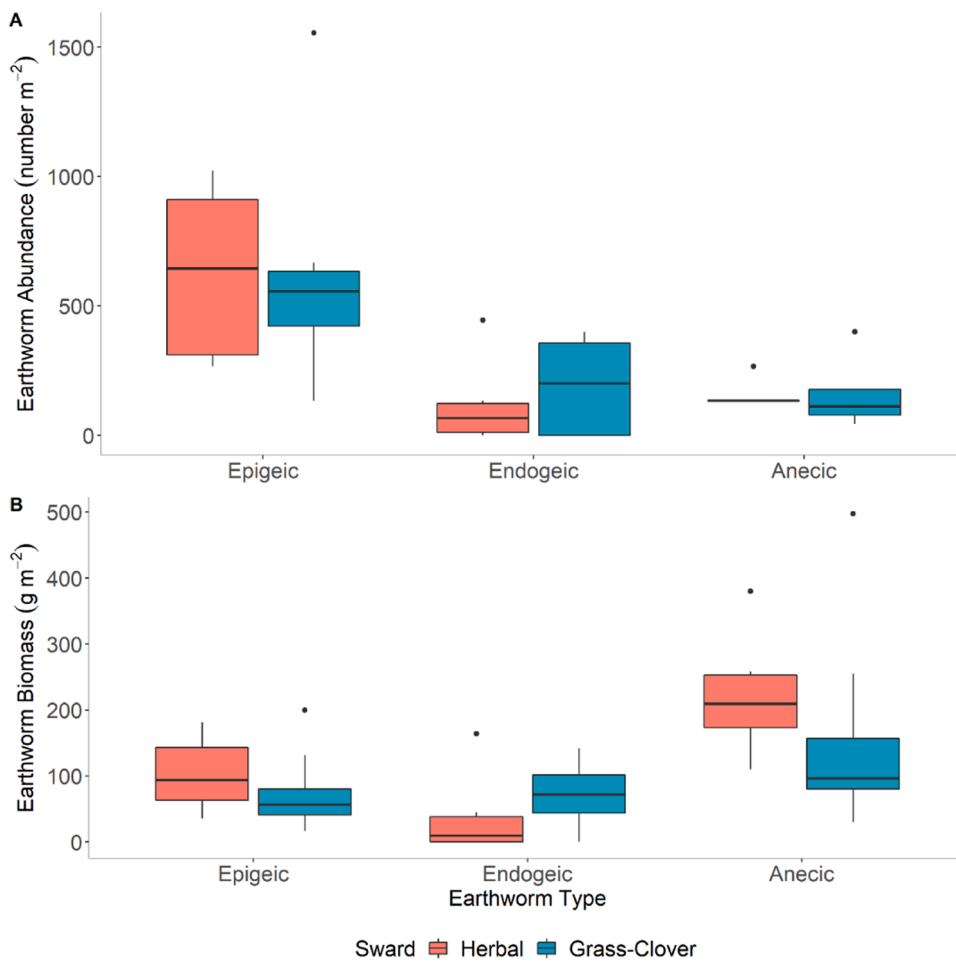
**Fig. 1.** Example X-ray  $\mu$ CT images of the ROI measured from intact soil cores; A = herbal - *Cichorium intybus*, B = herbal - *Plantago lanceolata*, C = herbal - grass-mix, D = grass-clover. Top row shows 2D binary slices of the middle of the ROI, black indicates pore space and white soil; scale 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.

$100.6 \pm 21.1 \text{ g m}^{-2}$  grass-clover) ( $T_{(12)} = -0.7708$ ,  $p = 0.456$ ) 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 (Fig. 3). However, anecic earthworms contributed most to biomass at  $222.8 \pm 38.0 \text{ g m}^{-2}$  and  $156.6 \pm 54.0 \text{ g m}^{-2}$  in the herbal and grass-clover ley, respectively.



**Fig. 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  $\pm$  SEM.



**Fig. 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.

### 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  $179,168 \pm 9945$  classified reads per soil 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 1). Bacteria in soil from both swards were dominated by Proteobacteria (50.5%), Actinobacteria (41.6%), and Planctomycetes (2.3%) (Supplementary Figure 4). 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 ( $K_{(1)} = 0.75$ ,  $p = 0.387$ ) (Fig. 4). (Supplementary Figure 7).

## 4. Discussion

### 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 ( $24 \text{ m m}^{-2}$ ) than *Triticum aestivum* grown after *Festuca arundinacea* ( $6 \text{ m m}^{-2}$ ). 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 differences between swards may 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. Firstly, 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  $\mu$ CT imaging. Instead, it is likely that rapid root turnover of fine roots ( $< 0.5$  mm diameter) occurred and produced micropores that contributed to producing a 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 is 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 2), with key taproot herbs *Plantago lanceolata* and *Cichorium intybus*

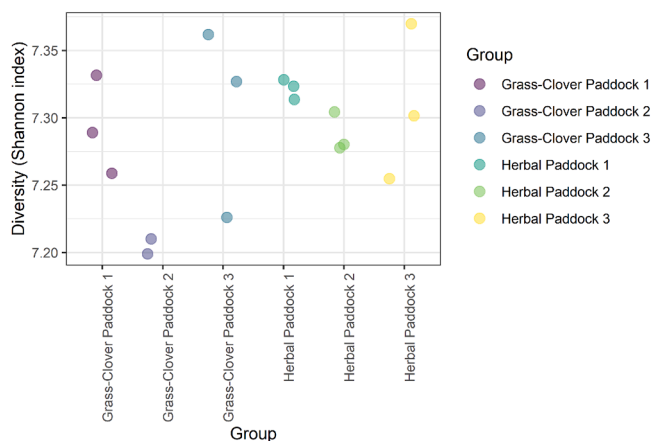


Fig. 4. Shannon Diversity index ( $H'$ ) at a species-level of the herbal and grass-clover ley, sampled 2-years after establishment,  $n = 8$  grass-clover,  $n = 9$  herbal ley.



comprising  $4.9 \pm 1.2\%$  and  $21.1 \pm 1.3\%$  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). 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 partially 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 16–44 years after the initial disturbance (Pohl et al., 2012), unlike after 2-years as in our study. It is important to note that it is difficult to compare our results to others, as there is limited information available for aggregate stability in improved grasslands, with the majority of previous research focused on arable-ley systems (e.g., Guest et al. 2022) with differing soil structure, soil texture, and initial soil organic matter content.

Finally, the high earthworm abundance recorded in the herbal (ca. 301 worms  $m^{-2}$ ) and grass-clover (ca. 311 worms  $m^{-2}$ ) ley after 2-years potentially biased pore characteristics determined via X-ray  $\mu$ CT. 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 (Fig. 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 3 and Supplementary Figure 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.

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). 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. 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 (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 a 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.

#### 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 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 (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* ( $3.1 \text{ t C ha}^{-1} \text{ yr}^{-1}$ ) than herb species such as *Cichorium intybus* ( $0.6 \text{ t C ha}^{-1} \text{ yr}^{-1}$ ) (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 ( $0.7 \pm 0.1 \text{ t C ha}^{-1} \text{ yr}^{-1}$ ) than monocultures of the same species ( $0.1 \pm 0.1 \text{ t C ha}^{-1} \text{ yr}^{-1}$ ) 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 ( $5.3\text{--}9.4 \text{ t ha}^{-1}$ ) containing *Cichorium intybus* and *Medicago sativa* increased topsoil (0–30 cm) carbon inputs by  $1.2 \text{ t C ha}^{-1}$  compared to the root mass of a conventional grass-clover ley ( $3.8\text{--}5.7 \text{ t ha}^{-1}$ ).

Although no measurement of root biomass or root turnover was made in this study, the similar topsoil SOC content within the herbal ( $26.1 \pm 1.1 \text{ t C ha}^{-1}$ ) and grass-clover ( $25.7 \pm 1.1 \text{ t C ha}^{-1}$ ) 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. 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. However, while there is limited data available for SOC sequestration in herbal leys, particularly when established 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<sup>-1</sup>, any additional topsoil SOC accrual produced via altering the sward composition likely occurs within the particulate organic matter 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). 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.

#### 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 only slightly 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). Aucubin can inhibit the enzymatic activity of ammonia monooxygenase and 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 reseeded 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., <sup>15</sup>N, <sup>18</sup>O or <sup>14</sup>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. 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 indicators of 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 provides a valuable insight into the effects of herbal leys on soil quality in a sheep grazed grassland.

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 utilising commercial herbal leys (with a typical seed cost of ca. £200–250 ha<sup>-1</sup>) compared to grass-clover leys (ca. £150 ha<sup>-1</sup>) is not currently rewarded 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.

### CRedit authorship contribution statement

**Jones Davey L.:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Cooleedge Emily C.:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sturrock Craig J.:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis. **Mooney Sacha J.:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Atkinson Brian S.:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Murphy Daniel V.:** Writing – review & editing, Funding acquisition. **Brailsford Francesca L.:** Writing – review & editing, Funding acquisition. **Chadwick David R.:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Leake Jonathan R.:** Writing – review & editing, Funding acquisition.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

Data will be made available on request.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.agee.2024.108928](https://doi.org/10.1016/j.agee.2024.108928).

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