Dairy soil bacterial responses to nitrogen application in simulated Italian ryegrass and white clover pasture

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ABSTRACT

Through clearing and use of fertilizer and legumes, areas of southwestern Australia's unique coastal sand plains can support relatively low-cost dairies. However, the ancient, highly weathered nature of the soils in this region makes the dairies susceptible to a range of threats, including nutrient leaching and erosion. Despite this, Western Australian dairy cows typically produce up to 5,500 L of milk per head annually supported by inorganic nitrogen (N) fertilizer (commonly 50:50 urea and ammonium sulfate) at rates up to <320 kg of N/ha per year. Where hotspots exist (up to 2,000 kg of N/ha per year), total N exceeds pasture requirements. We investigated plant and soil bacteria responses to N fertilizer rates consistent with Australian legislated production practices on dairy farms for pure and mixed swards of white clover (*Trifolium repens*) and Italian ryegrass (*Lolium multiflorum*) in a long-term pasture experiment in controlled glasshouse conditions. Although the soil bacterial community structure at phylum level was similar for white clover and Italian ryegrass, relative abundances of specific subgroups of bacteria differed among plant species according to the N fertilizer regimen. Marked increases in relative abundance of some bacterial phyla and subphyla indicated potential inhibition of N cycling, especially for N hotspots in soil. Ammonium concentration in soil was less correlated with dominance of some N-cycling bacterial phyla than was nitrate concentration. Changes in bacterial community structure related to altered nutrient cycling highlight the potential for considering this area of research in policy assessment frameworks related to nutrient loads in dairy soils, especially for N.

Key words: nitrogen management, dairy pasture production, soil bacteria, clover, ryegrass

INTRODUCTION

Nutrient use efficiency, especially for nitrogen (N), is facilitated by maximizing beneficial microbial processes in dairy soil leading to less nutrient leaching and runoff, lower costs, and better outcomes for farmers (Bolland and Guthridge, 2007b; Chen et al., 2018; Erisman et al., 2018). Australia's ecological sustainability legislation under which the dairy industry operates is outlined in Australia's Environment Protection and Biodiversity Conservation Act (Commonwealth Government of Australia, 1999). This legislation does not include the effects of microbial interactions associated with nutrient loads from the perspective of whole-ecosystem life cycle or environmental fate (Hemond and Fenchner-Levy, 2000; Svatos, 2018). Reports on soil quality, including N cycling, from New Zealand, the European Union, the United Kingdom, and the United States suggest that Australia's national sustainability legislation may need review in the light of new research linking production practices to microbial interactions in soil management with outcomes for ecosystems and primary producers (Anseeuw et al., 2012; Erisman et al., 2018). However, this area of research is significantly underrepresented in assessment frameworks and policy discussions, yet shows great potential (Bünemann et al., 2018).

The presence of legumes in dairy pastures can improve N use efficiency (van Eekeren et al., 2009; Lüscher et al., 2014), but excessive use of N fertilizer aimed at maximizing biomass for production gain is common practice in some places (Acosta-Martínez et al., 2010; Chapman et al., 2014; McDowell, 2017). The addition of high rates of N fertilizer to dairy pastures has potential to significantly reduce the effectiveness of soil microbial processes associated with efficient nutrient cycling, which may lead to overdose fertilization and downstream effects (Wepking et al., 2017; Duan et al., 2019). This may occur where excessive N accumulates and forms hotspots (e.g., urine patches), with total N entering ecosystems of up to 2,000 kg/ha per year (Cameron et al., 2013; Chadwick et al., 2018).

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Plant species composition can influence functional patterns of bacterial biodiversity in grassland soils (Leff et al., 2015). There may be benefits for pasture soil management specific to pasture plant species and associated bacterial groups (e.g., N fixation facilitated by bacteria within *Proteobacteria* (Pham et al., 2017; Wall et al., 2019). Dominance or subdominance of particular groups of soil bacteria and their functions are now being categorized quantitatively via “omics” technologies. However, their role in soils cannot be determined by merely considering the dynamics and statistical significance of common gene database sequences or bacterial species due to multilayered biological evolutionary selection (Rosselli et al., 2016; Schöler et al., 2017; Denef, 2018). For microbiota inhabiting rhizospheres, selection acts simultaneously on genes, individuals, cells, groups, and the holobiont (the plant host with its extended microbiome; Garcia and Kao-Kniffin, 2018). It is essential to minimize bias surrounding extrapolation (the typical approach in species-specific gene functional analysis) and focus on increasing data reliability (Alberdi et al., 2018). By limiting assessments to analyses with a proven, classical, empirical basis, (e.g., N fixation; Tsoy et al., 2016) and by limiting functional prediction to already-characterized groups within traditional physiological classes in combination with long-term experiments, extrapolations can almost be eliminated (Contosta et al., 2015; Damaso et al., 2018; Schlöter et al., 2018). We expected that persistent application of N fertilizer on the soil bacteria under swards of white clover (*Trifolium repens*) and Italian ryegrass (*Lolium multiflorum*; Van Dorland et al., 2008) grown in simulated glasshouse conditions would show changes in bacterial community structure related to altered nutrient cycling (Kasparovska et al., 2016; Melchior and Myer, 2018).

MATERIALS AND METHODS

This study examined the effect of N and pasture plant species on soil bacterial diversity and relative abundance at the end of a long-term (8 mo) pasture sward experiment set up in a controlled (20°C) glasshouse at The University of Western Australia (UWA).

We selected soil for this study from a dairy farm located in Cowaramup, Western Australia (33°50'35.5"S, 115°11'38.0"E) as part of a collaboration between Dairy Australia, UWA, and the Department of Primary Industries and Regional Development (McArthur, 2004; Dairy Australia, 2012). The soil collection methodology was consistent with the spatial sampling principles of Manter et al. (2010). We sampled the soil from the top 10 cm of the profile and transported it to UWA (at 0°C) on the same day, and potted it on the next day.

Before potting, the soil was screened, thoroughly sieved over a 5-mm fraction, and mixed to minimize bacterial spatial microclimate effects or bias and to ensure maximum achievable replication for all treatments. Soil bulk density was also determined (King, 1981; Bruand and Gilkes, 2002), as was the ratio of water to soil for watering up to (but not over) approximately 60% field capacity. The soil was placed in 10-cm plastic-lined pots, and the surface was covered with plastic beads to minimize moisture loss (0.6 kg of soil/pot).

There were 3 N treatments, and each was calculated for elemental N using yearly known farmer application rates supplied to the Department of Primary Industries and Regional Development and Dairy Australia specific for dairy district practice and for the highest rate to simulate “hotspot” urine-like effects (Cameron et al., 2013). The 3 treatments were 0, 180, and 912 kg of N/ha per year. The total amount received for each pot was the equivalent dose per week, calculated on daily doses of 0, 0.5, and 2.5 kg of N/ha per day for the 0, 180, and 912 kg of N/ha per year treatments, respectively. The inorganic N fertilizer consisted of a 50:50 mix of urea and ammonium sulfate dissolved in aqueous solution. Nitrogen was applied weekly (in aliquots) and adjusted with additional water during the same fertilization event to maintain pots at approximately 60% field capacity.

The pasture species treatments were (1) Italian ryegrass (*Lolium multiflorum*), (2) white clover (*Trifolium repens*), and (3) Italian ryegrass and white clover sown at 50:50 (30 plants per pot; Moorby et al., 2009). There were 3 replicates of each treatment and 2 cycles of plant growth consisting of 2 generations of pasture plants grown to full maturity and harvested at the height of 1 cm above the soil level (harvested shoots were removed).

After the first harvest, the original seed stock seeds were resown into the same soil profile at the same seeding rate without significant soil disturbance after a few weeks. Fertilizer treatments were maintained throughout the 2 cycles. No fertilizer was added between growth cycles. At the final harvest (after 2 × 4-mo periods of continual growth), soil cores (1 cm²) from the top 0 to 5 cm of each pot were taken and placed in separate vials and stored in a freezer (−20°C) for wet molecular chemistry, downstream sequencing, and bioinformatics.

At second harvest, shoots were harvested and dried, and the biomass was determined (shoot dry weight). Soil was oven-dried and analyzed by CSBP Laboratories in Bibra Lake, Western Australia, for (1) Colwell phosphorus (mg/kg) and Colwell potassium (mg/kg; methods 9B and 18A1, respectively; Rayment and Lyons, 2011), (2) KCl 40 sulfur (mg/kg; method 10D1, Rayment and Lyons, 2011), (3) organic carbon (%;

method 6A1, Rayment and Lyons, 2011), (4) nitrate N (mg/kg) and ammonium N (mg/kg; method 7C2b, Rayment and Lyons, 2011), (5) electrical conductivity (dS/m; method 3A1, Rayment and Lyons, 2011), and (6) pH (H₂O; method 4A1, Rayment and Lyons, 2011).

Genomic DNA was isolated from sample vials using the PowerSoil DNA Total Isolation Kit (Mobio Laboratories Inc., Solana Beach, CA). We optimized the manufacturer's protocol for our soil samples by using a bead-beating lysis procedure and by adding 200- μ m-diameter microbeads (0.2 g) to each sample vial (Abbott et al., 2012). The DNA extract quality and quantity were checked using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Ribosomal 16S rRNA amplicons were generated via PCR (Suzuki and Giovannoni, 1996; Vestergaard et al., 2017), and the resultant PCR products were then prepared for sequencing and analysis (Klindworth et al., 2013; Vestergaard et al., 2017). Sequencing was performed using an Ion PGM OT 200 semiconductor (Thermo Fisher Scientific). Briefly, within R (Lucent Technologies, Murray Hill, NJ) packages and implemented through the Anaconda distribution (Anaconda, Austin, TX), Phyloseq (<https://github.com/joey711/phyloseq>), and biocLite (<http://bioconductor.org/bioclite.r>; Bioconductor, Huber et al., 2015), FastQ files were checked for accuracy against the latest available benchmark standards (mid-2018 release). For this process, we used Windows OS (Microsoft Corp., Redmond, WA) running Ubuntu 14.04 LTS (Canonical Ltd., London, UK) through a Virtual Box (Oracle Corporation, Redwood Shores, CA) virtual machine within a Python 3.6.5 reconfigured QIIME pipeline (Caporaso et al., 2010). FastQ files were then separately checked for low-quality reads before dereplication, annotation, and phylogenetic identification (Meyer et al., 2008; Lallias et al., 2015; Jovel et al., 2016). The phyla representing nonclassical taxonomic groups or less than 1% of the entire sample size were also removed, and the final taxonomic data sets were compiled (Abbott et al., 2012).

Data sets were analyzed as follows. First, a range of statistical ANOVA correlations were performed in GenStat (VSN International Ltd., Hemel Hempstead, UK) for significance based on the least significant differences (LSD). This included each N treatment and plant combination (shoot and soil data). Next, relationships between bacterial community diversity and relative abundance and soil data (eigenvectors) were assessed using canonical correspondence analysis in Microsoft Excel add-in XLSTAT ADA (Addinsoft, Paris, France). Then, pie chart representations were made for major taxonomic phyla across plant associations and N treatments. Next, histograms were made representing

significant expanded phylum (class, order, and family) versus plant associations and N treatments. Finally, an assessment was then made in relation to all quantitative data sets with regard to the hypotheses (Lindgreen et al., 2016; Alberdi et al., 2018; Deneff, 2018; Schloter et al., 2018).

RESULTS

Effects of N Treatments on Pasture Plant Species (White Clover and Italian Ryegrass)

Application of N to the plant swards increased ($P < 0.001$) shoot growth for both white clover and Italian ryegrass when grown alone. The highest level of N application influenced the ratio of white clover to Italian ryegrass in the mixed sward (Table 1). When the equivalent of 180 kg of N/ha per year was applied, the ratio of white clover to Italian ryegrass was reduced from 0.16 to 0.08 due to an increase in biomass of Italian ryegrass, but there was no change in white clover biomass compared with 0 kg of N/ha per year (Table 1). When 912 kg of N/ha per year was applied, white clover was completely outcompeted by Italian ryegrass (Table 1). Soil ammonium was higher under white clover than under Italian ryegrass (Table 1). Nitrate was very low in the soil for all treatments (2 mg/kg of soil). Analysis of variance of soil data indicated that the application of N was highly significant ($P < 0.001$) in its effects on ammonium (LSD = 4.49 mg/kg), sulfur (LSD = 12.2 mg/kg), potassium (LSD = 18.8 mg/kg), and pH (water; LSD = 0.16; Figure 1).

Effects of N Application and Pasture Plant Species on Dominant Soil Bacterial Phyla and Physiologically Significant Subgroups (Order and Family)

Italian ryegrass grown alone with the highest N application supported an increase in the relative abundance of *Proteobacteria* (Figure 2) and α -*Proteobacteria* (Figure 3) and a slight decrease in the relative abundance of *Acidobacteria*, whereas the relative abundance of *Actinobacteria* and *Firmicutes* remained largely unaffected (Figure 2). For white clover grown alone with the highest N application, there was a decrease in the relative abundance of *Proteobacteria* and increases in the relative abundance of *Firmicutes*, *Acidobacteria*, and *Actinobacteria* (Figure 2). In soil from the mixed ryegrass and clover pasture sward, the relative abundance of *Proteobacteria* and *Acidobacteria* decreased slightly, whereas that of *Actinobacteria* and *Firmicutes* increased slightly with increasing N application. The

Table 1. Dry weight shoot biomass (g/pot) of white clover and Italian ryegrass and soil ammonium (mg of N/kg of soil; n = 3; ±SE) when grown in dairy soil for 4 mo with 1 control rate and 2 N treatment rates (0, 180, and 912 kg of N/ha per year) relative to the already-prevailing N soil concentrations, plus 5% least significant differences (LSD) for plant growth

Item	Application rate (equivalent; kg of N/ha per day)	White clover	Italian ryegrass	Ammonium
Italian ryegrass	0	—	2.75	16.67 ± 0.83
	0.5	—	3.55	18.33 ± 0.92
	2.5	—	6.34	23.00 ± 1.15
White clover	0	1.65	—	29.67 ± 1.48
	0.5	3.15	—	32.00 ± 1.60
	2.5	3.09	—	31.00 ± 1.55
Italian ryegrass + white clover	0	0.22	2.08	20.33 ± 1.02
	0.5	0.28	3.34	19.00 ± 0.95
	2.5	0	6.69	20.67 ± 1.03
5% LSD		1.56	1.13	

effects of N application and plant sward composition were minimal on bacterial groups that were recorded in the soil at low relative abundance.

Within *Proteobacteria*, application of N increased the relative abundance of *Caulobacterales* when white clover was grown alone, and the relative abundance of

Rhizobiales decreased (Figure 3). Within *Firmicutes*, N application to white clover reduced the relative abundance of some families of *Bacillales* and increased the relative abundance *Clostridiaceae* (Figure 4). Furthermore, there was an increase in the relative abundance of families *Veillonellaceae* and *Peptococcaceae* with N

Figure 1. Canonical correspondence analysis (eigenvector analysis) of soil data versus sequenced 16 S rRNA dominant phyla (italicized) shows the variance that the dominant soil phyla have based on N treatments and plant species associations (including 5% SE bars). The proximity of phylum (open circles) to data vectors (bold) Colwell P (mg/kg), Colwell K (mg/kg), KCl 40 S (mg/kg), organic carbon (%), nitrate N (mg/kg), ammonium N (mg/kg), electrical conductivity (dS/m), and pH (H₂O; red squares) indicates increasing correlation to that soil data point. Conversely, decreasing proximity to the data vectors indicates decreasing correlation (based on the soil data). The length of each vector indicates the significance of the relationship that the vector (soil property data) has to the overall community relative abundance and diversity makeup for the total sample size. In this canonical correspondence analysis (CCA), the first 2 axes of the eigenvector plots explain more than 77.1% of the total variance, which represents good sample separation about the (x, y) axis.

application when white clover was grown alone (Figure 4).

DISCUSSION

For white clover and Italian ryegrass grown either alone or in combination on dairy farms, 50:50 urea and ammonium sulfate application in accordance with industry standards and legislation is likely to have a marked effect on soil bacterial communities related to nutrient uptake by plants in addition to overall plant biomass effects when N exceeds 180 kg of N/ha per

year (Iannetta et al., 2016). Recent studies that highlight some of the bacteria effects on soil and plants in a similar N concentration range include Wepking et al. (2017) and Duan et al. (2019). We also expect that the bacterial community changes are associated with N cycling changes; however, because quantifying N cycling and N cycling efficiency is not as simple as assessing N required for pasture plant biomass yield (Holly et al., 2018), many of the microbiological responses occurring in these production environments are often overlooked. However, they remain highly beneficial sources for improved management (Bünemann et al., 2018).

Figure 2. Treatment-specific relative abundance pie charts for domain: bacteria (phyla). Each pie chart indicates the relative abundances of the most dominant phyla present in each of the specific nitrogen (N) treatment pots based on each plant association: Italian ryegrass (ryegrass), white clover (clover), and mixed (ryegrass + clover). Among all phyla displayed are 4 dominant phyla from all pots based on N treatment (0, 180, and 912 kg of N/ha per year for zero, low, and high, respectively), including *Proteobacteria*, *Acidobacteria*, and *Firmicutes*, with lesser abundant groups including the equally distributed across all treatments “bacteria–other” making up total species diversity and relative abundance within the entire sample set (all plant association pots and all treatments). The number 1 in the first pie chart and at the top of the phyla key is for color identification. The arrows indicate how to correlate pie slices with the phyla (clockwise-downward manner).

In this experiment, the concentration of ammonium under white clover was more significant in its effect on soil bacteria than nitrate (ammonia was less correlated than nitrate) based on eigenvector canonical correspondence analyses (Figure 1), which is consistent with the observations of Di et al. (2010), Chapman et al. (2014), and Sun et al. (2018). When N fertilizer was applied to clover grown alone, there was an increase in *Rhizobiales* (α -*Proteobacteria*) and *Clostridia* (*Firmicutes*; Minamisawa et al., 2004; Che et al., 2018). A dominance of alpha versus beta and gamma classes of *Proteobacteria* indicates oligotrophic soil conditions (Inceoglu et al., 2011; Morrison et al., 2017) synonymous with N-limited environments (Senechkin et al., 2010; Ren et al., 2016). Because *Rhizobiales* was the most dominant order present across all treatments, it needs to be mentioned that the importance of their presence is consistently underrepresented in linking soil quality to soil function and ecosystem services (although not measured for

functionality in our experiment; Albright et al., 2019; Wall et al., 2019).

There was a marked influence of the higher N application on pasture composition for white clover and Italian ryegrass grown together. Clover may mitigate production loss under increased stocking rate in sandy soils where constant N concentrations range from 180 up to (the maximum legislated) 320 kg of N/ha per year. Application rates above this range might trade off beneficial soil processes (especially around N hotspots) due to the inability of clover to compete with ryegrass (Kasparovska et al., 2016; Melchior and Myer, 2018). Interactions between N fertilizer, plant genotype, and soil bacterial community structure contribute to the effects of N on soil bacterial communities rather than N fertilizer alone.

The increase in the relative abundance of the *Caulobacteriales* (within the β -*Proteobacteria*) with N application for white clover grown alone but not in com-

Figure 3. Diversity and relative abundance within phylum *Proteobacteria* in response to 3 nitrogen (N) treatments (0, 180, and 912 kg of N/ha per year for zero, low, and high, respectively) and 3 plant treatments [Italian ryegrass (rye), white clover (clover), and the combination of Italian ryegrass and white clover (clover + rye)]. The phyla displayed correspond to the colors shown on the right side of the chart. The order in which the phyla appear for the treatments is consistent.

Figure 4. Diversity and relative abundance within phylum *Firmicutes* in response to 3 nitrogen (N) treatments (0, 180, and 912 kg of N/ha per year for zero, low, and high, respectively) and 3 plant treatments [Italian ryegrass (rye), white clover (clover), and the combination of Italian ryegrass and white clover (clover + rye)]. The phyla displayed correspond to the colors shown on the right side of the chart. The order in which the phyla appear for the treatments is consistent.

combination with Italian ryegrass or Italian ryegrass alone corroborates a previous hypothesis that *Caulobacterales* may dominate N-fixing environments associated with clover (Zahran, 1999; Revell et al., 2012; McDowell, 2017). The ability of bacteria within *Caulobacterales* to regulate and transcribe orthologs (through speciation) to associate with N fixation processes is an essential consideration for dairy soils, especially for efficient use of N (including N fixation), as it may include previously unidentified biological pathways and needs investigation (Negreanu et al., 2012; Tsoy et al., 2016; Bünemann et al., 2018).

Bacteria in the order *Clostridia* have an essential role in N cycling (Heller, 1921; Wiegel, 2005). The relative abundance of *Firmicutes* was largely unaffected across all N and plant treatments except within the order *Clostridia*. Increases in the relative abundance of *Veillonellaceae* and *Peptococcaceae* after N application to white clover raise the possibility of resistance to acidified soil environments. The link between the increasing negative correlation between N and soil pH variation and the correlation between the presence of *Firmicutes* and organic carbon and pH supports this assumption (Figure 1). *Veillonellaceae* decreased again, indicating a buffering capacity threshold for N applied at a rate of

180 kg of N/ha per year $< X < 320$ kg of N/ha per year (Bolland and Guthridge, 2007a; Dairy Australia, 2012; Chadwick et al., 2018).

CONCLUSIONS

Nitrogen fertilizer applied at current levels to dairy pastures can alter the structure of soil bacterial communities as well as pasture plant dominance. We showed a marked effect of weekly applications of N fertilizer on the relative abundance of soil bacteria for swards of white clover (*Trifolium repens*) and Italian ryegrass (*Lolium multiflorum*) in a long-term glasshouse experiment. There is evidence to support our hypothesis that sustained and elevated N application alters the dominance of N cycling bacteria in this dairy soil, with potential for reduced N use efficiency, which could lead to increased prevalence of oligotrophic soil conditions (İnceoğlu et al., 2011; Morrison et al., 2017). Nitrogen applied to this dairy soil affected bacterial communities associated with 2 pasture plant species in a variety of ways (Gschwendtner et al., 2014). Characterization of bacteria from the orders *Rhizobiales* and *Clostridia*, within the α -*Proteobacteria* and *Firmicutes*, respectively, may provide a litmus test for oligotrophic soil condi-

tions in environments of poor nutrient binding (Ren et al., 2016; Tsoy et al., 2016). Further understanding of how N fertilizer use influences the functional aspects of bacterial communities associated with N cycling in dairy soils could assist in the development of better sustainability policies related to N application that takes into account group-level concepts of the soil microbiome and surrounding holobiont to minimize related risk.

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