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Dung Beetle Activity Is Soil-Type-Dependent and Modulates Pasture Growth and Associated Soil Microbiome

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Abstract: The introduction of numerous exotic dung beetles across southern Australia in regions where native dung beetles are not generally efficient in processing livestock dung has resulted in significant reductions in the quantity of such dung on the soil surface in recent years. However, the direct impacts of such ecosystem services on pasture quality and soil nutrient mobility have not yet been investigated in the Riverina region of New South Wales (NSW), an area recognised for prime cattle and sheep production in Australia. Utilising 48 soil columns for lysimetry, we quantified the impact of a common introduced dung beetle (*Bubas bison*) in this region on water quality after permeation through four different soil types sown to winter annual pastures. Dung beetle treatments included dung plus dung beetles, dung alone and no dung beetles, and no dung and no beetles as a control. Dung beetles and soil type impacted on the performance of improved overseeded annual pastures as measured by biomass accumulation over a four-month growing season. The four soil types, namely, Chromosol, Kandosol, Rudosol, and Vertosol, differed considerably with respect to their water-holding capacity and nutrient profiles, as assessed by initial soil testing and soil leachate evaluation following rainfall plus simulated rainfall events. The concentration of *Escherichia coli* resulting from cattle dung, cattle dung plus beetles, and the control soils without dung or beetles was assessed in collected leachates over a three-month period. *E. coli* numbers were significantly increased following *B. bison* activity, when compared to the dung-only and control treatments. Evaluation of the soil microbiome, by assessing genomic DNA in soils sampled 10 cm below the soil surface where dung beetles remained active following tunnelling, revealed significant differences among soil types with respect to bacterial and fungal communities. Within each soil type, dung beetle activity impacted the fungal community structure, but not the bacterial community. Pasture performance as assessed by biomass accumulation was significantly improved following dung beetle activity in later stages of pasture growth, while *E. coli* numbers and total coliforms appeared unaffected by beetle presence.

Keywords: lysimeter; annual pasture; dung beetles; livestock dung; *Escherichia coli*; pollution; soil microbiome; insect; arthropod; nutrient cycling

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1. Introduction

Subsequent to the introduction of domestic livestock to Australia in the late 18th century, accumulation of livestock dung on grazing lands became prevalent, owing to the absence of native detritivores capable of processing ruminant dung effectively [1]. This resulted in widespread pasture fouling, slower soil carbon and nutrient turnover, and increased livestock pest infestation, particularly with respect to flies and nematodes. As a potential biocontrol solution, exotic dung beetles that feed on livestock dung were

introduced periodically, from the early 1960s onward [1,2]. Dung beetles are coprophagous insects of order Coleoptera and are the dominant species in dung-associated communities, usually showing specificity in preference to dung types and certain environmental conditions [3,4]. Dung beetles are also considered ecosystem engineers, regulating the availability of resources to other organisms by altering physical and chemical properties of the soil environment [1,5,6]. To date, over 60 species of exotic dung beetles originating from both temperate and tropical climates have been introduced to livestock production regions in Australia [7,8]. The majority of established exotic dung beetles exhibit tunnelling behaviour, and are capable of processing herbivore dung, excavating tunnels in soil, and burying dung deep in the soil profile [7–9].

Although introduced dung beetles have successfully established in Australian grazing lands over time, their ecosystem services and perceived socioeconomic benefits have not yet been fully evaluated, due to a lack of consistency in experimental methods and difficulty in monitoring their impact below ground [1]. However, several studies using different species and methodologies have highlighted the ability of introduced dung beetles to deliver several beneficial ecological functions including enhancement of soil fertility and pasture growth, and suggested a significant reduction in livestock pests in Australian pastures [1,6,10–12].

In general, dung beetles enhance soil quality in several ways, including increased aeration through extensive tunnelling, dung shredding, and dung incorporation [13], which results in improved soil moisture infiltration [14] and nutrient cycling [15], leading to reduced soil nitrification and, in some cases, improved pasture growth. In the saturated–unsaturated soil zones, drainage is tied to the textural qualities of the soil, in addition to gas exchange [16]. Typical soil profiles demonstrate the effect of soil texture on plant available water content, which is influenced by the water-holding capacity of the soil, water held in the soil after free drainage, and water remaining in the soil profile after crop uptake [17]. Therefore, understanding soil water-holding capacity and gaseous movement in soils with contrasting physical properties is key to elucidating the impact of dung beetle activity in Australian pastoral ecosystems.

The geographical distribution of dung beetle assemblages may be determined by variations in soil structure and textural properties [18]. Therefore, to further investigate behaviour and impact of dung beetles, it is necessary to evaluate a variety of soil types, as the presence of clay in a soil profile may be a significant factor affecting the diversity of dung beetles [19]. Soil depth and clay content may also influence dung beetle tunnelling as tunnel depths may exceed one metre [20]. Sandy soils typically encourage tunnelling behaviour as these soils remain less compacted than other soil types despite livestock movement across pastures [21]. Australian soil classification separates various soil types based on several soil properties including texture and structural features [22]. For example, Chromosol is made up of a duplex soil profile, Kandosol has a mostly uniform clay depth and contains non-expanding clay, and Vertosol is made up of smectite clay with a predominantly 2:1 layer of silicates [22]. The tunnelling, nesting, and dung burial behaviour of dung beetles in diverse soil types in Australia has not been studied in detail.

A major concern related to the presence of introduced dung beetles in pastures and rangelands is associated with their perceived impact on the leaching of microbial contaminants from dung deposited on the soil surface into ground water, through tunnelling and the deep burial of dung within the soil profile [23]. However, dung beetles have also been shown to reduce microbial pollution of waterways by removal of pathogenic zoonotic microorganisms from the soil surface by burial of dung over time. For example, dung burial by dung beetle species *Bubas bison* during the winter months substantially reduced the numbers of *Cryptosporidium* oocysts detected on the soil surface, which would otherwise be washed into waterways following winter rains [12]. Similarly, *Onthophagus hecate* has been shown to contribute to the suppression of pathogenic *Escherichia coli* strain O157:H7 in agricultural landscapes [24]. A recent study conducted in New Zealand to assess the quality of water leaching from soil cores containing dung beetles demonstrated

that dung beetle tunnelling and burial of dung did not impact the quality of water leaching through an allophanic soil [23]. In Australia, the impact of dung beetle activity on soil water contaminants including *E. coli* in soil leachates is currently unknown.

Although the changes in soil physical and chemical properties due to tunnelling and burial of dung by dung beetles have been relatively well established for several different ecosystems around the world [21,25,26], the impact of dung beetles' activities on the soil microbiome has yet to be explored. Beetle-related changes in the soil profile could potentially be expected to influence soil biota dynamics, thus affecting plant–microbial interactions in the rhizosphere and soil nutrient cycling, which, in turn, impact pasture productivity. Moreover, the population dynamics of zoonotic pathogens in pasture soils are likely to differ depending on soil type, thus potentially impacting both herd and human health.

Currently, there exist only a limited number of studies investigating the changes in soil microbiome in the presence of active dung beetles. Slade et al. [27] demonstrated that there are significant interactions between dung beetles and soil microorganisms, and the diversity and composition of microbial communities in both the dung pats and underlying soils were significantly affected by the presence or absence of dung beetles. More recently, Kaleri et al. [28] showed that the dung beetle activity increased soil bacterial diversity and soil enzyme activity contributing to enhanced plant growth. However, the effect of dung beetle activity on the soil microbiome and its function in diverse soils underlaying pastures has not yet been reported, specifically in Australia.

The periodic introduction of various exotic dung beetle species to grazing lands in Australia has been justified by the perceived economic benefits of dung beetle activity in agricultural soils attributed to the multiple ecosystem services provided including reduced pasture fouling, loss of nitrogen, and livestock parasites; increased soil moisture retention and availability; pasture growth plus biomass accumulation [1,6,15,29]. Beynon, Wainwright, and Christie [5] estimated that the impact of ecosystem services provided by dung beetles to the UK cattle industry is ~GBP 367 million per year. Although it is estimated that introduced dung beetles in Australia process about 300 million tons of livestock dung annually [30], and provide ecosystem services worth ~AUD 1 billion a year [30], a systematic assessment of the economic impact of the introduced dung beetles in Australia is yet to be conducted. Therefore, one of the objectives of the Australian dung beetle project (Dung Beetle Ecosystem Engineers) is the assessment and quantification of dung beetle ecosystem services with respect to on-farm economic services, and off-farm environmental and social benefits [30].

The multi-institutionally supported Dung Beetle Ecosystem Engineers (DBEE) project was launched in Australia in 2018, to assess the impact of both existing and introduced dung beetles on soil ecosystems. A field mesocosm study conducted at three locations across NSW on silty clay loam soils showed a significant impact of dung beetles on dung decomposition resulting in greater pasture growth in contrast to dung alone or soil-only controls [31]. However, there remain several key aspects related to the environmental impact of dung beetles on pastoral ecosystems that overlay diverse soil types in Australia, which have not yet been investigated. We hypothesised that soil type may affect dung beetle activity and subsequent pasture growth and modulate the associated soil microbiome over time.

In this study, we employed a lysimeter assembly similar to that of Aislabie, McLeod, McGill, Rhodes, and Forgie [23], with slight modifications to create structured columns of four different soil types under simulated field conditions to assess the (i) soil water movement through the soil profile, (ii) presence and potential leaching of pathogenic *E. coli* and coliform bacteria, (iii) differences among the soil microbiome and associated microbial communities, and (iv) pasture performance, in four diverse soil types from the Riverina NSW with (i) no treatment, (ii) supplementation with cattle dung, and (iii) supplementation with both cattle dung and a large local winter-active, tunnelling dung beetle, *Bubas bison*, on a pasture mix of grass and legume species grown under natural environmental conditions.

2. Materials and Methods

2.1. Facility Construction, Preparation of Lysimeter Columns, and Experimental Design

The lysimeter facility was constructed following broadly the design described by Aislabie, McLeod, McGill, Rhodes, and Forgie [23], on a level site located at the Charles Sturt University (CSU) livestock research farm in Wagga Wagga, NSW (35°02′00.5″ S 147°21′40.6″ E). Mean annual precipitation at this site is 571 mm [32] (Supplementary Table S1). Forty-eight lysimeter columns, each 0.5 m in height and 250 mm in diameter with a surface area of 0.05 m^2, were constructed on a raised platform that was 7.34 m long, 1 m high, and 1 m wide to accommodate all columns, surrounded by crushed gravel to maintain soil temperatures similar to field conditions, in a north–south direction (Supplementary Figure S1).

Soils and treatment combinations of dung and dung beetles were randomly allocated to the lysimeter columns in a factorial design with four replicates of each soil type and of each treatment arranged in a randomised complete block design. Each soil type was placed in three columns in each of the four blocks and then the three treatment combinations were allocated randomly within each block. Three dung and/or beetle treatments were applied on each soil type, i.e., Control (soil only), Dung on soil, and Dung on soil with dung beetles. Altogether, 48 soil lysimeter columns were maintained from June through November 2021 in this experiment.

Each column was filled with approximately 80 L of soil. The four soil types selected for use were a Red Chromosol, Grey Vertosol, Red Kandosol, and Brown Rudosol [33], which were collected from field sites within 100 km of the CSU campus. Once collected, soils were air-dried and maintained in separate containers, and then sieved using a 2 mm sieve and later repacked in the columns in three distinct layers in the order of their collection, i.e., 0–15 cm, 15–30 cm, and 30–45 cm, into each lysimeter column at a similar soil bulk density as those of original field soils. The Red Chromosol and Red Kandosols were collected from a location close to Charles Sturt University farm (−35.052748, 147.332736) (Supplementary Figure S2). The New South Wales Department of Primary Industries' research station at Yanco NSW provided the Vertosol for this study (−34.604503, 146.358754). The Rudosol (with a dominant loamy sand) was obtained close to Marrar NSW (−34.867899, 147.354228). Basic properties of the soils are shown in Table 1.

Table 1. Initial chemical properties of bulked soil types representative of four soils collected from 0–45 cm depths and bulked by field coring at each collection site, prior to column assembly.

Parameter	Chromosol	Kandosol	Rudosol	Vertosol
Phosphorus (mg/kg P)	7.22	12.79	11.26	87.03
Nitrate Nitrogen (mg/kg N)	4.35	2.26	0.49	12.74
Ammonium Nitrogen (mg/kg N)	6.64	7.45	5.80	12.05
Sulphur (mg/kg S)	4.64	6.94	4.17	22.29
pH (1:5 water)	6.91	6.80	7.00	7.66
Electrical Conductivity (dS/m)	0.04	0.04	0.03	0.11
Estimated Organic Matter (% OM)	1.33	1.40	0.62	1.61
Effective Cation Exchange Capacity (ECEC) (cmol+/kg)	3.71	6.87	2.97	29.46
Calcium (%)	70.31	62.13	69.61	66.16
Magnesium (%)	14.27	21.73	16.48	28.56
Potassium (%)	13.60	15.09	10.94	4.43
Sodium—ESP (%)	1.63	0.94	2.83	0.82
Aluminium (%)	0.20	0.11	0.13	0.03
Zinc (mg/kg)	0.60	0.65	<0.5	0.82
Manganese (mg/kg)	37.22	27.27	24.17	11.02
Iron (mg/kg)	29.47	38.05	14.57	39.67
Copper (mg/kg)	0.45	1.01	0.31	2.00
Boron (mg/kg)	0.51	0.82	0.33	1.22
Silicon (mg/kg Si)	42.45	74.65	36.98	46.31
Total Carbon (%)	0.76	0.80	0.35	0.92
Total Nitrogen (%)	0.11	0.11	0.07	0.14
Carbon/Nitrogen Ratio	6.86	7.24	4.68	6.54

After the installation of soil columns, the space between the cores was backfilled with a washed gravel sand mix to reduce the temperature fluctuation. The entire facility was covered by a porous shade cloth protection supported by aluminium scaffolding to protect the experimental site from severe weather conditions including excessive rain, heat, and wind.

2.2. Establishment of Pasture

Upon construction, all lysimeter columns were allowed to settle for 30 days under field conditions. In early June, a mixture of dual-purpose oats (cv. Mannus) and purple vetch (cv. Popany) inoculated with a commercially available rhizobia (Type F; New Edge Microbials, Albury, NSW, Australia) was sown at a rate of 30 kg/ha (equivalent to 30 oat seeds and 20 vetch seeds per column). At this time, Diammonium Phosphate (DAP) fertiliser (17.7% nitrogen, 20% phosphorus; Incitec Pivot, Southbank, VIC, Australia) at a rate of 40 kg/ha or 10 g/m^2 was applied to the soil and incorporated at a depth of 2 cm, as per recommendations [34,35], to provide a starter dose of N and P. Following seeding, 1 L of reverse osmosis (RO) laboratory water, equivalent to 20 mm of rainfall, was supplied to each column to promote seed germination and seedling establishment. After 30 days, seedling stands of oat and vetch were thinned to 10 and 12 plants per column, respectively. All columns were overseeded with annual ryegrass at the rate of 15 kg/ha and an additional top dressing of DAP at 20 kg/ha, resulting in approximately 50 ryegrass plants per column. Post-seeding irrigation was provided as mentioned earlier.

2.3. Introduction of Dung Beetles and Cattle Dung

One hundred healthy and mature matched male and female pairs of *Bubas bison* were collected and sorted from the CSU cattle farm for use in the lysimeter experiment. Upon experimental initiation, 50 kg of fresh dung was collected from undrenched cattle at the Charles Sturt University commercial cattle farm. Freshly collected and homogenised dung was applied to lysimeter columns assigned to dung-only, and dung and beetle treatments, at a rate of 750 mL/column in mid-August 2021. Five pairs of *B. bison* were then added to each column assigned to the dung plus beetle treatment. Only sexually mature dung beetles were sourced for this experiment to achieve maximum tunnelling and dung burial activity. After beetle addition, each column was covered with muslin cloth secured with elastic tape to prevent beetle escape. Residual dung left on the soil surface was removed after 28 days.

2.4. Irrigation of Soil Columns

Throughout the plant growth period, each column was manually supplied with 1 L of RO water, equivalent to 20 mm of rainfall, applied at weekly intervals. During this time, each core was exposed to natural rainfall events and, as necessary, soil moisture was supplemented by application of RO water on a weekly basis when natural rainfall was not sufficient to maintain soil moisture levels at field capacity. Once the pasture crop was established, RO water was supplied at varying levels for each soil column following major rainfall and irrigation events to generate leachate. All rainfall (Supplementary Table S1) and RO water received were recorded for each soil column individually over time until experimental termination. Three major rainfall and irrigation events occurred approximately one month apart in September (22 mm), October (44 mm), and November (158 mm).

2.5. Collection of Soil Leachates

Leachates from each lysimeter column were collected over 3-month at 30-day intervals following rainfall or irrigation events in September, October, and November 2021. All leachates were allowed to leach through soil columns until equal volumes were obtained from each soil column and were collected in covered 10 L plastic buckets placed under each lysimeter column. Each sample was mixed by shaking and divided into two subsamples, for enumeration of total coliform and *E. coli* bacteria, and for analysis of total N, total P, and

NO_x. Leachate samples for chemical analysis were stored at −20 °C until processing, while leachates for microbial enumeration were processed within four hours of collection.

2.6. Optimisation Protocols for Testing for E. coli and Coliform Bacteria

Prior to establishment of the field lysimeters, four lysimeter columns containing each of the four different soil types were used in a glasshouse trial as treatments to determine the water-holding capacity and drainage time and rate required for each soil type, as well as to determine the dilution factor for leachates from each soil type suitable to obtain an estimated total coliform and *E. coli* concentration. RO water was applied manually to determine the amount of water held at field capacity for each soil type, and to quantify the water input required for each soil type to result in sufficient leachate for analysis of total coliform and *E. coli* concentration in each soil type. RO water and rainwater were used as treatment controls for enumeration of coliform and *E. coli* in the water supply. The dilution factor for each soil type was determined using a commercial testing kit to enumerate total coliform and *E. coli*, i.e., IDEXX Colilert testing kit using the Colilert Quanti-tray technique (IDEXX Laboratories, Inc., Westbrook, ME, USA), following the manufacturer's instructions [23].

2.7. Enumeration of Total Coliforms and E. coli in Soil Leachates

Enumeration of total coliforms and *E. coli* in soil leachates was conducted within 4 h of collection, using the IDEXX Colilert kit as mentioned previously. A sample of soil leachate (100 mL) was used to enumerate coliform and *E. coli* leached out of each lysimeter column, diluted in sterile RO according to the estimated dilution factor for each soil type.

2.8. Collection of Pasture Biomass

Above-ground pasture biomass was collected at 30-day intervals to simulate livestock grazing, first in August 2021 at the time dung and dung beetle treatments were introduced, and then at monthly intervals in September, October, and November 2021, at the same time leachates were collected, by trimming foliage to 40 mm above the soil surface in all lysimeter columns. Pasture biomass samples were stored at 4 °C until drying at 70 °C in a forced-air-drying oven for 72 h before weighing.

2.9. Analysis of Total N and P in Soil Leachates

Three sets of soil leachate samples collected from lysimeter columns at each sampling were stored at −20 °C prior to total N and P analysis. The first set of leachate samples collected following the first major rainfall and irrigation event, from the lysimeter columns with dung-only, and dung and dung beetle treatments, were analysed at the Environmental and Analytical Laboratories (EAL) in the National Life Sciences Hub at Charles Sturt University. Total P was analysed using the standard methods APHA 4500-P B5 and 4500-P E/F, and total N was estimated by combining the results of the total Kjeldahl nitrogen (TKN) and the NO_x-N analyses. The standard TKN methods used were APHA 4500-Norg B and 4500-NH_3 C, and the NO_x-N method was APHA 4500-NO_3 F.

2.10. Microbial DNA Extraction from Soils and Marker Gene Sequencing

Following the final harvest of the pasture crop, five soil cores were sampled from each lysimeter column at a depth of 5–10 cm using a handheld soil corer with a core diameter of 5 cm. A composite soil sample was prepared by combining the soil cores from within each column. Soil genomic DNA extraction, and subsequent phylogenetic marker gene sequencing of a 300 bp paired-end run targeting the 16 S V3–V4 and ITS regions for bacteria and fungi, respectively (Table 2), on an Illumina MiSeq next-generation sequencing platform (Illumina, Melbourne, VIC, Australia) were performed at the Australian Genome Research Facility (AGRF, Adelaide, SA, Australia).

Table 2. Primers used for phylogenetic marker gene sequencing of bacteria and fungi.

Primer Name	Primer Sequence
341F—Universal 16S	5′-CCTAYGGGRBGCASCAG-3′
806R—Universal 16S	5′-GGACTACNNGGGTATCTAAT-3′
1F—Universal ITS	5′-CTTGGTCATTTAGAGGAAGTAA-3′
2R—Universal ITS	5′-GCTGCGTTCTTCATCGATGC-3′

2.11. Analysis of Quantitative Data

Irrigation water supplied for each column was measured in litres and converted to millimetres for data analysis. Total coliform and *E. coli* counts were obtained as MPN per 100 mL for each leachate sample and were corrected for any dilutions. Total N, total P, and NOx in leachates were measured as milligrams per millilitre. All data were transformed for homoscedasticity, and statistically analysed using analysis of variance (ANOVA) and factorial analysis in R version 4.0.2. Treatment means were compared using the Bonferroni pair-wise comparison method.

2.12. Analysis of Marker Gene Sequence Data

Data processing and statistical analysis to generate OTU tables were performed by the Australian Genome Research Facility (AGRF). Briefly, paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.95, Exelixis Lab, Heidelberg, Baden-Württemberg, Germany). Primers were identified and trimmed, with the trimmed sequences then processed using Quantitative Insights into Microbial Ecology (QIIME2) [36], USEARCH (version 8.0.1623) [37], and UPARSE software [38]. Utilising the tools within USEARCH, sequences were quality-filtered, and full-length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtering using "Unite" as the reference database. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. QIIME2 software was used to assign taxonomy with UNITE database as the reference database (version 7.2) [39] for subsequent generation of absolute abundance values for each OTU. All subsequent microbial analyses were performed using the MicrobiomeAnalyst platform [40]. For data that were used through this platform, low abundant features were filtered based on mean values, with a minimum count size of 4, with low-variance features excluded based on the interquartile range. Following data filtering, features were normalised using the total sum scaling method prior to further analysis. Alpha diversity was calculated using the Shannon index, while the PCoA ordination method was used for the beta diversity analysis, with the Bray–Curtis index used for significance testing.

3. Results

3.1. Soil Water Content

The average quantity of water required to reach field capacity within each soil type after collection from field sites was estimated to be 41 mm, 39 mm, 33 mm, and 22 mm for Chromosol, Vertosol, Kandosol, and Rudosol, respectively. Measurements of soil water content and related soil physical properties are shown in Table 3.

Table 3. Initial soil moisture content of specific soil types at the time of column assembly, included in lysimetry experimentation performed in Wagga Wagga, NSW, measured as gravimetric water content.

Soil Type	Packed Soil Water Content (L/L)	Gravitational Water Content $^{w}/_{w}$	Bulk Density Gcm^{-3}
Chromosol	0.090	0.109	1.187
Kandosol	0.073	0.093	1.165
Rudosol	0.065	0.067	1.142
Vertosol	0.087	0.276	1.337

3.2. Rainfall

Total rainfall received from May to November 2021 following the establishment of lysimeters was 462.6 mm, while the total of the long-term average (years 1942–2022) rainfall during this period was estimated at 358.7 mm [32], suggesting that the overall rainfall experienced at Wagga Wagga during this period was above average for pasture establishment and growth (Figure 1).

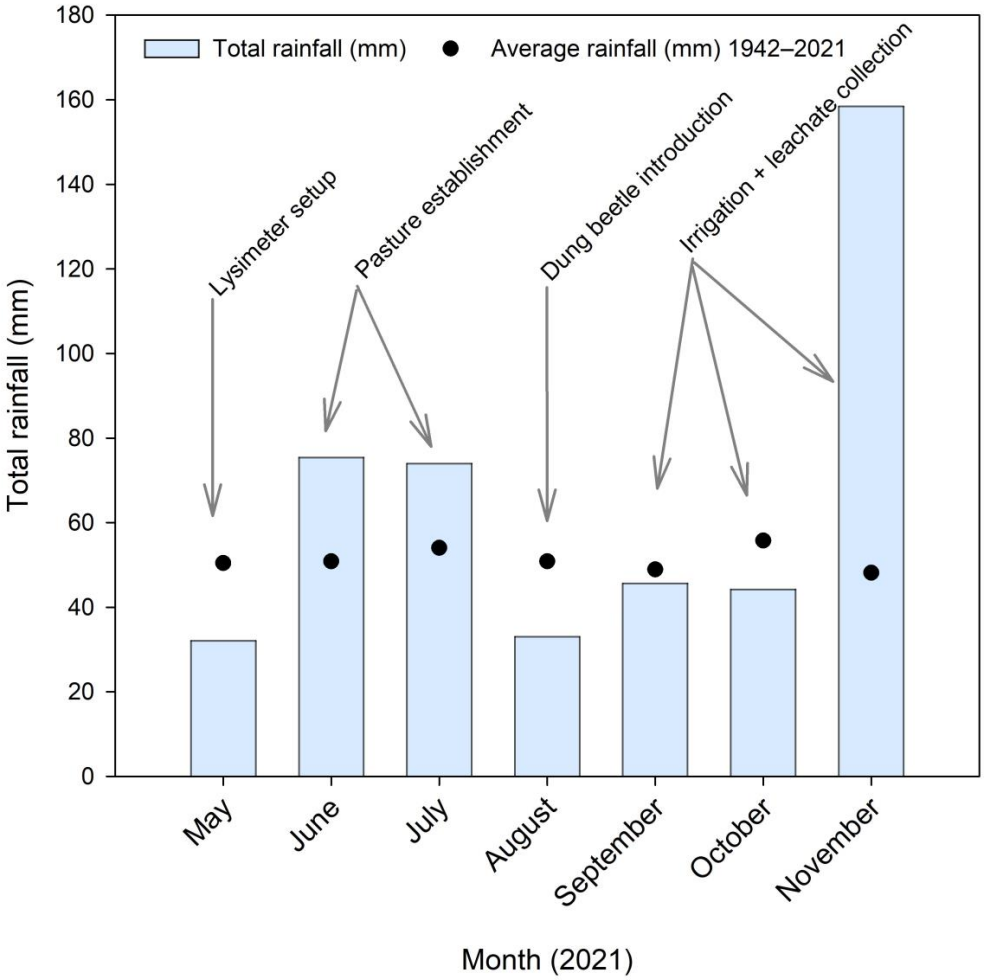

Figure 1. Total rainfall as contrasted with long-term average rainfall in Wagga Wagga, NSW over the pasture growing season of May–November 2021 [32].

Although 2021 experienced ~160 mm rainfall in November due to a La Nina condition experienced across southern Australia atypical of historic winter rainfall patterns, additional manual irrigation events (20 mm each) were required between rain events from May to July 2021 to ensure sufficient soil moisture for pasture growth in lysimeters.

3.3. Irrigation

The average volume of RO water required to irrigate each soil type in order to generate an equal volume of leachate is presented in Figure 2. We observed significant ($p < 0.01$) differences among the four soil types in soil moisture-holding capacity at each of the three-monthly irrigation events, which could be attributed to inherent differences in water-holding capacity of each soil type, as well as porosity, and other physical properties of soil types. Within the soil types, the volume of water applied was similar for each column, except for Kandosol, requiring 2.8 L of irrigation for individual soil columns in the third irrigation event, when compared to the previous two irrigation events (1.8 and 1.9 L for irrigation 1 and irrigation 2, respectively). In general, Rudosol required the least volume of

applied water (1.25–1.9 L) to generate the required volume of leachate, due to the low soil water-holding capacity of this sandy soil type [41].

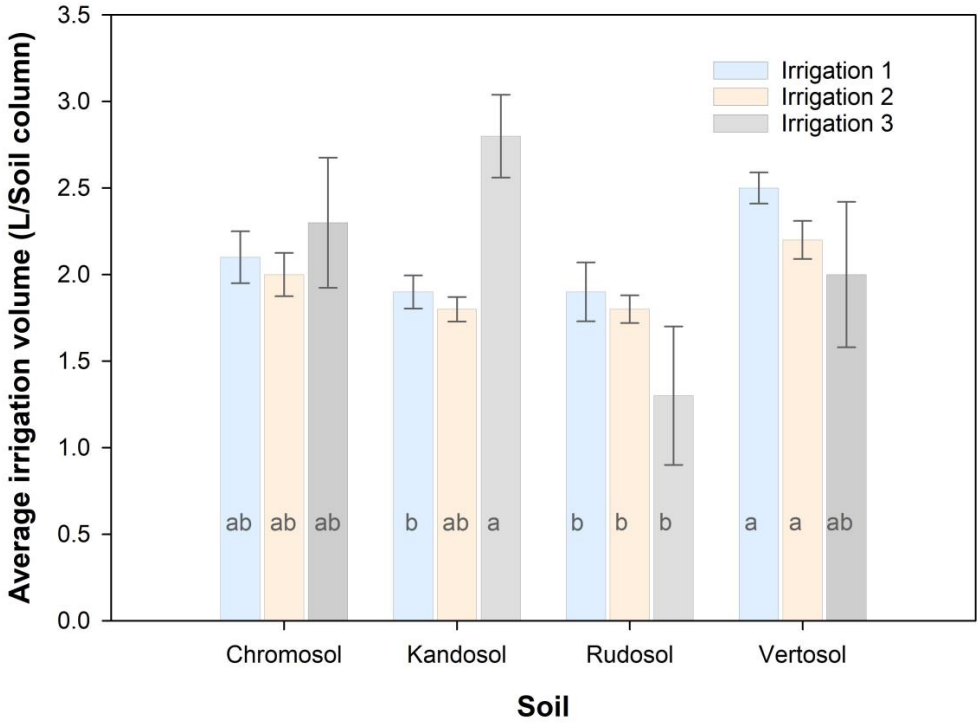

Figure 2. The average volume of irrigation applied to lysimeter columns to generate an equal volume of leachate. Error bars represent standard error. Different letters represent significant differences between treatment means. Bars sharing the same letters are not significantly different within each soil type at $\alpha = 0.05$.

3.4. Microbial Contamination in Leachates

Colorimetric determination of the total coliform bacteria from soil leachates was attempted using a commercially available testing kit, designed for environmental water testing. The appropriate leachate dilution ranges for each soil type were determined using leachates collected in the preliminary lysimeter experiment conducted under controlled environment conditions. However, when translated to the field-based lysimeter setup, these dilutions were inadequate to generate most probable numbers for total coliform due to saturation of the spectrophotometer beyond the quantifiable value in all soil types tested (data not shown). However, the dilution series chosen for each soil type was adequate for the quantification of *E. coli*.

There was no significant impact of soil type on *E. coli* leaching. Therefore, sample results for each soil type were pooled to assess treatment effects. In isolation, the soil-only treatment contained <10 MPN/mL of *E. coli* at the first collection, which is equivalent to numbers expected in water used for agricultural irrigation of non-edible crops (Figure 3) [42]. Addition of dung at the soil surface increased the leached soil *E. coli* number to ~20 MPN/mL but was not significantly greater than the soil-only control. However, dung beetle activity increased the *E. coli* number over 10-fold when compared to the soil-only and dung-only treatments ($p < 0.05$). Successive leaching events produced less *E. coli* MPN/mL potentially through flushing of the soil columns after the first collection of leachates followed by the removal of residual dung pat at that time, and were typically below 30% when compared to the number in the previous collection time.

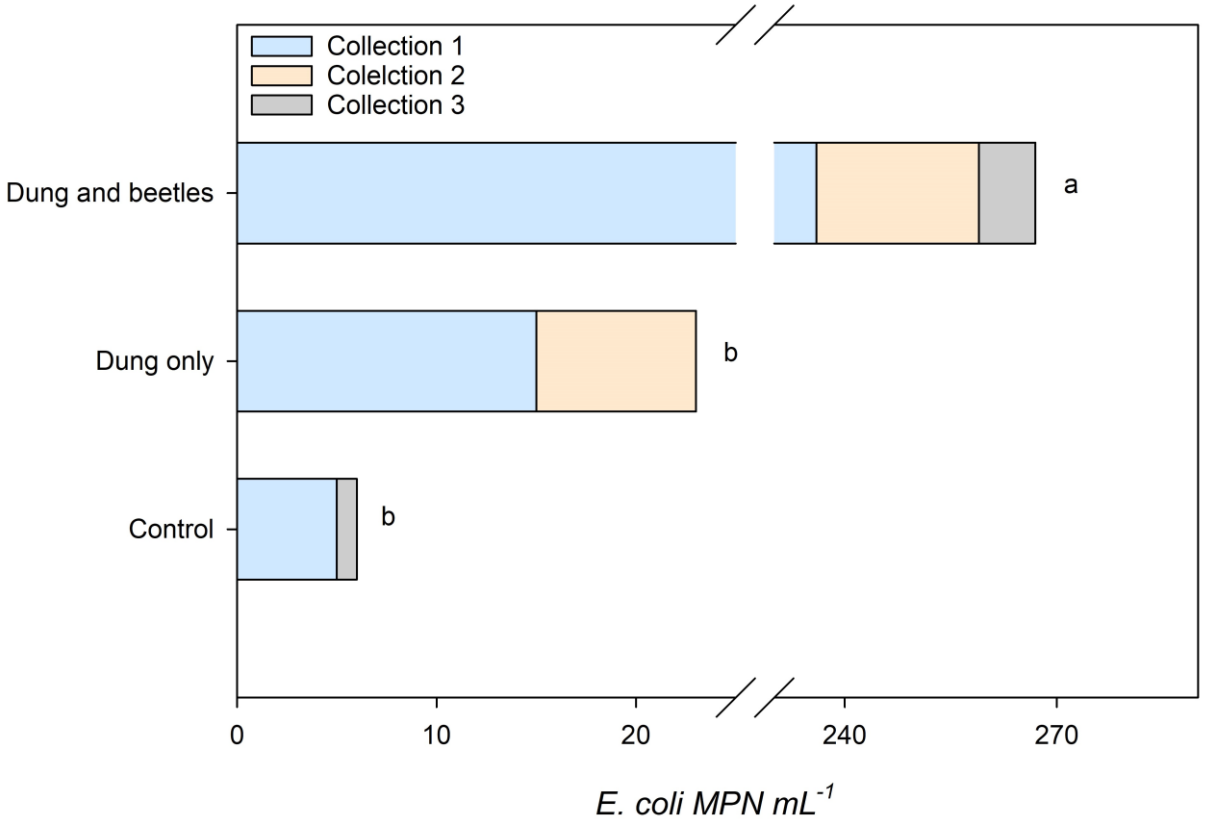

Figure 3. Cumulative leaching of *E. coli* bacteria from lysimeter columns measured at each leachate collection. Error bars represent standard error. Different letters represent significant differences between means of treatments. Bars sharing the same letters are not significantly different within each treatment at $\alpha = 0.05$.

3.5. Total N, Total P, and NOx in Soil Leachates

Total N and total P in all leachate samples analysed were negligible, ranging between zero and 0.1 mg/L (Supplementary Table S2). However, available NOx varied between zero and 23 mg/L, showing a significant ($p < 0.05$) difference between the soil types (data in Supplementary Table S2). Vertosol and Rudosols leached more NOx than Chromosol and Kandosol, with equal amounts of dung applied to all four soil types in lysimeters, suggesting more extensive elution in those soil types. There was no significant difference among the dung-only treatment versus dung plus dung beetle treatment, suggesting that dung beetle activity did not increase NOx leaching from soil.

3.6. Soil Microbiome Assessment

The bacterial microbiome was mostly dominated by the presence of actinobacteria in all soil types when assessed at the phylum level (Figure 4A). However, the proportion of Actinobacteria was less abundant in Vertosol, exhibiting a 10–20% reduction in comparison to other soil types. The relative abundance of other phyla including Chloroflexi, Actinobacteria, and Acidobacteria was higher in Vertosol. Importantly, the alpha diversity of rhizobacteria was not impacted by the management practices in any of the soil types assessed (data not shown). However, PERMANOVA analysis revealed that the soil type significantly impacted the beta diversity as the bacterial community profiles differed between the soil types assessed ($p < 0.001$), with those in Vertosol clustering separately to the other soil types (Figure 4B).

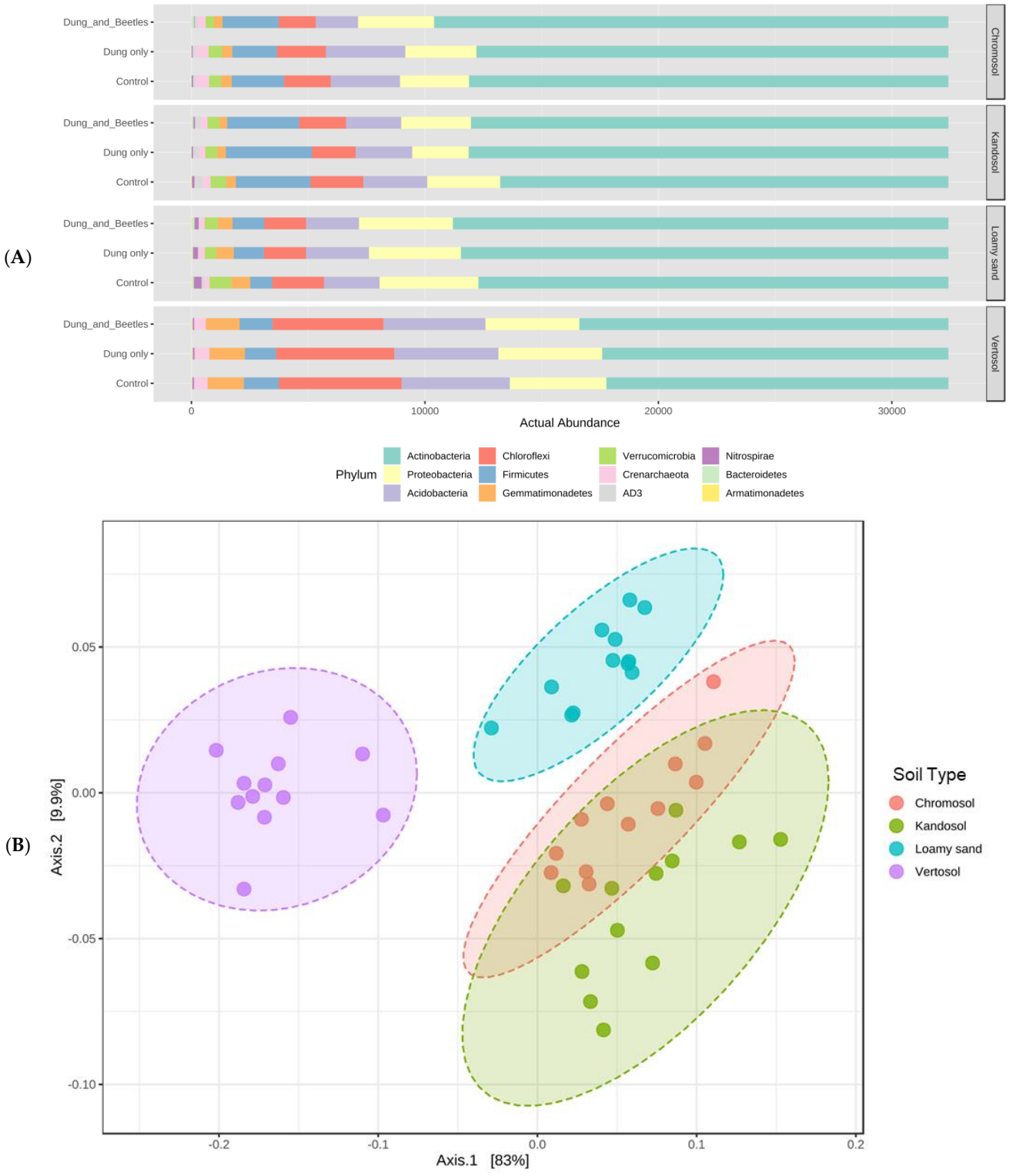

Figure 4. The rhizobacterial community assembly in the different soil types assessed in response to the control, dung, and dung plus beetle treatments. (**A**) The taxonomic distribution of bacterial communities under different pasture management practices. (**B**) Principal coordinate analysis of 16 s rRNA diversity in the rhizosphere of the different soils assessed (data from all treatment types combined due to similarity of the treatment effect).

The fungal phyla were mostly dominated by the presence of Ascomycota in Vertosol and Rudosol for all management practices, and for the control and dung plus beetle treatment in Kandosol (Figure 5). In contrast, Chromosol was dominated by Mortierellomycota for all treatment types, as well as the dung-only treatment for Kandosol. Importantly, the fungal diversity was not impacted by the management practices at the phylum level for all soil types except for Vertosols, where the dung plus beetle treatment resulted in a significant increase in the Shannon index ($p < 0.05$; Supplementary Figure S2). PERMANOVA revealed that the soil type impacted the beta diversity most, with the fungal community profiles differing significantly between the soil types assessed ($p < 0.001$). Taxa associated with Vertosol clustered separately to the other soil types.

In soil samples associated with dung beetle activity, we observed an increase in the bacterial class Clostridiaceae, which contains various genera with known pathogenicity to humans and animals, as well as potential to fix nitrogen in the soil (Figure 6A) [43]. We also observed that the fungal class Agaricaceae was associated closely with dung beetle presence (Figure 6B). Most genera of this class of fungi are saprophytic, decomposing lignin and cellulose [44]. Collectively, these observations suggest that the burrowing behaviour of beetles may have enhanced the movement of water along with surface-dwelling exogenous microorganisms deeper into the soil profile.

(**A**)

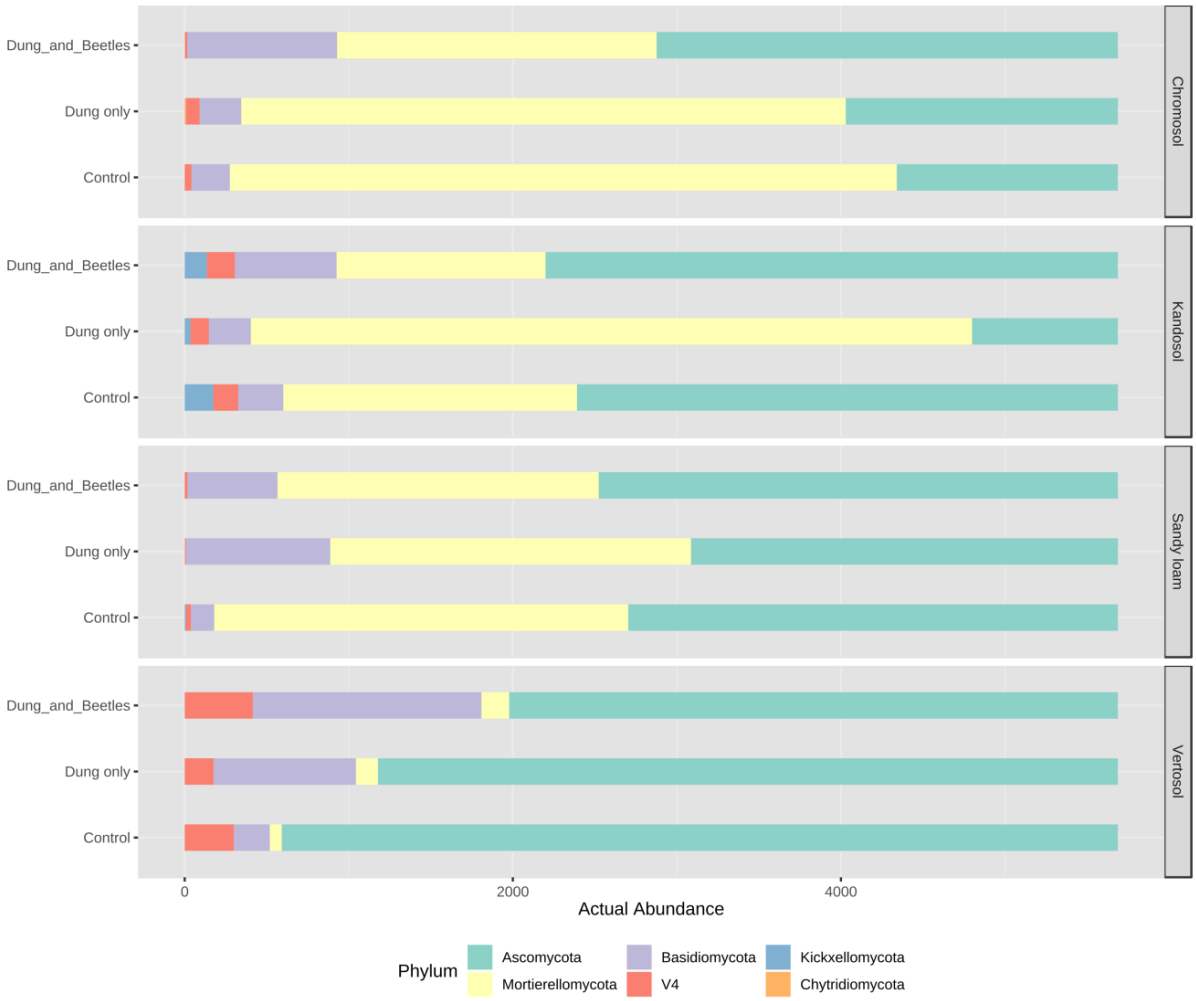

Figure 5. *Cont.*

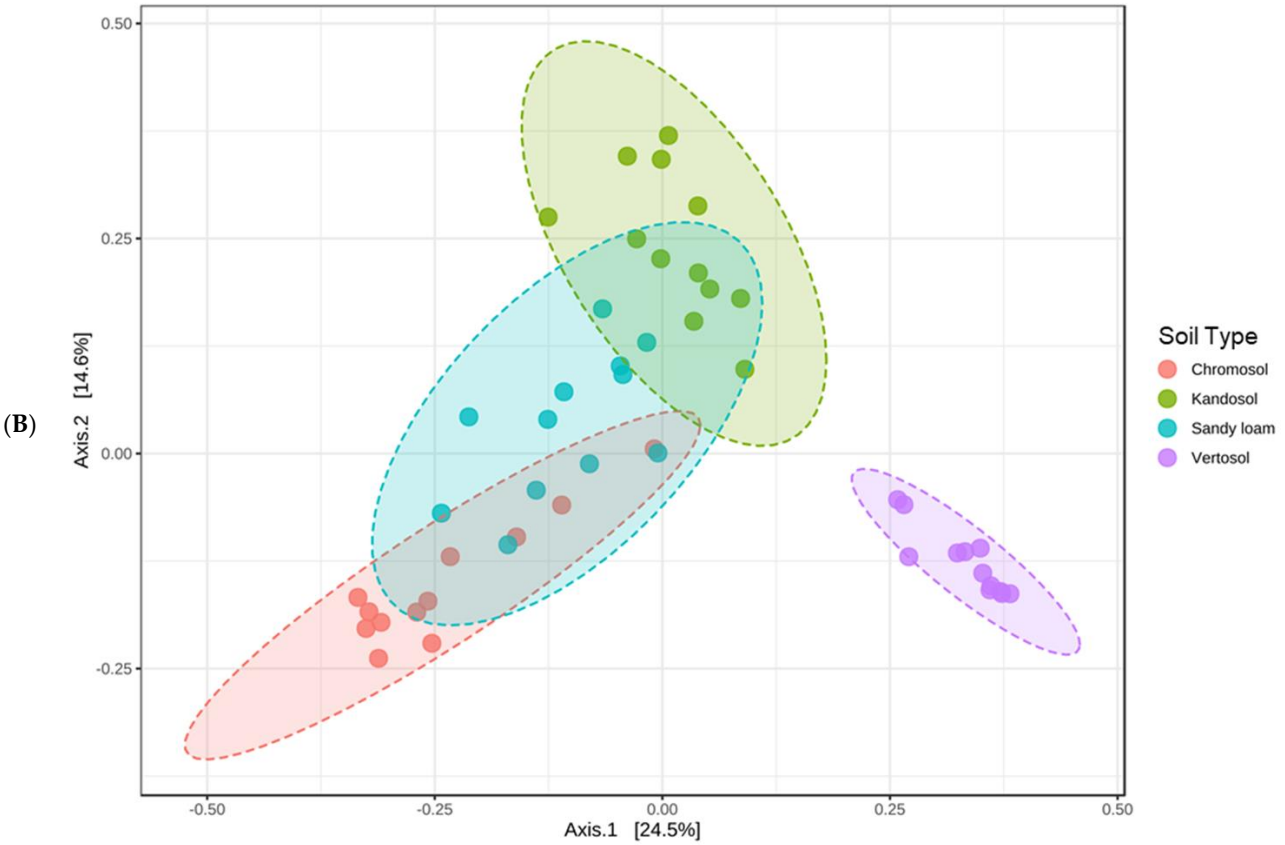

Figure 5. The soil fungal community assembly in the different soil types assessed in response to the control, dung, and dung plus beetle treatments. (**A**) The taxonomic distribution of fungal communities under different pasture management practices at the feature level. (**B**) Principal coordinate analysis of 16 s rRNA diversity in the rhizosphere of the different soils assessed (data from all treatment types combined due to similarity of the treatment effect).

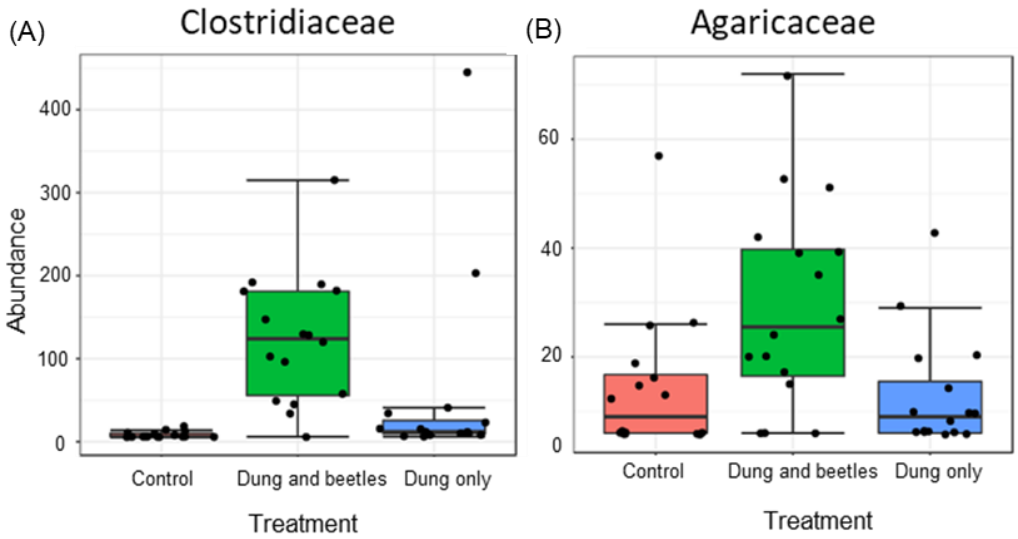

Figure 6. Linear discriminant analysis effect size, performed on bacterial and fungal classes, revealed a significant effect size of dung beetle activity for (**A**) the bacterial class *Clostridiaceae* and (**B**) fungal class *Agaricaceae*. Dark dots represent individual data points.

3.7. Pasture Biomass

Average dry weights of pasture biomass harvested at four consecutive samplings in 30-day intervals are presented in Figure 7A,B, grouped by the soil type and the treatments, respectively. Significant differences in pasture biomass growth among different soil types were observed initially, evident at the first and second pasture harvests at 30 days after planting (30 DAS) and 60 days after planting (60 DAS), respectively, likely to reflect the inherent nutrient content of different soil types. The effect of dung treatment on pasture biomass growth was evident from the third pasture harvest at 90 days after planting (90 DAS) and continued to the fourth pasture harvest at 120 days after planting (120 DAS), despite the removal of residual dung pat at 60 DAS. A highly significant effect of dung beetles on pasture biomass growth was observed at the fourth pasture harvest at 120 DAS, indicating long-term turnover of dung beetle activity on pasture soils, potentially reflecting the gradual mineralisation of nutrients from dung. Cumulative pasture biomass at 120 days after planting is presented in Figure 7C,D, grouped by the soil type and the treatments, respectively. In summary, Vertosol generated the largest pasture biomass, followed by Chromosol, Rudosol, and Kandosol ($p < 0.001$). Total pasture biomass generated over time was significantly ($p < 0.01$) higher for dung and dung beetle treatments, compared to the dung-only treatment.

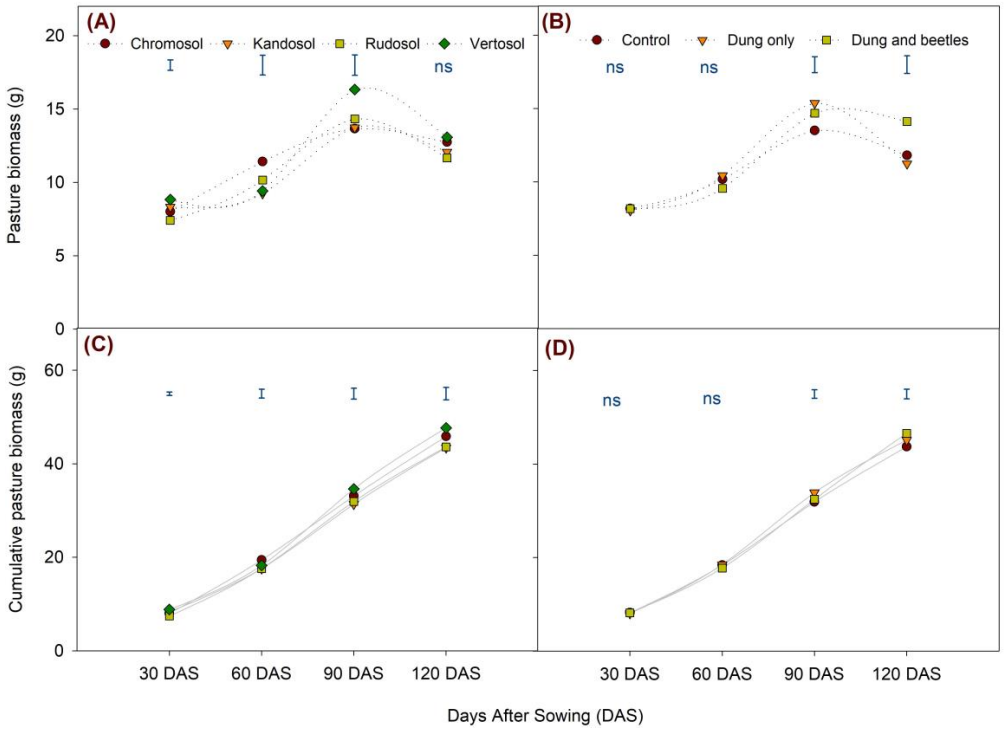

Figure 7. Average pasture biomass at four consecutive harvests, by soil type (**A**) and treatment (**B**). Cumulative pasture biomass at four consecutive harvests by soil type (**C**) and treatment (**D**). Error bars indicate least significant difference at $\alpha = 0.05$. ns represents no significant difference.

4. Discussion

Lysimetry-based experimentation enables the collection of soil leachates for the evaluation of soil chemical and biological dynamics in response to changes as impacted by soil moisture, nutrient application, and crop management [45], and fills the gap between laboratory and full-scale field experimentation under controlled conditions [46,47]. The inclusion of a microbial community profiling approach then enabled the quantification of the impact of both soil and agronomic treatments on the abundance and diversity of soil bacteria and fungi. Following on from statistical analysis, we observed that soil type and textural properties significantly impacted nitrate and total N leaching while dung incorporation by

dung beetles generally had no effect upon nitrate leaching in collected soil water samples. The soil type affected nutrient leaching, while dung incorporation by dung beetles did not. However, the addition of cattle dung and dung beetle activity initially increased soil water contamination with *E. coli*, which is a common indicator of groundwater contamination with faecal coliforms, but was below the hazardous threshold [48]. Clearly, both soil type and the dung incorporation impacted the diversity of soil microfauna.

The water-holding capacity (WHC) of the four soil types evaluated differed greatly, as expected, providing the opportunity to study the impact of dung and dung beetle activity on leachate quality, in representative soils for high and low water-holding scenarios. We used four different soil types underlying Australian pastures, namely, Vertosol with generally high water-holding capacity, Chromosol and Kandosol with moderate WHC, and Rudosol with low WHC [22]. To our knowledge, this is the first study of its kind conducted in the southern hemisphere to assess the impact of dung beetles on soil leachates over various soil types.

A previous study conducted in New Zealand using a similar lysimetry setup assessed leachate properties in allophanic soils, a common soil type encountered in New Zealand pastoral properties [23]. Soil type and its effect on leaching of pathogenic *E. coli* were not addressed in the present study. Instead, pasture management with dung and dung beetles increased the *E. coli* concentration in leachates. This is a similar observation to Aislabie et al. [23] who reported an increase in *E. coli* by up to 8 MPN 100 mL^{-1} following the introduction of dung plus dung beetles. In our study, we observed approximately 2.5 MPN 100 mL^{-1} of *E. coli* in leachates, which is similar to values encountered in bore or rain-fed water tanks in the Riverina region [49]. While the World Health Organisation considers only 0 MPN 100 mL^{-1} of *E. coli* to be safe, a range of 1–10 MPN 100 mL^{-1} is regarded as low-risk [48]. Overall, our results provide an insight into the relatively limited potential for soil water contamination following the introduction of dung beetles on pastoral properties, for the specific soil types evaluated in this study.

Cattle and other livestock dung types are known to improve soil nitrogen levels when used as manure or fertilisers [50]. In pastoral lands, however, the long-term presence of dung pats on the soil surface reduces productivity for the underlying pasture while also deterring grazing activity of livestock [15]. There is also growing environmental concern with respect to volatilisation, run-off, and leaching of nutrients present in high concentrations in dung [51]. Our results demonstrated that nitrate leaching was prevalent in the lower-clay-containing Rudosol as well as the high-clay-containing Vertosol soils. Vertosols, rich in layered sheet silicates [52], characteristically contain microcracks, providing channels for solutes to move through the soil profile [53]. Kandosol and Chromosol in comparison are less porous and are less prone to cracking [54], thereby reducing the movement of water and solutes through the soil profile. Importantly, dung beetle activity was not found to exacerbate nitrate leaching in any of the soil types when compared to the control group. This observation is in agreement with a similar lysimetry study [23] where dung beetle activity in volcanic allophanic soils showed no impact on nutrient leaching over time.

The identity of dung beetle species may have an impact on the ecosystem services provided in an agricultural system. For example, in a recent study conducted by Maldonado et al. [55] evaluating the nutrient cycling benefits of dung beetles in mid-western Argentina, it was found that under moisture-controlled conditions in pots, a native-tunnelling beetle *Sulcophanaeus imperator* incorporated more organic material, 10–20 cm below the soil surface when compared to an introduced tunnelling beetle *Digitonthophagus gazella*, a native lifter *Eucranium arachnoides*, and a roller *Malagoniella* (Megathopomina) *puncticollis*. However, no impact was observed for soil nitrate levels to a depth of 20 cm between any of the species. The present study used the introduced tunnelling dung beetle, *Bubas bison*, due to its potential to process livestock dung over autumn, winter, and spring seasons coinciding with the winter annual pasture growing season [56]. *B. bison* has also been shown to alleviate drought stress in a winter Brassica crop through potential soil conditioning achieved by increasing the permeability of soil to water [6]. It is also very likely that the increase in

pasture biomass production by the dung + dung beetle treatment at 120 days after sowing was associated with increased water permeability of the soil following beetle activity over the drier spring months.

Soil, in the absence of ameliorants and extra nutrients, is considered an oligotrophic environment, with a small cohort of active microorganisms, in the generally low-nutrient matrix [57]. However, the modification of this environment by abiotic and biotic factors may affect the active component of the microbiome [58]. Indeed, in the soil types we assessed, the abundance of organic carbon, nitrogen, sulphur, and phosphorus levels differed. Accordingly, these soils were found to have dissimilar microbiomes. As cattle dung is a source of intestinal microorganisms, and it modulates soil nutrient levels once incorporated [59], it was expected that the soil microbiome profile may differ following the addition of dung and following dung beetle activity incorporating dung in the soil. Our results showed, however, that at the phylum level, the addition of the dung and dung + dung beetles had no impact on the bacterial microbiomes for each soil type. It is likely that many of the exogenous bacteria from dung did not survive and colonise the soil rhizosphere. In a previous study, Semenov et al. [59] demonstrated that 78% of manure-sourced microbes did not survive over two weeks in soil, with approximately 95% becoming non-viable over the course of the experiment (44 weeks). Interestingly, members of the Clostridia class were found to be dominantly represented in the surviving cohort of dung-sourced bacteria. Similarly, we observed that Clostridia was the most differentially abundant class of bacteria in dung and dung + beetle treatments.

In contrast to the relative abundance of bacteria, the relative proportions of fungal phyla differed among soil types and the beetle management strategy applied. Previous studies demonstrated that the aeration of rhizosphere soils typically increased the diversity and abundance of soil fungal communities [60,61]. Soil fungi are also stratified in undisturbed cropping soils, with diversity reducing with increasing depth [62]. However, these layers can be homogenised by soil mixing, including processes such as ploughing of soil [63]. It is, therefore, likely that the aeration of the deeper soils by dung beetle tunnelling activity and movement of organic material by the beetles may have altered the fungal communities in the soil.

Incorporation of manure into the soil substrate generally improves soil organic carbon content and other macronutrient levels, and increases soil pH over time, while also enhancing crop yields when compared to synthetic fertilisers, particularly when used over a longer time frame [64]. The process of dung incorporation can be further facilitated through dung beetles, particularly those that tunnel deeply into the soil profile [65]. For example, *B. bison* have been shown to bury up to 90% of cattle dung lying on the soil surface within five days of being introduced to a site [12]. In the absence of dung beetles, the duration for soil incorporation of dung could exceed four months [66]. It is likely that the later but positive response in pasture growth at the final pasture evaluation was associated with the transfer of soil nutrients to the soil following dung incorporation by *B. bison*; however, this requires further investigation under field conditions.

5. Conclusions

Dung beetles contribute to various and diverse ecosystem services, positively impacting soil function, fertility, and livestock pest management. Using a soil lysimetry approach, we demonstrated that soil leachate nutrient quality in terms of soluble nitrogen was impacted by local soil type, but not initially by dung presence or dung beetle activity in four representative soils from the Riverina region of Southeastern Australia. Interestingly, leachate quality deteriorated following dung beetle activity in all soils assessed, with significant increases in *E. coli*. However, observed levels of *E. coli* were very similar to those encountered regionally in local natural groundwater sources. Interestingly, regional soil type impacted the composition of the soil microbiome found after experimental termination. The soil community structure was generally not impacted by the presence of surface dung or dung beetle activity. Our results also demonstrate that the survival of saprophytic fungi

associated with the degradation of dung is enhanced by dung beetle activity over time. Accumulation of pasture biomass was initially dependent on soil type, and increased in association with dung beetle activity, particularly in later stages of pasture growth.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020325/s1, Figure S1: Schematic representation of the general assembly of a lysimeter column and leachate collection system, established in Wagga Wagga NSW; Figure S2: The impact of soil management strategies on the diversity of (A) bacterial and (B) fungal phyla; Table S1: Monthly Climate Statistics for 'WAGGA WAGGA AMO' [072150], NSW, Australia; Table S2: Nutrients in leachates collected from water percolating through four different soil types in lysimetry experimentation performed in Wagga Wagga, NSW during May–November 2021

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Data Availability Statement: Data are maintained at Charles Sturt University for at least 7 years following experimental completion and publication.

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