Soil Nitrogen Treatment Alters Microbiome Networks Across Farm Niches

XinYue Wang

University of Otago

Kerri Reilly

AgResearch Ltd

Ambarish Biswas

AgResearch Ltd Grasslands Research Centre

Linda Johnson

AgResearch Ltd

Suliana Teasdale

AgResearch

Gwen Grelet

Landcare Research New Zealand

Ana Podolyan

Landcare Research New Zealand

Pablo Gregorini

Lincoln University

Sergio Morales (✉ sergio.morales@otago.ac.nz)

Department of Microbiology and Immunology, University of Otago, PO Box 56, Dunedin 9054, New Zealand <https://orcid.org/0000-0002-5862-0726>

Graeme Attwood

AgResearch Ltd Grasslands Research Centre

Research

Keywords: Agricultural microbiomes, 16S, 18S, ITS, amplicon sequencing, Microbiome networks

Posted Date: January 13th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-142739/v1>

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Version of Record: A version of this preprint was published at Frontiers in Microbiology on February 14th, 2022. See the published version at <https://doi.org/10.3389/fmicb.2021.786156>.

Abstract

Background

Agriculture is fundamental for food production, and microbiomes support agricultural through multiple essential ecosystem services. Despite the importance of individual (i.e. niche specific) agricultural microbiomes, microbiome interactions across niches are not well-understood. To observe the linkages between nearby agricultural microbiomes, multiple approaches (16S, 18S, and ITS) were used to inspect a broad coverage of niche microbiomes. Here we examined agricultural microbiome responses to 3 different nitrogen treatments (0 kg/ha/yr, 150kg/ha/yr and 300kg/ha/yr) in soil and tracked linked responses in other neighbouring farm niches (rumen, faecal, white clover leaf, white clover root, rye grass leaf, rye grass root)

Results

Nitrogen treatment had little impact on microbiome structure or composition across niches, but drastically reduced the microbiome network connectivity in soil. Networks of 16S microbiomes were the most sensitive to nitrogen treatment across amplicons, where ITS microbiome networks were the least responsive.

Conclusions

Nitrogen enrichment in soil altered soil and the neighbouring microbiome networks, supporting our hypotheses that nitrogen treatment in soil altered microbiomes in soil and in nearby niches. This suggested that agricultural microbiomes across farm niches are ecologically interactive. Therefore, knock-on effects on neighbouring niches should to be considered when management is applied to a single agricultural niche.

Background

A farm is a combination of multiple agriculturally relevant ecological niches split by locations, including above-ground, below-ground and animal-associated niches each harbouring unique microbiomes. Microbiomes are essential as providers of ecosystem services including biogeochemical nutrient cycling, greenhouse gas emission, and host-microbiome interactions [1-5]. The interactions within and between niche microbiomes are the drivers for nutrient cycling through microbial metabolic processes, such as bacterial-plant symbioses and nitrogen cycling [1, 6-8]. Saprotrophic organisms in soil break down organic matter into composites inducing fertility [9-11]. Microbiomes from phyllosphere (above-ground parts associated with plants) and rhizosphere (below-ground parts associated with roots) provide benefits to host growth and well-being, for instance helping with nutrient acquisition from soil, defence against plant-pathogens or adapting to environmental stresses [7, 12, 13]. These benefits are also seen in animal microbiomes through nutrient acquisition mediated by gut microbiomes [14]. Physical and functional connections at a multi-niche scale are poorly understood in farm ecosystems but are

established in the human microbiomes. Significant co-occurrence and co-exclusion are found between human microbiomes from different body sites, where most relationships are niche-specific but multi-niche linkages are also discovered [15].

Fertilizers are commonly used for better agricultural yields, but intensive fertilizations could also introduce environmental and biological impacts on soils and other related niches. Application of fertilizers supplies a large amount of nutrients which could lead to increased greenhouse gas emissions, nutrient leaching, and disturbances to microbiomes [13, 16-27]. Many studies focused on fertilization impacts on microbiomes in individual niches (e.g. soil or rhizosphere [26, 28-33]), but impacts on surrounding niches were unexplored. Apart from microbiome diversities and compositions, both organic and inorganic fertilization influenced microbiome networks, consequently affected microbiome community stability [34, 35]. Liu et al. showed that soil organic fertilization was linked to more connective soil microbiome networks compared to that without fertilisation [36], but Wang et al. found that organic fertiliser treated soil had less interactions among bacteria and fungi [21]. Overall, findings on fertilization effects in agricultural ecosystem were complex and usually from a narrow focus (in individual niches). Thus more attention is required to investigate knock-on effects by single-niche disturbances on a multi-niche scale to capture a more complete view on the entire ecosystem.

Therefore, the current study examined microbiome responses to nitrogen treatment applied to soils across different agricultural niches within the same farm. The objectives of this study were 1) to better understand microbiomes in individual farm niches, and 2) to investigate linkages between microbiomes across farm niches, using nitrogen treatment (i.e. urea based fertilization) as disturbances. In order to obtain representational characteristics of microbiomes across farm niches (i.e. soil, ryegrass root, ryegrass leaf, white clover root white clover leaf, faecal, and rumen), multiple approaches (16S, 18S and ITS amplicon sequencing) focusing on specific organisms were used. Alpha diversities, beta diversities, and microbiome networks analyses were used to assess both microbiomes and their linkages across farm niches. We hypothesised that 1) nitrogen treatment in soil reduces microbiomes richness, composition, or networks in soil and nearby niches in prokaryotes; 2) nitrogen treatment will have unique responses across each microbiome subpopulation (i.e. prokaryotes, eukaryotes and fungi)

Methods

Farm site and urea treatment

The experiments were carried out at perennial ryegrass and white clover swards at Lincoln University's Ashley Dene Research and Development Station, Canterbury, New Zealand (-43.6468, 172.3467). Urea was applied to separate sampling sites during the farm season (1 June 2017 to 31 May 2018) to achieve designed nitrogen treatment (no-nitrogen [0 N kg/ha/yr], medium-nitrogen [150 N kg/ha/yr], and high-nitrogen [300 N kg/ha/yr]). The three levels of nitrogen treatment were selected based on the range of nitrogen application rates in pastoral dairy farms in Canterbury.

The experimental swards were grazed by 30 lactating dairy cows (10 cows per nitrogen treatment) to allow the normal grazing practice for pasture and animals. Same amount of herbage intake was controlled across cows (~30kg DM/cow/day) by break feeding. To stabilise the environmental microbiomes, the experimental sites were grazed by cows two weeks before sampling. The allocated swards were examined daily before and after grazing using a rising-plate meter (RPM) to estimate pasture intake during sampling by using the equation RPM reading $140 + 500 \text{ kg DM/ha}$.

Sample collections

Seven types of samples (bulk soil, cow rumen content, cow faeces, white clover leaf, white clover root, ryegrass leaf, and ryegrass root) were taken from three ecological niches (soil, plant, and animals). A total of 169 samples were used in the current study where metadata details can be found in Table S1.

Soil cores were taken using a 110mm wide (internal measurement) corer, with a 100mm wide (internal measurement) plastic sleeve inserted. Plant materials were gently twisted into the centre of the core and sleeve to minimise damage during sampling. The corer was driven into the soil to a depth of 150mm to ensure that at least the top 100mm soil is recovered intact. Once the depth was reached, the corer was gently twisted and lifted out of the ground. Two smaller plastic sleeves were used to transfer the soil from the corer. The soil sample was then wrapped in clingfilm and stored at 4°C. Bulk soil samples were taken after removing stones and plant materials.

For both white clover and ryegrass, individual plants were gently removed from the soil core and split into root and leaf parts with a sterile scalpel blade. After removing loose soil particles, the plant samples were then snap froze with liquid nitrogen and stored at -80°C until DNA extractions.

Rumen samples were collected by stomach tubing of animals. Faecal samples were taken with digital collection from cow anuses at the same time of rumen content collection. Both types of animal samples were snap froze with liquid nitrogen and stored at -80°C until further progress.

DNA extractions and amplicon sequencing

DNA was extracted from individual sample (300 mg/sample) with a Macherey-Nagel NucleoSpin 96 Soil kit. The manufacturer's protocol was followed but with the following modifications. Buffer SL1 (700 µL) and enhancer SX (300 µL) was pre-combined and added to a 1.7ml tube. Grounded sample was then transferred to the tube and vortexed for 1 min. The mixture was then centrifuged for 4 mins at $11,000 \times g$. After removing the supernatant, 150 µL buffer SL3 was added to the tube. The tube was then incubated at 4°C for 20 mins and centrifuged for 2 mins. The clear supernatant was then processed following manufacture protocols and eluted twice with 70 µL of buffer SE each time.

Next generation sequencing was conducted with an Illumina MiSeq V2 platform at Massey University. The total community genomic DNA for each sample was sequenced targeting 16S ribosomal RNA (rRNA), 18S rRNA, and internal transcribed spacer (ITS) regions with primers following the Earth Microbiome Project (www.earthmicrobiome.org).

The demultiplexed paired end sequence reads were then quality filtered and merged using dada2 package V1.12 [37] in R V3.6.1 [38] following the Dada2 tutorial pipelines (16S and 18S <https://benjjneb.github.io/dada2/tutorial.html>, ITS https://benjjneb.github.io/dada2/ITS_workflow.html) with default settings. The merged sequence reads were then denoised, chimera removed, and dereplicated into Amplicon Sequence Variant (ASV). Silva v132 [39] and UNITE databases [40] were used to assign taxonomy to each ASV. For each amplicon, ASV table, metadata (Table S1), and taxonomy table were converted into a phyloseq object with phyloseq version 1.28.0 [41]. For 16S phyloseq object, chloroplasts sequences were removed. Each phyloseq object (16S, 18S, and ITS) was then rarefied and trimmed prior to statistical analyses.

Statistical analyses and visualization

Alpha-diversity, beta-diversity

Species richness across farm niches and in response to nitrogen treatments was calculated with packages dplyr version 0.8.3 [42], plyr version 1.8.4 [43], reshape2 version version 1.4.3 [44], vegan version 2.5.6 [45]; visualizations were created with packages ggplot2 version 1.28.0 [46], Biostrings version 2.52.0 [47], forcats version 0.4.0 [48], ggpubr version 0.2.3 [49], lemon version 0.4.3 [50], RColorBrewer version 1.1.2 [51], scales version 1.0.0 [52]. Kruskal-Wallis test was used to compare species richness across farm niches and nitrogen treatments. Microbiome dissimilarities between samples were determined using the Bray-Curtis distance, with significant changes in community structure or variance tested via ANOSIM and ADONIS respectively. Differences in communities were visualized using NMDS plots.

Exact test, heatmap and volcano plot

Exact tests were used to determine differential abundant ASVs for each individual niche between nitrogen treated and untreated microbiomes illustrated with a dot plot. Packages edgeR version 3.26.8 [53], ggplot2 version 1.28.0 [46], ggpubr version 0.2.3 [49], ggrepel version 0.8.1 [54], lemon version 0.4.3 [50] and phyloseq version 1.28.0 [41] were used.

Heatmap and volcano plot were used to supplement Exact tests. Heatmaps provided the presence and absence information on responsive ASVs across individual niches between samples. Volcano plots illustrated the changing direction (positive or negative) for ASVs responses across niches and between nitrogen treatments. R packages ggplot2 version 1.28.0 [46], ggpubr version 0.2.3 [49], lemon version 0.4.3 [50], and phyloseq version 1.28.0 [41] were used.

Correlation network analyses

Networks for each niche under each nitrogen treatment were constructed with packages igraph version 1.2.4.1 [55], networkD3 version 0.4 [56], and tidyr version 1.0.0 [57] to provide insight into the microbial interactions responses to different nitrogen managements. In each niche, ASVs were chosen based on two criteria: 1) were present in at least 60% of samples; and 2) were significantly ($p \leq 0.05$) differentially

abundant in response to nitrogen treatments in any niche, or were the top 200 most abundant across niches. Spearman correlations were conducted between all chosen ASV pairs in each niches with package psych version 1.8.12 [58]. ASV pairs having strong significant correlations ($r \geq 0.8$ or $r \leq -0.8$, $p \leq 0.05$) were included in the networks. The force-directed layout algorithm was then used to calculated the network layout. Nodes were coloured by phylum represent ASVs and edges were coloured by correlation positiveness represent significant microbial coefficients.

Niche microbiome metapopulation models

Occupancy-frequency distribution of ASVs in each niche was made with packages ggplot2 version 1.28.0 [46], vegan version 2.5.6 [45] and scales version 1.0.0 [52] following previous study [59]. In the current study, the occupancy of an ASV is defined as the fraction of samples it occupied in each niche. The frequency denotes the existence number of unique ASVs in a certain proportion of samples in each niche.

Results

We examined microbiomes using 3 approaches focusing on specific microbiome subpopulations, namely prokaryotes (16S rRNA), eukaryotes (18S rRNA) and fungi (ITS rRNA). Next-generation sequencing data were captured from 7 farm niches, specifically 2 animal-associated niches (Rumen [R] and Faecal [F]), 3 below-ground niches (WhiteCloverRoot [WCR], RyeGrassRoot [RGR], and Bulk Soil [BS]), and 2 above-ground niches (WhiteCloverLeaf [WCL] and RyeGrassLeaf [RGL]). For each of these microbiomes, community responses were compared across 3 different soil nitrogen treatments (control or no-nitrogen [0 N kg/ha/yr], medium-nitrogen [150 N kg/ha/yr], and high-nitrogen [300 N kg/ha/yr]).

Microbial richness and composition were distinct across niches, but shared similar responses to soil nitrogen treatment.

To compare the microbiome richness across niches and nitrogen treatment, Alpha diversities were calculated and analysed across niche microbiomes with ASVs (amplicon sequence variants). For all 3 amplicons, richness of microbiomes were distinct across farm niches ($p < 0.001$, Kruskal-Wallis rank sum test) (Table 1 and S2), but were not significantly differentiated between nitrogen treatments (Fig. 1A, S1A-S2A, and Table S2). In general, leaf microbiomes were the least diverse where BS microbiomes richness under no-nitrogen were the highest across amplicons, with an average of 788, 357 and 385 observed ASVs from 16S, 18S and ITS respectively. In contrast, phyllosphere microbiomes richness were relatively low. For instance WCL microbiome richness under no-nitrogen were low across amplicons, with an average of 36, 20 and 149 observed ASVs from 16S, 18S and ITS respectively. Nitrogen treatment did not alter microbiome richness except for ITS of below-ground niche WCR (Fig. S2A), where microbiome richness was decreased with soil nitrogen treatment ($p = 0.045$, Kruskal-Wallis rank sum test).

Taxonomic compositions were distinct across niches, but did not change in response to soil nitrogen treatment (Fig. 1B, S1B-2B, Table S3a-c). For 16S communities, regardless of nitrogen treatment, the phyllosphere microbiomes in both RGL and WCL were dominated by *Proteobacteria* (82% and 79% on

average respectively). Similarly in soil and rhizosphere niches (BS, RGR, and WCR), *Proteobacteria* dominated at 28%, 36% and 47% on average respectively. Animal-associated microbiomes R and F were both dominated by *Bacteroidetes* and *Firmicutes* (Table S3a). Trends (i.e. changes across niches, with no treatment effects) were similar for 18S and ITS microbiomes (Fig. S1B-2B, Table S3b-c) with a couple of exceptions. For 18S, *Phragmoplastophyta* dominated in all phyllosphere and rhizosphere niches, while *Cilliophora* dominated the 2 animal-associated niches (Fig. S1 and Table S3b). For ITS, *Ascomycota* dominated across all niches except for R, where *Neocallimastigomycota* was the most abundant (Fig. S2 and Table S3c).

Bray-Curtis distances were calculated and plotted with NMDS ordinations to examine microbiome beta diversities across niches. Significant differences in community composition were observed across niche microbiomes (16S: ANOSIM: $R=0.758$ and ADONIS: $R^2=0.505$. 18S: ANOSIM: $R=0.246$ and ADONIS: $R^2=0.248$. ITS: ANOSIM: $R=0.647$, ADONIS: $R^2=0.325$, $p<0.01$ for all cases), but not in response to nitrogen treatment (Fig. 1C, S1c-2c, and Table 1) except for WCR (16S community) and R (18S community). For 16S, animal-associated microbiomes were more distinct compared with other niches (Fig. 1C). Samples from the rhizosphere were clustered primarily by niches, but partial overlaps were observed between BS, RGR, RGL, WCR and WCL microbiomes. Microbiome compositions for 18S and ITS were also significantly different across niches, but broad overlaps across niches were found. Nitrogen treatment had minimal impact on microbiome compositions within each individual niche except for 16S microbiomes in WCR (ANOSIM $p=0.004$, $R=0.317$ and ADONIS $p=0.01$, $R^2=0.189$), and for 18S microbiomes in R (ANOSIM $p=0.027$, $R=0.043$ and ADONIS $p=0.048$, $R^2=0.056$).

To compliment the microbiome compositional and structural change in response to nitrogen treatment, microbiome structural dynamics were also measured for each amplicon across niches with frequency and occupancy plots (Fig. 2 and S3-4). Microbiomes occupancy-frequency distributions across amplicons in both of the phyllosphere niches showed similar skewed patterns. The number of shared ASV declined with increasing number of samples within niche, followed by an increase in species occupying all or most sites, illustrating a core-satellite pattern [59, 60]. Compared to the phyllosphere microbiomes, rhizosphere microbiomes showed variations in distribution dynamics across amplicons. Core-satellite patterns were found in ITS and 18S communities, but not in 16S, suggesting variations in community assembly mechanisms and selective pressure across sub-populations and niches.

Nitrogen treatment affected ASV abundance, but only for certain taxa in animal associated niches.

To identify changes in ASV abundance in response to nitrogen treatment, an Exact test was performed for each niche between two nitrogen treatment pairs: no-nitrogen verses medium-nitrogen, or no-nitrogen versus high-nitrogen. For each niche, responsive ASVs (Exact test with $\log_{2}FC \geq 2$ or $\log_{2}FC \leq -2$, and BH adjusted $p<0.05$) (defined as N-responsive ASVs) were identified. Scattered plots were used to illustrate abundance changes of N-responsive ASV across niches (Fig. 3, Fig. S5-6). Volcano plots (Fig. S7-9) and heatmaps (Fig. S10-12) were used to provide complementary details on foldchanges of N-responsive ASVs across niches and nitrogen treatments.

All N-responsive ASVs were linked with animal-associated niches (Fig. 3, Fig. S5-6), but their presences were detected in other niches (Fig. S10-12). In 16S communities for example, 126 ASVs were originally identified as N-responsive in animal-associated niches (Table S4a, Fig. 3), but none in other niches. Interestingly, over one third (47 out of 126) of N-responsive 16S ASVs were classified under the genus *Prevotella*. Moreover, all of the N-responsive *Prevotella* ASVs had positive foldchanges under medium- or high-nitrogen (Table S4a). Similarly in 18S and ITS communities, the majority of N-responsive ASVs of (18S: 1692 out of 1694, ITS: 139 out of 140) were only responsive in animal-associated niches (Fig. S8-9, Table S4b-c). Surprisingly, no ASV from BS niche was N-responsive across amplicons. In addition, close to a third of 18S N-responsive ASVs (495 out of 1694) were unclassified, suggesting a lack of reference sequences for N-responsive 18S sequences.

Nitrogen treatment drastically reduced microbial network connectivity in soil but the knock-on effects on other niches were random.

To investigate microbiome network changes in response to nitrogen treatment across niches, network analyses were performed for each niche individually with a pool of unique ASVs. The ASV pool was formed by a conjunction of N-responsive ASVs and the 200 most abundant ASVs (Table S5) from each niche. Pairwise Spearman correlations were conducted between selected ASVs (i.e. ASVs found in ASV pool) to calculate their correlations. Only strong correlations ($r \geq 0.8$ or $r \leq -0.8$, $p \leq 0.05$, defined as connections) were included in the networks (Table S6). Networks in each niche were formed by all connections found with each nitrogen treatment, each network connection was formed by two ASVs (i.e. nodes) and a line (i.e. edge) in between. Overall, microbiome networks were responsive to nitrogen treatment across niches, but responses were inconsistent between niches and across amplicons.

Nitrogen treatment drastically reduced or eliminated network connections in BS across amplicons (Fig. 4, Fig. S13-14 and Table S7). On average, BS networks had 4.94, 9.43 and 16.21 edges per node in 16S, 18S and ITS respectively under no-nitrogen. BS network connections were drastically reduced for ITS under medium- and high-nitrogen (2.86 and 3.03 edges per node respectively), and were eradicated for 16S and 18S under medium- and high-nitrogen.

Nitrogen treatment had knock-on effects on other microbiome networks, but resulted in random changes across niches and amplicons (Fig. 4, Fig. S13-14). For 16S, faecal networks under medium-nitrogen (108 nodes and 149 edges) were the most dense compared to networks under no- or high-nitrogen (no-nitrogen: 22 nodes and 20 edges; high-nitrogen: 76 nodes and 59 edges. Fig. 4 and Table S7). Similar trends were noted in the other 16S animal-associated networks, but not for other amplicons (Fig. S13-14 and Table S7). Rhizosphere networks responded to nitrogen treatment differently between niches and amplicons. Network connections of WCR in both 16S (18 nodes and 24 edges) and ITS (94 nodes and 700 edges) under high-nitrogen were the most dense compared to that under no- or medium-nitrogen (Fig. 4, Fig. S14, and Table S7). In contrast, RGR networks only showed notable changes in 18S, where networks (39 nodes and 55 edges) under medium-nitrogen were the most dense (Fig. S13). Phyllosphere microbiome networks across amplicons showed no response to nitrogen treatment.

Discussion

Despite the growing interest of agricultural microbiomes and their responses to different agricultural management systems, the focus has been usually within specific niches, such as soil [61, 62], rumen [63, 64], or rhizosphere [28, 65]. However, microbial interactions also play a critical role in many ecosystem process, especially nutrient cycling [66], that may have carry over effects into other niches. Focusing only on the managed niche limits the ability to understand the consequences of management decisions and disturbances to other nearby niches. Therefore, the current study focused on microbiomes of soil under nitrogen treatment, as well as microbiomes in the nearby niches, to investigate potential relationships between niche microbiomes.

Nitrogen fertilization had no impact on microbial diversity and composition.

Microbiome diversity and community composition had no response to nitrogen treatment in the soil niche itself or in any other niche within the same farm. This is consistent with previous studies [67-69], but not with many others [29-31]. Zhou et al. suggested that long term nitrogen fertilization significantly decrease fungal diversity and alter fungal compositions in soil [70], but our results suggested much weaker effects. The disagreement could be caused by the heterogeneity of soil microbiomes between different research sites. Unlike rumen or gut microbiomes, which were relatively more defined, soil microbiomes varied both spatially and temporally [71-73]. Besides, certain organisms could be more sensitive to nitrogen treatments than others. Zhao et al. suggested that the overall fungal community composition and diversity were not affected by nitrogen treatments, but around 10% of taxa were sensitive to nitrogen treatment [67]. Furthermore, only a small number of soil samples were taken in the current study. This potentially reduced the statistical power to detect significant distinctions between microbiomes under different nitrogen treatments.

Plant associated (phyllosphere and rhizosphere) microbial community compositions and diversities were not responsive to nitrogen treatments except 16S microbiomes in WCR. Ledgard et al. suggested that nitrogen additions (400kg/ha/yr) reduced the productivity of nitrogen fixation in white clovers [74]. The symbiosis between white clover root and *Rhizobium* are fundamental for nitrogen fixation [75], and nitrogen fertilization can make this capability redundant, thus leading to compositional changes in root communities, such as a lower proportion of nitrogen fixers.

Our results showed that soil nitrogen treatment did not affect microbiome richness and compositions in animal-associated niches. It was unlikely that animal-associated microbiomes were experiencing direct changes of nitrogen availability. In contrast, the soil nitrogen treatment would most likely influence ruminants by changing the plant materials grazed on the pasture [76]. Applications of nitrogen fertilizer was proven to favour pasture growth while the production of clover was unfavourable [77], leading to a higher ryegrass : white clover ratio. This vegetation change would then lead to changes in nutrient intake to a higher fibre and less protein diet for ruminants [78]. Smith et al. and Bowen et al. showed that changes in sward types (i.e. ryegrass only versus combined ryegrass and white clover) altered the rumen microbiome composition in dairy cows [79, 80]. In contrast, O'Callaghan et al suggested that changes in

feedings between ryegrass only and mixture of ryegrass and white clover had no impact on rumen microbiome composition, but altered the rumen metabolome [81]. Unlike the previous microbiome studies on sward type alterations (i.e. with or without white clover) [79, 80], we did not expect to see a huge change in ryegrass and white clover ratio between control and fertilized ground (i.e. about 5 – 10% reduction in white clover on fertilized ground [77]). This small change in vegetation might not lead to a detectable change in animal microbiomes.

N-responsive ASVs were observed in animal associated niches, but not in other niches.

Many N-responsive ASVs were present across niches (Fig. S10-12), but most of N-responsive ASVs only had strong correlations in animal-associated niches across amplicons, and few N-responsive ASVs in phyllosphere or rhizosphere niches (Fig. S5-6). Zancarini et al. suggested that soil nitrogen treatments only affected rhizosphere microbiomes with certain host plant genotypes [82]. Therefore, our results could mean that white clover and rye grass rhizosphere microbiomes were insensitive to soil nitrogen treatments. Beattie and Lindow stated that phyllosphere microorganisms were generally oligotrophs which can tolerate low-nutrient conditions or formed symbiosis with host plant to obtain more nutrients [83]. When obtaining nutrient from soil, physical movement of nutrients, most likely from host roots to leaf surface, was inevitable for phyllosphere microbiomes. Hence, phyllosphere microbiomes might not experience much knock-on effects caused by soil nitrogen treatments due to physical barriers.

Among 16S N-responsive ASVs, over one-third (47 out of 126) were classified as *Prevotella* spp., all of which were enriched under medium- or high-nitrogen compared to no-nitrogen, suggesting that *Prevotella* spp. were potentially benefited from soil nitrogen treatment, or vegetation change (i.e. 5 – 10% reduction of white clover). Compared to white clover, ryegrass contained less protein and more soluble and structural carbohydrates [78]. *Prevotella* was known to be an abundant and essential group of catabolic generalists in the rumen [84, 85], due to the ability of degrading a variety of carbohydrate substrates, including hemicellulose, xylan and pectin [86]. Therefore, the enrichment of N-responsive *Prevotella* spp. was most likely due to increases in carbohydrates, or in other words higher ryegrass intake by cows. Unlike *Prevotella* spp., patterns were random for other N-responsive taxa. This suggested that the organisms under the same taxa groups could respond to the same disturbance differently. For example, *Trichostomatia* is a common group of rumen protozoa fermenters involved in degrading non-structural polysaccharides and soluble sugar [87]. This aligned with our results as 696 out of 1199 N-responsive ASVs were *Trichostomatia* ASVs. Furthermore, because the range of carbohydrates metabolized was genus-dependent [87], it was unsurprising to see inconsistent change in N-responsive *Trichostomatia* ASVs.

Microbiome networks were responsive to nitrogen treatments but with no consistent pattern.

Our results showed that for 16S and 18S communities, network connections in soil were completely eradicated under medium- and high-nitrogen, whereas ITS networks complexity and connectivity were drastically reduced but still detectable. Our findings aligned with a previous study showing agricultural fertilization associated with decreases in microbial network complexity [69]. The dramatic reduction in

network linkages in response to nitrogen could be due to the redundancy of nitrogen-fixation functions. When nitrogen was limited in soil, microorganisms formed networks to transform inaccessible nitrogen into an accessible form for non-nitrogen fixers, supporting community growth [88]. Under medium- or high-nitrogen, potentially non-nitrogen fixers would no longer require nitrogen fixation from nearby nitrogen fixers, leading to an alteration of microbiome networks. Instead, alternative nitrification pathways, such as aerobic ammonia oxidation, could potentially be induced and consequently led to population and activity increases of ammonia-oxidizing microbes [89, 90]. However, further investigations on the transcriptome are required to corroborate the conjecture.

Other than reduction of soil network connectivity, ITS community networks were unresponsive to nitrogen treatment across niches, suggesting that fungal communities were less sensitive to nitrogen treatments compared to prokaryotes and other eukaryotes in general. However, since the current study was only based on amplicon sequencing, the presence of 'relic fungal DNA', such as dormant fungal spores, was potentially interfere with detection of relevant fungi and confound the results.

Limitations Of The Study

One limitation of the current study was the low statistical power in non-animal niches due to a small sample size. Other than rumen (n = 52) and faecal (n = 62) samples, no other niche had more than 12 samples (i.e. WhiteCloverLeaf: n = 12, WhiteCloverRoot: n = 11, P.RyeGrassLeaf : n = 9, P.RyeGrassRoot : n = 12, BulkSoil : n = 11), making the statistical tests less reliable. Another limitation was the potential existence of 'relic DNA' in soil [91], especially for fungal communities. Depending on the organisms, Carin et al. suggested that 'relic DNA' could persist in soil for weeks to years depending on the type of dead organisms [91]. Unlike most prokaryotes or plants, fungal spores were known to be long-lived [92], and widely dispersed [93]. This complicated our results, as we could not be certain all the ITS sequences detected in the study were from live local fungal species. Therefore, further RNA work is necessary to resolve the potential confounding factor caused by potential 'relic DNA'.

Conclusions

In conclusion, our results showed that urea-based nitrogen fertilization of soils did not affect microbiome diversity or composition in soil and neighbouring niches, but does affect microbiome networks and potential interactions. Soil microbiome networks from all three amplicons were drastically reduced under medium- and high-nitrogen. Microbiome networks across neighbouring niches, such as rumen and faecal, were also responsive to nitrogen treatments despite no direct management. However, changes of microbiome networks were varied between niches, suggesting differentiations in reconstruction mechanisms between microbiomes across niches. The present study showed that treating one single niche in a farm could lead to knock-on effects on neighbouring microbiomes. This study would be of value to further evaluate the effects of treatments or disturbances in agricultural land and potentially in other various ecosystems.

Abbreviations

ASV: Amplicon sequence variant

BS: Bulk soil

DM: Dry matter

F: faecal

ITS: Internal transcribed spacer

N: Nitrogen

R: Rumen

RGL: Ryegrass leaf

RGR: Ryegrass root

RPM: Rising-plate meter

rRNA: Ribosomal RNA

WCL: White clover leaf

WCR: White clover root

Declarations

Ethics approval and consent to participate

This study was conducted at the Lincoln University's Ashley Dene Research and Development Station, Canterbury, New Zealand (-43.6468, 172.3467). This experiment was approved in accordance with the requirements of Animal Welfare Act 1999 via Lincoln University.

Consent for publication

Not applicable

Availability of data and material

The raw amplicon (16S, 18s and ITS) sequencing files are available from SRA database (SRA number TBC). R scripts for analyzing data are available in GitHub (https://github.com/Cecilia-Wang/OLW_amplicon_analyses.git)

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by the National Science Challenge—Our Land and Water: Innovative Agricultural Microbiomes Project Number A24085.

Authors' contributions

G.A., G.G., P.G., and S.E.M. conceived this study and participated in its design. K.R., L.J., G.G., S.T., P.G., A.P., A.B. and G.A. collected samples and conducted the experiments. X.W. analysed data, wrote and illustrated the paper with input from all authors.

Acknowledgements

We thank Dr. Markus Lindh for technical support in conducting metapopulation analyses.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

16S Microbiome diversities and composition comparisons across farm niches under three levels of nitrogen treatments (0, 150N/ha/yr, 300N/ha/yr). A) Microbiome richness based on number of observed ASVs are shaped by nitrogen treatments. Open circle ○, plus symbol + and closed triangle ▲ represent 0, 150N/ha/yr and 300N/ha/yr nitrogen managements respectively. Statistical significances shown in figure were calculated with Kruskal-Wallis test. B) Relative abundance of microbiome taxa across farm

niches coloured at Phylum level. C) NMDS plot using Bray-Curtis distance coloured by niches and shaped by treatment levels. ANOSIM statistic R: 0.7534, significance: 0.001; ADONIS R2: 0.52944, significance: 0.001.

Figure 2

Microbiome frequency and occupancy plot (16S). Different number of populations occupying different number of samples at each niche are illustrated where X-axis illustrates number of sample sites panelled by each individual niche and Y-axis illustrates accumulated number of present ASV in certain number of samples. (For example, example in panel Rumen, occupancy of 1 and frequency of 2299 indicate there are 2299 unique ASVs only present in a single sample out of all rumen samples, where there are 2 unique ASVs found in 50 rumen samples.)

Figure 4

Microbiome networks (16S) across niche and N treatments. Pair-wise Spearman correlation is calculated for each ASV pair. Only strong significant correlations with $r > 0.8$ were kept. Each dot represent an ASV, coloured by phylum. Purple edges represent negative correlation, olive yellow edges represent positive correlation.

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