

Assessing antimicrobial resistance in pasture-based dairy farms: a 15-month surveillance study in New Zealand

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ABSTRACT Antimicrobial resistance is a global public and animal health concern. Antimicrobial resistance genes (ARGs) have been detected in dairy farm environments globally; however, few longitudinal studies have utilized shotgun metagenomics for ARG surveillance in pasture-based systems. This 15-month study aimed to undertake a baseline survey using shotgun metagenomics to assess the relative abundance and diversity of ARGs in two pasture-based dairy farm environments in New Zealand with different management practices. There was no statistically significant difference in overall ARG relative abundance between the two dairy farms ($P = 0.321$) during the study period. Compared with overseas data, the relative abundance of ARG copies per 16S rRNA gene in feces (0.08–0.17), effluent (0.03–0.37), soil (0.20–0.63), and bulk tank milk (0.0–0.12) samples was low. Models comparing the presence or absence of resistance classes found in >10% of all feces, effluent, and soil samples demonstrated no statistically significant associations ($P > 0.05$) with “season,” and only multi-metal ($P = 0.020$) and tetracycline ($P = 0.0003$) resistance were significant at the “farm” level. Effluent samples harbored the most diverse ARGs, some with a recognized public health risk, whereas soil samples had the highest ARG relative abundance but without recognized health risks. This highlights the importance of considering the genomic context and risk of ARGs in metagenomic data sets. This study suggests that antimicrobial resistance on pasture-based dairy farms is low and provides essential baseline ARG surveillance data for such farming systems.

IMPORTANCE Antimicrobial resistance is a global threat to human and animal health. Despite the detection of antimicrobial resistance genes (ARGs) in dairy farm environments globally, longitudinal surveillance in pasture-based systems remains limited. This study assessed the relative abundance and diversity of ARGs in two New Zealand dairy farms with different management practices and provided important baseline ARG surveillance data on pasture-based dairy farms. The overall ARG relative abundance on these two farms was low, which provides further evidence for consumers of the safety of New Zealand’s export products. Effluent samples harbored the most diverse range of ARGs, some of which were classified with a recognized risk to public health, whereas soil samples had the highest ARG relative abundance; however, the soil ARGs were not classified with a recognized public health risk. This emphasizes the need to consider genomic context and risk as well as ARG relative abundance in resistome studies.

KEYWORDS antimicrobial resistance, shotgun metagenomic sequencing, antimicrobial resistance genes, resistome, dairy farm environment, pasture-based system, New Zealand

The development and transmission of antimicrobial resistance (AMR) is a serious global public and animal health concern. Antimicrobial resistance genes (ARGs) have been identified across numerous environments globally, including urban sewage (1–3),

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hospital wastewater (4), agricultural environments including dairy farms (5, 6), and soil samples from pristine environments (7). Traditional methods for AMR surveillance have focused on culture-based screening of specific bacterial pathogens such as *Escherichia coli* (8) or polymerase chain reaction (PCR) of a limited number of target ARGs (9). Advances in next-generation sequencing technologies and a reduction in their cost (10) have led to an increase in the number of studies utilizing shotgun metagenomic sequencing to study AMR. This has enabled a deeper understanding of the bacterial taxa and ARGs present in various ecosystems. An advantage of short-read shotgun metagenomic sequencing methods is the depth of sequencing data that can be achieved, allowing for the detection of genes of interest that may be present at low levels (11). Additionally, if the sequencing depth is sufficient, the genomic context of ARGs can be determined (10, 12). This contextual inference is crucial as it is important to assess (i) whether the ARGs are able to be transferred and (ii) which bacterial species harbor the resistance gene(s) to assess the public and animal health risks associated with each specific ARG (13). Acquired ARGs can be transferred via horizontal gene transfer and are of particular concern due to their ability to disseminate within bacterial populations.

The use of antimicrobials in food-producing animals has become a consumer concern (14–16), and potential transmission pathways of antimicrobial-resistant bacteria between animals, humans, and the environment have been proposed (17, 18). Globally, ARGs have been detected in the dairy farm environment including in feces, manure effluent, wastewater, and soil (5, 6, 19, 20) as well as raw bulk tank milk (21). Antimicrobial resistance is a global threat to human and animal health, driven by complex interactions across human, animal, and environmental compartments that can increase the transmission and prevalence of microorganisms with antibiotic resistance genes (22–26). For example, heavy metal (27–30) and biocide use (31) may co-select for AMR, and farm management practices such as buying in cattle (32) and feeding waste milk to calves (33, 34) may influence the prevalence and shedding of antimicrobial-resistant bacteria in the dairy farm environment. Other modifiable factors influencing AMR include seasonality, farm management practices such as the use of teat sealants, indoor housing, and antimicrobial use (17). The majority of antimicrobial usage (AMU) on New Zealand (NZ) dairy farms is for mastitis treatment and prevention (35), and therefore, AMU is likely to be higher post-lactation. Single time point sampling or collecting samples from a limited number of locations on a farm may be insufficient to accurately estimate the prevalence of antimicrobial-resistant *E. coli* (36); therefore, longitudinal study designs may be more suitable for AMR surveillance in agricultural environments, as they account for changes in AMR prevalence, seasonality, AMU, and farm management practices.

NZ dairy farm management practices differ when compared with international systems in that they are largely pasture-based (37), have a smaller average herd size of 431 cows (38), and have a low prevalence of coliform mastitis (39). The New Zealand Veterinary Association (NZVA) has announced aspirations that by 2030, antimicrobials will not be required for the maintenance of animal health and welfare (40), and instead, their use will be reserved for the treatment of disease. In NZ, prophylactic antibiotic use is only permitted with a veterinarian prescription and antibiotics of importance to human health are not permitted for growth promotion in animals (41). Compared with many international systems, NZ uses low amounts of antimicrobials in food-producing animals (42, 43) and like AMR from NZ food animals (44), the prevalence of ARGs in the NZ dairy farm environment is likely to be low. Thus, the aim of this study was to undertake a baseline survey using shotgun metagenomics to assess the relative abundance and diversity of ARGs in two pasture-based dairy farm environments in NZ.

RESULTS

In this study, 123 metagenomic libraries were sequenced consisting of feces ($n = 30$), soil ($n = 30$), effluent ($n = 28$), milk ($n = 24$), and waste milk ($n = 1$) samples collected over a 15-month period plus controls ($n = 10$) (Table S1). Both positive and negative control DNA preparations were included in this study comprising of a mock community (Table

S2; $n=3$), mock community log distribution (Table S3; $n=3$), blank reagent ($n = 2$), and PBS controls (Table S4; $n=2$). The shotgun metagenomic libraries were analyzed to determine their microbiome composition, relative abundance, and distribution of ARGs within the farm environment as well as identifying the bacterial hosts carrying ARGs.

Rarefaction analysis was used to determine whether the sequencing depth was sufficient to detect the resistome (defined as the collection of all resistance genes and their precursors, including those associated with both non-pathogenic and pathogenic bacteria [45]) in each sample, including resistance genes present in low relative abundance. This study used a sequencing depth of ≥ 40 million read pairs per sample, and rarefaction analysis suggested that this depth was sufficient to study the resistome in the effluent, feces, milk, and soil samples sequenced in this study. However, the relative abundance of ARGs was higher in three effluent samples (DF0145, DF0176, and DF0188), and the waste milk sample (DF0167) where the number of unique genes was still increasing after sub-sampling of all reads (Fig. S1), suggesting that the sequencing depth was not sufficient to detect the total resistome in these four outlier samples. The three effluent samples identified as outliers were collected from Dairy 1 from the second effluent collection point in the study. The remaining samples collected from this location ($n = 3$) had lower antimicrobial, heavy metal, and biocide resistance genes identified (< 200 genes). The waste milk sample contained a high number of ARGs, even when a low proportion of reads was sub-sampled.

Microbial community composition of dairy farm samples

At the taxonomic class level, the microbiome composition was similar across the fecal samples ($n = 30$) from both farms, with four predominant classes (*Clostridia*, *Bacilli*, *Gammaproteobacteria*, and *Actinobacteria*) (Fig. 1), which were also commonly detected in the effluent samples. However, the total relative abundances were more varied, and the taxonomic composition was more diverse across the different effluent samples (Fig. 1). On Dairy 1, two different collection points were used for effluent sampling, which may account for the diverse range of taxonomic classes detected within these sample types. The microbial community composition was relatively similar across the soil samples (*Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, and *Gammaproteobacteria*) (Fig. 1). Two distinct microbiome profiles for the milk samples were observed: *Clostridia* followed by *Bacilli* predominated in eight samples (Dairy 1 $n = 2$; Dairy 4 $n = 6$), and *Alphaproteobacteria* and *Betaproteobacteria* in the remaining sixteen samples (Fig. 1). Samples with the latter microbiome profile had a low number of sequencing reads post-processing (106,376–335,148; Table S1) compared with the milk samples where *Clostridia* dominated (21,028,390–34,666,238; Table S1). Therefore, these contrasting microbiome profiles are likely an artifact of deep sequencing of milk samples with low DNA yields and associated with exogenous contamination from the DNA extraction kit and/or buffer solution used in sample preparation (Table S4). The waste milk sample (DF0167; collected from Dairy 1 in October 2019) had a distinct microbiome profile, which was dominated by *Gammaproteobacteria*, *Bacilli*, and *Clostridia* (Fig. 1).

At the order level, the microbiome profiles clustered by sample type, irrespective of farm (Fig. 2). PERMANOVA analysis showed no statistically significant associations for both the soil and feces microbiomes ($P > 0.05$) when compared with “farm” and “season.” The fecal and soil microbiomes were homogeneous, clustering together between the farms, but milk samples clustered into two groups, independent of farm or collection date and likely associated with sequencing reads (Fig. 1). The single waste milk microbiome profile clustered separately from the other sample types. The effluent microbiome profiles were more diverse across both farms, reflecting the complex composition of effluent and different treatment methods between the two farms. PERMANOVA analysis showed both “farm” ($P = 0.001$) and “season” ($P = 0.001$) had a statistically significant effect on the effluent microbiomes. On Dairy 1, the clustering of effluent microbiome profiles was independent of the two collection sites.

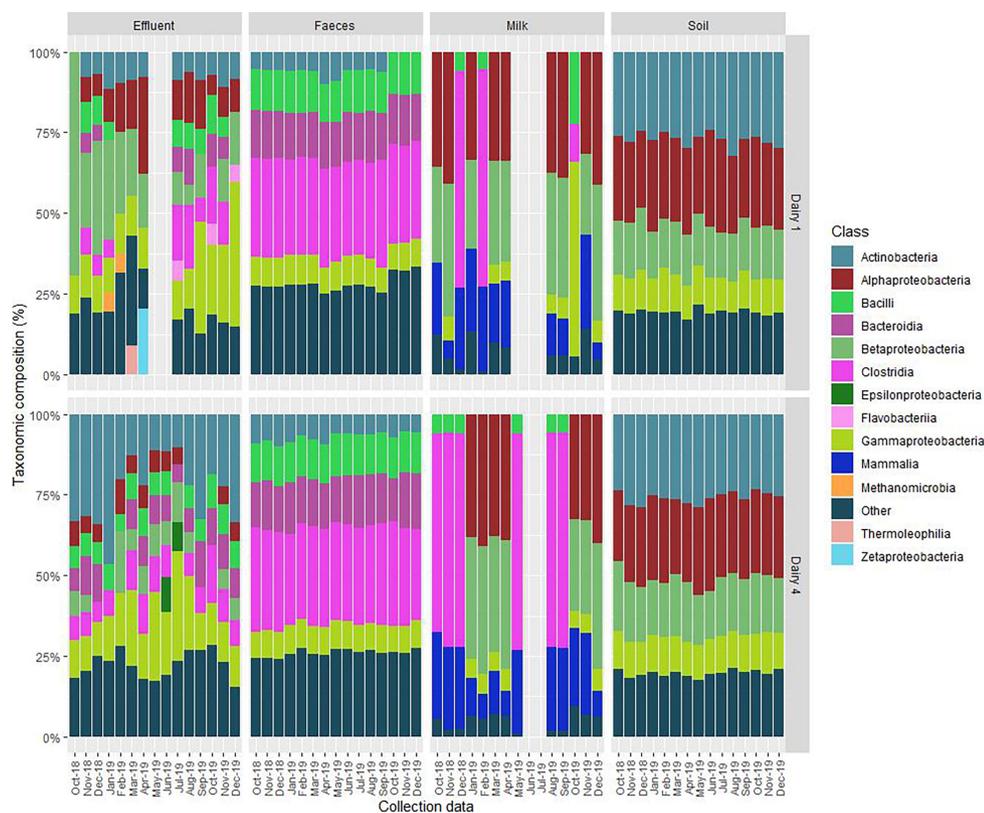


FIG 1 Taxonomic classification of sequencing reads at the class level from feces, farm dairy effluent, soil, and milk. Classes present in $\leq 5\%$ total relative abundance were grouped together as “other.” No milk samples were collected during winter when the cows were not being milked (Dairy 1, May–July 2019; Dairy 4, June–July 2019). The October 2019 milk sample from Dairy 1 was waste milk. Effluent samples were not collected from May–June 2019 on Dairy 1.

Low relative abundance of ARGs across two NZ dairy farms

Shotgun metagenomic sequencing of feces, effluent, soil, milk, and waste milk samples from Dairy 1 and Dairy 4 identified 372 unique ARGs, representing 37 resistance classes belonging to the drugs, biocide, metal, and multi-compound classes (46). ARGs belonging to the drug and multi-compound classes (excluding biocide and heavy metal classes) ranged from 0.03 to 0.37 ARG copies per 16S rRNA gene in effluent samples, 0.08–0.17 in feces, and the lowest and highest relative abundance in milk and soil, respectively (0.0–0.12; 0.20–0.63) (Fig. 3). ARG relative abundance in soil was higher than that in effluent and feces, despite harboring fewer unique ARGs. Three effluent samples with a higher ARG relative abundance were identified as outliers (Fig. 3A), two collected from Dairy 1 in October 2018 (DF0009) and December 2019 (DF0188) and one from Dairy 4 in July 2019 (DF0115). Differences in the total ARG relative abundance (ARG copies per 16S rRNA gene) were not statistically significant between Dairy 1 and Dairy 4 over the 15-month period ($P = 0.321$). Differences in the ARG relative abundance between the effluent samples on Dairy 1 and Dairy 4 were statistically significant ($P = 0.02$), but differences in the ARG relative abundance between soil ($P = 0.32$), feces ($P = 0.13$), and milk ($P = 0.6$) samples from Dairy 1 and Dairy 4 were not statistically significant. When comparing the presence and absence of resistance classes found in $>10\%$ of all feces, effluent and soil samples at the “farm” and “season” levels, only multi-metal ($P = 0.020$) and tetracycline ($P = 0.0003$) resistance was significant at the “farm” level. There was a higher association of resistance genes in the multi-metal class on Dairy 1 and a lower odds of the tetracycline resistance class on Dairy 1 compared with Dairy 4. No statistically significant associations were found at the season level or for the other resistance classes at the farm level ($P > 0.05$). Overall, the ARG relative abundance for the singleton waste

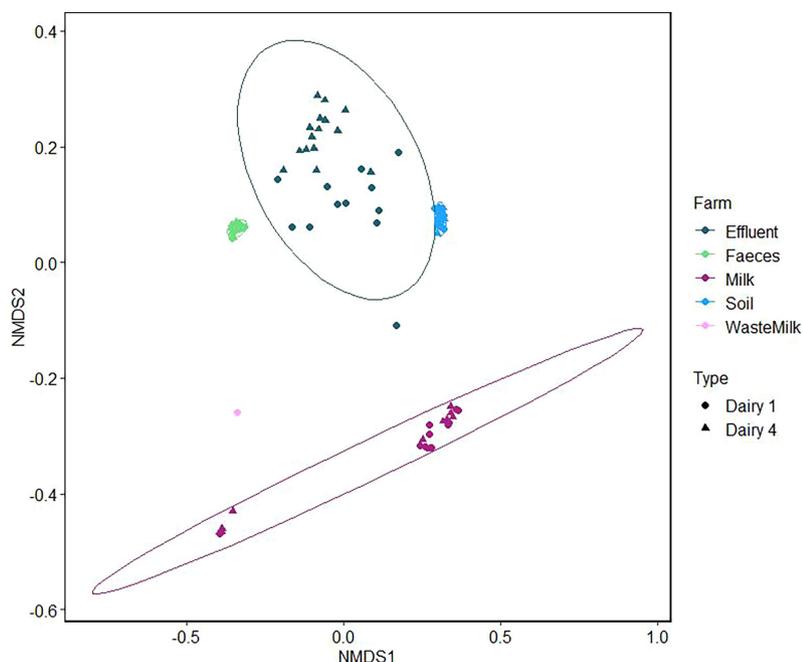


FIG 2 NMDS of microbiome profiles at the order level of farm samples based on Bray-Curtis dissimilarity matrix ($k = 3$; stress = 0.037). Samples are colored by type and shapes represent farm, as indicated in the figure key.

milk sample was greater than that from fecal, effluent, bulk tank milk, and soil samples (Fig. 4). Of the five sample types, effluent samples harbored the most diverse range of ARGs, with 164 unique ARGs representing 19 resistance classes, followed by feces and soil with 51 and 30 ARGs, representing 10 and 11 resistance classes, respectively (Fig. 5). The fewest unique ARGs ($n = 2$) were identified in milk, representing only two resistance classes (Fig. 5).

The ARGs detected in feces on both farms predominantly belonged to those conferring resistance to the β -lactam or macrolide, lincosamide, and streptogramin (MLS) antibiotic classes. The predominance of ARGs belonging to the β -lactam or MLS antibiotic classes is likely due to the presence of the *cfxA* and *InuC* genes, respectively, which were identified in all fecal samples ($n = 30$; Fig. 5). The relative abundance of *cfxA* ranged from 0.07 to 0.14 ARG copies per 16S rRNA gene. *InuC* was detected at a lower relative abundance than *cfxA* ranging from 0.01 to 0.03 ARG copies per 16S rRNA gene and encodes a lincosamide nucleotidyltransferase, which was first characterized in a clinical *Streptococcus agalactiae* on a transposon-like element (47).

The effluent samples contained the most diverse range of ARGs with resistance to antimicrobials including β -lactams, MLS, and aminoglycoside classes being predominant (Fig. 5). Resistance genes likely conferring resistance to multi-compounds were also identified (drug/biocide resistance and biocide/metal resistance). Although the ARGs identified in effluent were more diverse, they were generally detected at low relative abundance when compared with the soil resistome that had the highest ARG relative abundance. The *cfxA* gene group was identified in 27 of 28 effluent samples (0.0–0.10 ARG copies per 16S rRNA gene), and *InuC* and *sodB*, belonging to the MLS and peroxide resistance classes, were identified in 27 of 28 and 25 of 28 effluent samples, respectively, but at a low relative abundance (0.0–0.01 and 0.0–0.06 ARG copies per 16S rRNA gene, respectively). In soil samples, identified resistance classes were comparatively similar between both farms, with multi-drug resistance, drug/biocide resistance, glycopeptides, aminocoumarins, MLS, and rifampin resistance classes (Fig. 5). ARGs were detected in a limited number of milk samples (1 of 11 on Dairy 1; 2 of 13 on Dairy 4) and belonged to the trimethoprim and aminoglycoside resistance classes, and the relative abundance of

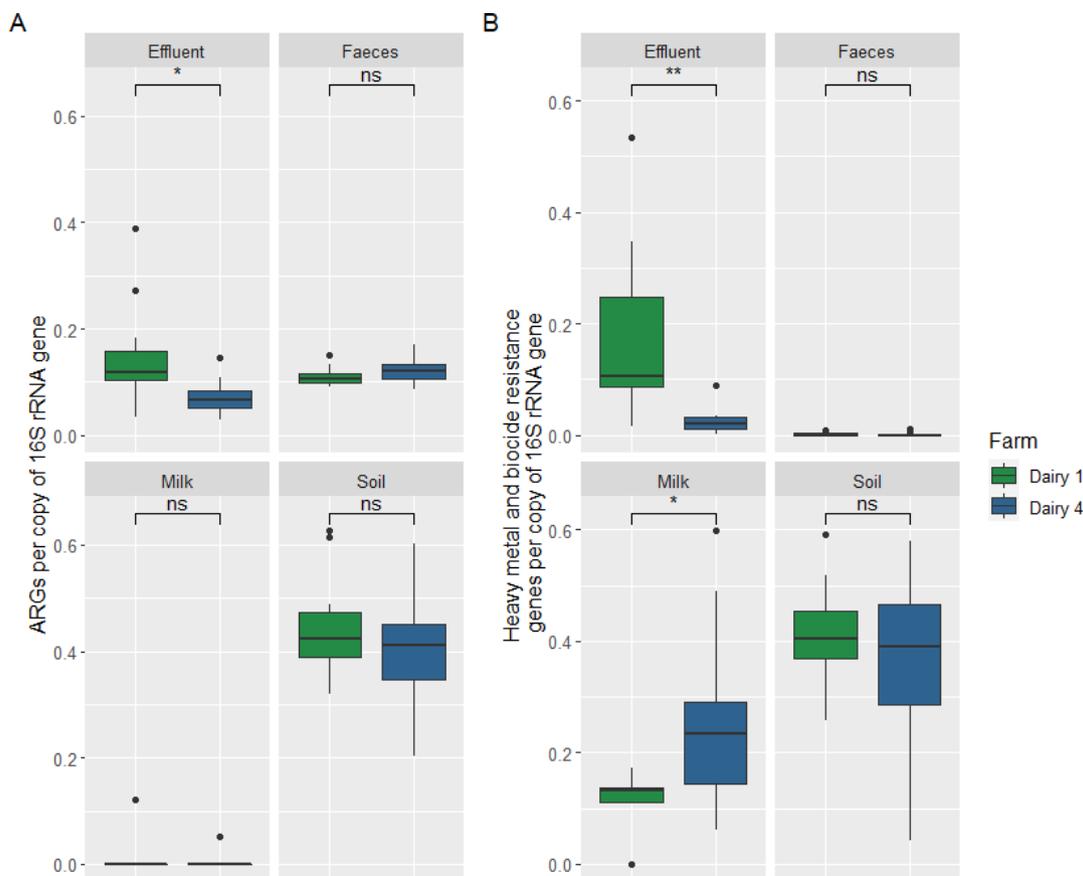


FIG 3 (A) Normalized antimicrobial resistance gene relative abundance (ARG copies per copy of 16S rRNA gene) and (B) heavy metal and biocide resistance gene relative abundance (heavy metal and biocide resistance genes per copy of 16S rRNA gene) in farm dairy effluent, feces, soil, and milk samples collected over a 15-month period on Dairy 1 and Dairy 4. The boxes show the median and upper and lower quartiles. The whiskers show the minimum and maximum values within the interquartile range, and the outliers are indicated by a black dot.

these genes was very low (0.00–0.12 and 0.00–0.05 ARG copies per 16S rRNA gene, respectively).

Differences in heavy metal and biocide resistance gene relative abundance in effluent were significant between the farms ($P = 0.0073$), with a higher relative abundance detected in Dairy 1 (0.01–0.33 ARG copies per 16S rRNA gene) when compared with Dairy 4 (0.00–0.08 ARG copies per 16S rRNA gene). For effluent samples, 149 unique heavy metal and biocide resistance genes representing 14 resistance classes were detected (Fig. S2). The *qac* gene encoding resistance to quaternary ammonium compounds, which are commonly used in disinfectants, was detected in effluent sporadically on both farms. In the milk samples, a higher relative abundance of heavy metal and biocide resistance genes was identified in Dairy 4 (0.09–0.59 ARG copies per 16S rRNA gene) when compared with Dairy 1 (0.00–0.19). Peroxide resistance (*sodB*) was detected on Dairy 4, and the remaining resistance genes all conferred mercury resistance (Fig. S2). The relative abundance of heavy metal and biocide resistance genes in feces was very low on both farms (0.00–0.01 ARG copies per 16S rRNA gene; $P = 0.94$). Heavy metal and biocide resistance gene relative abundance were higher in soil samples compared with the other sample types, and their relative abundance between soil on Dairy 1 (0.18–0.43 ARG copies per 16S rRNA gene) and Dairy 4 (0.00–0.37 ARG copies per 16S rRNA gene) did not differ ($P = 0.39$). Genes representing a diverse range of resistance classes were identified from soil, with most resistance genes conferring resistance to copper, peroxide, and iron.

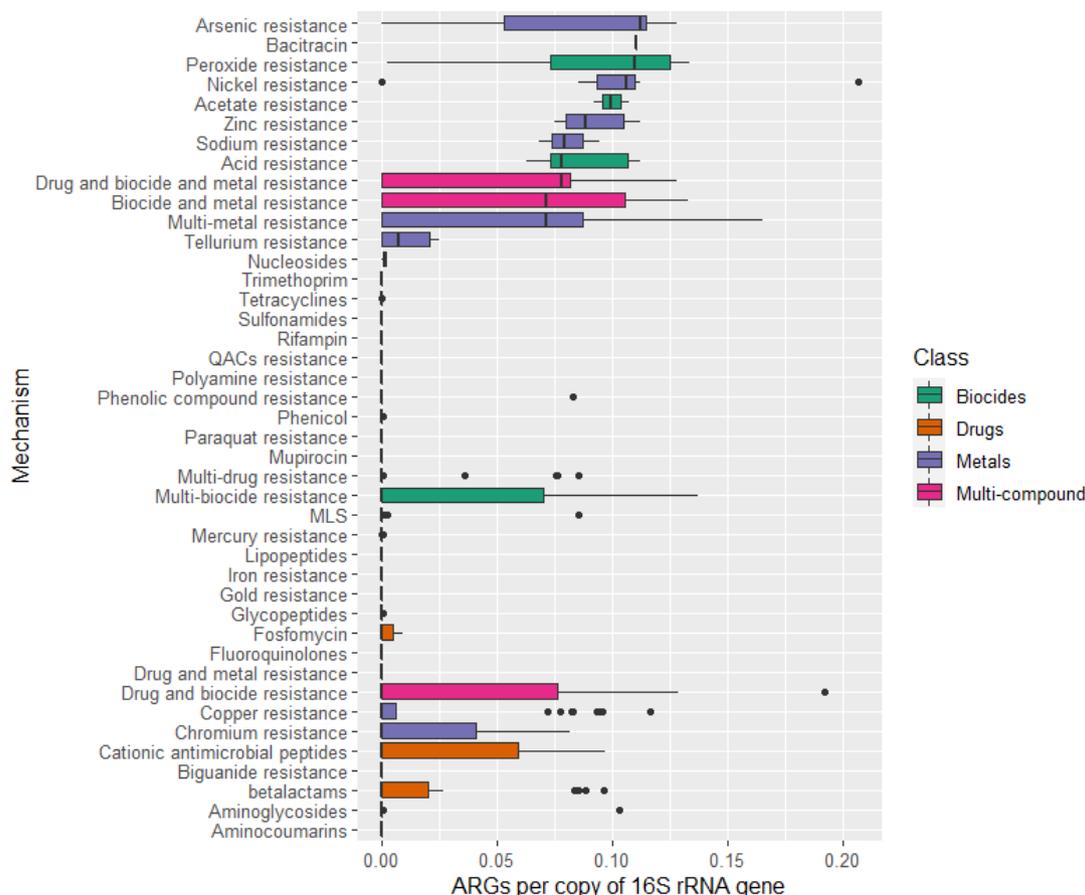


FIG 4 Antimicrobial, heavy metal and biocide resistance gene relative abundance (ARG copies per 16S rRNA gene) identified in the waste milk sample.

Compared with all other sample types, the single waste milk sample had a higher relative abundance of antimicrobial, heavy metal, and biocide resistance genes, with 0.0–3.05 ARG copies per 16S rRNA gene (Fig. 4). From the single waste milk sample, 103 unique antimicrobial, heavy metal, or biocide resistance genes were identified, representing 14 resistance classes including copper, acid, nickel, zinc, arsenic, and peroxide resistance (Fig. S3). The most abundant resistance gene classes in the waste milk were often multi-resistant such as multi-metal (3.05 ARG copies per 16S rRNA gene), drug/biocide (2.97 ARG copies per 16S rRNA gene), biocide/metal (1.18 ARG copies per 16S rRNA gene), and multi-biocide (0.78 ARG copies per 16S rRNA gene). Drug/biocide/metal (0.70 ARG copies per 16S rRNA gene) and multi-drug (0.27 ARG copies per 16S rRNA gene) classes were also relatively abundant.

Bacterial host range harboring contigs containing ARGs

Across the sequenced farm samples ($n = 113$), 147,822,721 contigs were assembled. Of these, 1,014 contigs (0.0007%) harbored at least one ARG and were assembled from 78.8% (88 of 113) metagenomic samples, but only 19.7% could be classified at the phylum level (200 of 1,014). The number of contigs that were taxonomically assigned reduced as the classification levels decreased down to the class (140 of 200; 70.0%), order (67 of 200; 33.5%), family (58 of 200; 29.0%), genus (42 of 200; 21.0%), and species (12 of 200; 6.0%) levels.

Within the 200 contigs originating from 70 metagenomic samples (Fig. 6), 57 unique ARGs were identified, representing resistance to 14 antibiotic classes. Several contigs (7 of 200; 3.5%) co-harbored multiple ARGs. Interestingly, the *bla*_{ACC} (plasmid-mediated class C β -lactamase) and *crp* (repression of multidrug efflux pump expression) genes

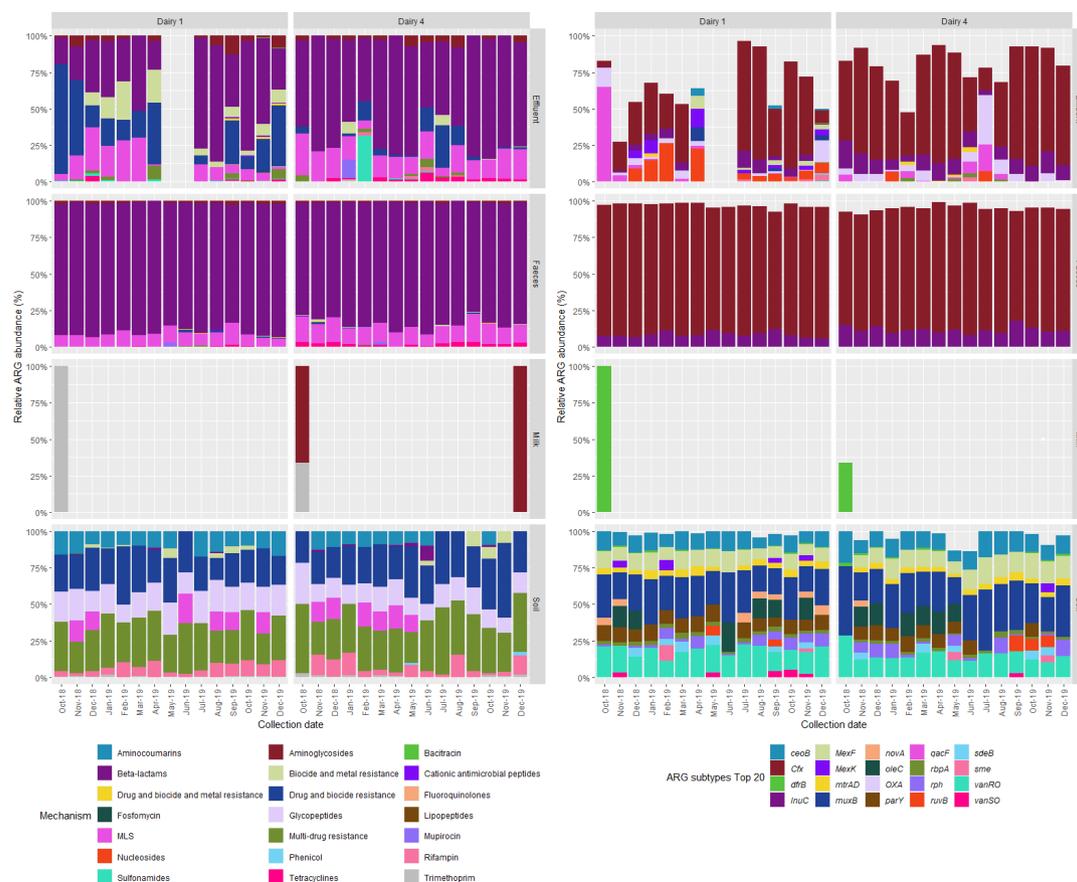


FIG 5 (A) Relative abundance of antimicrobial resistance genes and (B) the top 20 most abundant antimicrobial resistance genes identified from farm dairy effluent, feces, milk, and soil samples. Resistance genes are classified at the class level.

were identified on one contig assembled from the waste milk sample and were assigned to the bacterial order *Enterobacterales*. Most of the ARGs were on contigs assigned to Proteobacteria (30 genes) and Firmicutes (19 genes), followed by Actinobacteria (six genes), Bacteroidetes (three genes), and Fibrobacteres (one gene). At the phylum level, the majority of the contigs harboring ARGs were assembled from effluent samples ($n = 102$ contigs), with more contigs assembled from Dairy 1 ($n = 57$) compared with Dairy 4 ($n = 46$). ARGs identified in contigs assembled from effluent samples potentially confer resistance across 10 antimicrobial classes, with the highest number of gene groups assigned to resistance to drug and biocides ($n = 9$), β -lactams ($n = 7$), MLS ($n = 5$), and aminoglycosides ($n = 4$). These genes were predominantly found in Proteobacteria but were also detected in contigs assigned to Actinobacteria, Bacteroidetes, and Firmicutes. In addition, *qac* genes were found in contigs assigned to Proteobacteria from both farms. In total, 52 contigs harboring ARGs were assembled from soil samples with a similar number of contigs from each farm (Dairy: 1 $n = 27$; Dairy 4: $n = 25$). Fewer contigs with ARGs were assembled from fecal samples ($n = 26$), with more from Dairy 4 ($n = 17$) compared with Dairy 1 ($n = 9$). No contigs containing ARGs were assembled from milk samples. From the single waste milk sample, 19 contigs containing ARGs were assembled, with one contig co-harboring two resistance genes (*bla_{ACC}* and *crp*; Table S5). The ARGs identified in the waste milk sample potentially confer resistance to β -lactams, including *bla_{CTX-M}* identified in a contig belonging to the *Enterobacterales*, drug and biocide resistance and MLS classes from contigs assigned to the phylum Proteobacteria, as well as resistance to β -lactam, fosfomycin, glycopeptide, multi-drug, MLS, and nucleoside classes detected in contigs assigned to Firmicutes.

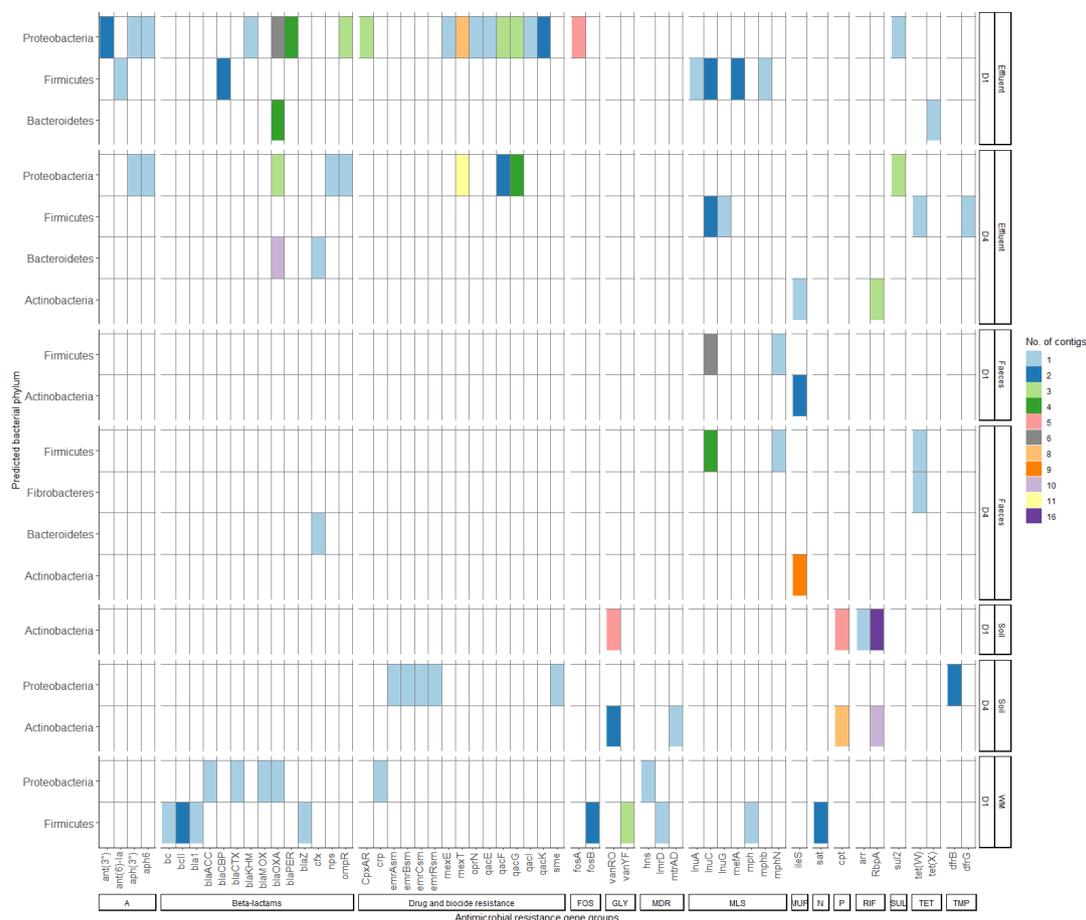


FIG 6 The predicted bacterial phyla of 200 contigs harboring antimicrobial resistance genes (ARGs). ARG groups were grouped per class of antibiotic and the contigs were grouped by sample type and farm. The number of contigs with the unique phyla and ARG combination is indicated by the color key on the figure legend. A, aminoglycoside; FOS, fosfomycin; GLY, glycopeptides; MDR, multi-drug resistance; MLS, macrolide, lincosamide and streptogramin; MUP, mupirocin; N, nucleosides; P, phenicol; RIF, rifampin; SUL, sulfonamides; TET, tetracycline; TMP, trimethoprim; and WM, waste milk.

The phylum Proteobacteria harbored the most diverse range of ARGs across the sample types. Proteobacteria was one of the predominant phyla found in the effluent samples (average: 45.1%), and the number of contigs containing ARGs classified as Proteobacteria across effluent samples from both farms was high (Dairy 1: $n = 43$; Dairy 4: $n = 26$). ARGs from contigs assigned to Proteobacteria were found in the waste milk sample ($n = 4$ contigs) and soil samples ($n = 4$ contigs), although the number of contigs was lower from these sources. Samples that had a high ARG relative abundance at the sequencing read level also had an increased number of contigs with ARGs assigned to Proteobacteria (effluent: DF0176 and DF0188; waste milk: DF0167). Despite being a predominant phylum detected in the fecal microbiome composition analysis, no Proteobacteria contigs containing ARGs were detected in fecal samples from either farm during the study.

The phylum Firmicutes also harbored a diverse range of ARGs ($n = 19$), and ARG-containing contigs were assembled from effluent ($n = 14$ contigs), feces ($n = 13$ contigs), and waste milk ($n = 14$ contigs) samples. *InuC* was identified from all fecal samples ($n = 30$) and most effluent samples (27 of 30 samples), and 14 contigs across effluent ($n = 4$) and feces ($n = 10$) samples. Four contigs could be classified at the family level and were assigned to the *Lachnospiraceae*, *Ruminococcaceae*, and *Sporomusaceae* families (Fig. S4). ARGs were identified in contigs assigned to Bacteroidetes ($n = 17$ contigs) from effluent ($n = 13$ samples) and feces samples ($n = 1$ sample). Of these, 14 contigs harbored the *bla_{OXA}* gene, and five contigs could be classified at the family level, assigned to

Flavobacteriaceae. One contig harbored *tetX* and was assigned to the *Sphingobacteriaceae* family. Two contigs assembled from feces and effluent ($n = 1$ each) harbored *cfx* (encodes a class A beta lactamase), and *cfxA* was identified in all fecal samples ($n = 30$) and most effluent samples ($n = 27$; 96.4%) at the sequencing read level; however, neither of these two contigs could be classified at a lower taxonomic rank.

The phylum Actinobacteria, prevalent in the soil and effluent, had a high number of contigs containing ARGs across the soil samples on both farms (Dairy 1: 13 of 15 samples; 27 contigs; Dairy 4: 11 of 15 samples; 21 contigs), as well as in feces (Dairy 1: 2 of 15, two contigs; Dairy 4: 9 of 15, nine contigs) and only effluent on Dairy 4 (4 of 15, four contigs). Effluent samples from both farms harbored the most diverse host range of contigs (Fig. 6).

DISCUSSION

The role of the pasture-based dairy farm environment in the development and spread of AMR is not fully understood. Due to the comparatively low use of antimicrobials in food-producing animals in NZ (42–44) and the largely pasture-based dairy farm system, it was hypothesized that the relative abundance of ARGs in the dairy farm environment would be low. To test this hypothesis, shotgun metagenomics with deep-sequencing was utilized to examine and compare the resistome of environmental samples (feces, effluent, soil, and milk) collected over a 15-month period from two NZ dairy farms with contrasting farm management practices. ARG relative abundance from effluent, feces, and milk collected on Dairy 1 and Dairy 4 was relatively low. These findings suggest that pasture-based dairy farms in NZ have low levels of AMR, which provides further evidence for consumers of the safety of New Zealand's export products. Binomial generalized linear models comparing the presence or absence of resistance classes found in >10% of all feces, effluent, and soil samples were used to test the associations of AMR with "farm" and "season," and no statistically significant associations were observed ($P > 0.05$), except multi-metal ($P = 0.020$) and tetracycline ($P = 0.0003$) resistance were significant at the "farm" level.

Effluent harbors a diverse range of ARGs (9, 48), and applying effluent to pasture has been associated with higher detection of AmpC-producing *E. coli* on beef farms in the United Kingdom (32). A study comparing the resistome from Chinese dairy farm environments found that the farm wastewater had the most diverse ARG subtypes compared with feces and soil (49); however, soil was collected from vegetable fields fertilized by dairy manure, compared with soil from recently grazed paddocks that were analyzed in this study. The diverse number of ARGs detected in effluent may reflect the complex composition and microbiota of this sample type (50) as well as temperature (50, 51) or storage conditions (52). The effluent management strategies differed between the two farms; effluent from Dairy 4 was stored conventionally in a large pond prior to being applied to pasture, compared with Dairy 1 where no effluent was applied to pasture during the study period but instead was filtered prior to waste-water discharge using an effluent filtration system. PERMANOVA analysis of the microbiome at the order level showed that "farm" had a statistically significant effect on the effluent microbiome ($P = 0.001$), which is likely due to the different effluent management strategies between farms. "Season" also had a statistically significant ($P = 0.001$) impact on the effluent microbiome, which may have been due to variations in ambient temperature and microbial composition (such as different feed types and negligible fecal and urine inputs from the milking shed into the effluent pond during winter when cows are not milked). There was a statistically significant difference ($P = 0.02$) in the ARG relative abundance in effluent samples from the two farms, with a higher ARG relative abundance in effluent from Dairy 1 potentially caused by the contrasting effluent treatment systems or farm level factors such as the higher levels of AMU on Dairy 1 compared with Dairy 4 during this study (53). However, when modeling of the data comparing the presence or absence of resistance classes found in >10% of all feces, effluent, and soil samples was under-

taken, no statistically significant associations ($P > 0.05$) with “season” were observed due to the more aggregated level of analyses undertaken.

ARG relative abundance in feces sampled in this study ranged from 0.08 to 0.17 ARG copies per 16S rRNA gene. These data are lower compared with the total ARG relative abundance in young calf feces (0.77–5.14 ARG copies per 16S rRNA gene) (54). A study comparing the fecal resistome of pre-weaned calves compared with lactating dairy cows on 17 commercial farms in the USA found a significantly higher ARG relative abundance in calves (0.43–2.9 ARG copies per 16S rRNA gene) compared with dairy cows (0.11–0.6 ARG copies per 16S rRNA gene) (55). ARGs belonging to the β -lactam resistance class were most abundant in feces due to the presence of *cfxA*, which encodes a β -lactamase and is associated with a transposon (56, 57) that may be involved in the horizontal transfer of the *cfxA* gene between *Bacteroides* and *Prevotella* species (57–59). Therefore, the high relative abundance of this gene in bovine feces may be due to Bacteroidetes being a predominant phylum detected in fecal microbiome of lactating dairy cows (60). The relative abundance of heavy metal and biocide resistance genes in feces was extremely low on both farms (0.00–0.01 resistance gene copies per 16S rRNA gene), suggesting that these genes are not prevalent in the fecal microbes from healthy dairy cattle in NZ.

The relative abundance of ARGs in milk was very low (0.00–0.12 ARG copies per 16S rRNA gene) on both farms and was only detected in three milk samples. A study comparing raw retail milk samples from across the USA found that the relative abundance of ARGs (0.0–1.0 ARG copies per 16S rRNA gene) varied from different locations and likely due to differences in the milk microbiota between samples (61). In this study, compared with ARGs, bulk tank milk samples (to be pasteurized prior to human consumption) from both Dairy 1 and Dairy 4 had a higher relative abundance of heavy metal resistance genes (0.00–0.59 resistance gene copies per 16S rRNA). All the metal resistance genes detected in milk potentially conferred mercury resistance. Other studies utilizing shotgun metagenomics to study the resistome of retail raw milk have not investigated the relative abundance of heavy metal and biocide resistance genes (21, 61). Genes conferring mercury resistance have been identified in numerous bacteria, including among members of the Firmicutes (62).

Exogenous contamination and its impact on microbiome analyses from low biomass samples such as bovine milk have been well documented (63–65). A study analyzing 16S rRNA gene sequence data from bovine milk and mammary epithelium samples found that >75% of the sequence data generated were from contaminating DNA, and for bovine milk samples, the source of this exogenous contamination was predominantly from DNA extraction kits (65). In this study, the bulk tank milk samples clustered into two distinct groups according to milk microbiome composition, which was likely an artifact of deep sequencing (40 million read pairs per sample) milk samples with low DNA yields. For these samples, the predominant genera identified were associated with exogenous contamination from the sample preparation DNA extraction kit and/or buffer solution (Table S4). Most of the genera detected in the negative controls in this study (except for *Diaphorobacter* and *Agrobacterium*) have been previously reported as contaminants of DNA extraction kits and laboratory reagents (64). ARGs were detected at a low frequency in the negative controls; however, this underscores the critical need to include such controls in studies of samples with low microbial biomass, such as bulk tank milk and anticipated low prevalence of ARGs. The presence of the two ARGs (*dfxB* and *ant6*) in bulk tank milk samples and negative controls further highlights the need for careful analysis of negative control samples in parallel with environmental samples to determine the origin of ARGs in low biomass samples. Further work including long-read sequencing is needed to elucidate the genomic context and thus significance of the ARGs detected in the bulk tank milk samples.

Feeding waste milk, which may contain low concentrations of antimicrobials (66), to young calves has been suggested as a risk factor for shedding and transmission of antimicrobial-resistant bacteria (67) and may be a source of pathogenic bacteria

(66, 68). It has been suggested that feeding waste milk to calves may increase ARG relative abundance in feces (69). The waste milk disposal strategy on farm may depend upon the volume of waste milk, the criteria used to designate the milk as waste, farm management practices, as well as local regulations. Disposal of waste milk generally includes either discharge into the effluent pond (or similar storage area) or it may be fed to calves (70), which may facilitate the dissemination of antimicrobial, heavy metal, and biocide resistance genes. The inclusion of waste milk in this study was serendipitous and provided useful preliminary insights into the ARG relative abundance in waste milk on this farm. According to individual antimicrobial animal treatments recorded, five cows were receiving antimicrobial treatment for mastitis and/or between claw/footrot with products containing either β -lactam, procaine penicillin G and penethamate compounds, within 6 days prior to the sampling date. The single waste milk sample had a higher relative abundance of ARGs as well as heavy metal and biocide resistance genes (0.0–3.05 resistance gene copies per 16S rRNA gene) compared with soil, effluent, feces, and milk samples. The most abundant resistance mechanisms identified were ARGs belonging to multi-compound classes including several ARGs encoding efflux pumps or regulators, highlighting that this waste milk sample may be a source of ARGs and genes conferring resistance to other compounds such as heavy metals and biocides. However, the extent to which other waste milk samples may have been contaminated with ARGs over the course of this study is unknown. At the contig level, the detection of clinically relevant ARGs, such as *bla*_{CTX-M}, which encodes an ESBL enzyme in *Enterobacteriales* from waste milk, is a concern due to the potential transmission of AMR in the dairy farm environment.

Soils, including those with low anthropogenic impact, have been shown to harbor a diverse range and relative abundance of ARGs (71, 72), and many antimicrobial compounds are naturally produced by soil microorganisms (72). ARG relative abundance as well as heavy metal and biocide resistance gene relative abundance was highest in the soil samples throughout this study (0.20–0.63 ARG copies per 16S rRNA gene and 0.00–0.43 resistance gene copies per 16S rRNA gene) compared with the effluent, feces, and milk sample types. The soil microbiome composition can be highly complex (73), and both ARG and bacterial diversity were significantly correlated in a study comparing soil from three distinct ecosystems (tundra, temperate, and tropical) (71), suggesting that microbiome sample variation drives ARG diversity. In this study, soil sample microbiomes from both farms were relatively homogeneous, with the two predominant phyla being Proteobacteria and Actinobacteria, congruent with previous studies from feedlot soils ($n = 4$) in Canada (74) and soil samples collected from five US dairy farms (19). There was no statistical difference ($P = 0.32$) in ARG relative abundance in the soil samples between the two dairy farms, which is not unexpected, given the close geographical proximity of the farms (< 5 km) and the similarity in microbiome between the soil samples. ARG relative abundance in soil samples collected in this study (0.20–0.63 ARG copies per 16S rRNA gene) was comparatively low compared with soil microcosms with/without compost manure collected from cows treated with specific antibiotics (75) and ARG relative abundance in soil collected from pens housing untreated ($n = 3$) and florfenicol treated calves ($n = 3$) (76), which ranged from 0.62 to 4.53 ARG copies per 16S rRNA gene. ARG relative abundance in soil defined as pristine with little anthropogenic impact had a relative abundance of 0.05–0.28 ARG copies per 16S rRNA gene (7), which is less than the agricultural soils in this study.

The soil samples had the highest ARG relative abundance and were the third most diverse sample type (after effluent and feces), with 30 ARG groups belonging to 11 resistance classes. Although soil samples ($n = 4$) from feedlots in Canada had a smaller number of unique ARG groups compared with this study, with only nine ARG groups belonging to six classes detected (74), the sample size was considerably smaller. The ARGs from soil belonging to the multi-drug resistance class encoded multi-drug efflux pumps (*muxB* and *tap*) or regulators (*mtrAD*). Multi-drug efflux pumps are also important for functions other than AMR such as detoxification of intracellular metabolites, cell

homeostasis, and bacterial virulence within plant and animal hosts (77). Several drug/biocide resistance genes were identified in soil, all of which encode efflux pumps or regulators. Despite ARGs being detected at a higher relative abundance in soil, some ARGs identified in soil have been shown to share relatively low sequence similarity to the corresponding ARG in clinical pathogens (71), although a limited number of clinical ARGs were analyzed and the phenotypic consequence of this finding is unknown.

The bacterial host and genomic context of ARGs are crucial to assess the health risk posed. In addition, some ARGs, such as efflux pumps, can have alternative functions and be involved in physiological processes unrelated to AMR. Human-associated mobile ARGs were identified as the highest risk in a recent risk framework study (13). Analysis of the ARGs used in the risk framework found that 70% (1,816 of 2,579) of ARGs were not human-associated (enriched in environments with anthropogenic impact), were classified as the lowest risk, with many not associated with mobile genetic elements (13). A diverse range of ARGs ($n = 372$) belonging to the drugs, biocide, metal, and multi-compound classes were identified in this study, many of which were identified in effluent. Despite a large number of unique ARGs being identified, the majority of these were not classified as the highest risk gene families (13). At the sequencing read level, the high-risk ARGs were *aac6'* identified in feces; *dfrB*, from one milk sample (*dfrB1* highest risk ARG) and *bla_{OXA}*, *bla_{CTX}*, and *lnuA* from effluent. Soil samples had the highest ARG relative abundance in this study; however, no high-risk ARGs were identified, suggesting that although the overall ARG relative abundance in these samples was high compared with feces, effluent, and milk, the human and animal health risk associated with these ARGs is low. The number of contigs containing ARGs that could be taxonomically classified was low, likely due to the limitations of short-read sequencing. This issue may be improved by using long-read sequencing technologies as long reads often encompass multiple genes, enhancing classification (78).

ARGs potentially conferring resistance to critically important antimicrobials in human medicine (79), including plasmid-mediated *mcr* genes conferring resistance to colistin (80), carbapenem resistance genes (81), or the resistance gene *mecA* from methicillin-resistant *S. aureus* (82), were not identified in this study. Of the ARGs detected in contigs assembled from the metagenomic samples, some high-risk ARGs were identified. Of particular concern, the *bla_{OXA}* gene was detected in a contig assigned to the *Campylobacteraceae* family from effluent, and *bla_Z* and the extended-spectrum β -lactamase resistance gene *bla_{CTX-M}* were identified in *Bacillaceae* and *Enterobacterales* from waste milk, respectively. The *tet(W)* gene, which is classified as high-risk rank II gene (which includes resistance emerging in non-pathogens), was detected in *Fibrobacteraceae* and *Lachnospiraceae* from feces and effluent samples on Dairy 4, respectively. However, it is important to note that the presence of an ARG does not always correlate with a resistant phenotype. These findings suggest that although a diverse range of ARGs was detected across the feces, effluent, soil, and milk samples, albeit at a comparatively low relative abundance, the majority of the ARGs detected do not pose a high public and animal health risk. These results highlight the importance of determining the bacterial host and mobility of ARGs to assess the relevant public and animal health risk posed.

Conclusion

ARG relative abundance in feces, effluent, soil, and bulk tank milk samples across two NZ dairy farms over a 15-month surveillance period was low compared with overseas studies. This research provides important baseline data for ARG surveillance and indicates that pasture-based systems commonly associated with NZ dairy farms may be associated with lower levels of AMR. Sustainable agricultural practices such as forage-feeding of dairy cattle can be associated with improved animal health and welfare, reduced antibiotic treatment, and AMR (17). No statistically significant difference in overall antimicrobial, heavy metal, and biocide resistance genes was observed between Dairy 1 and Dairy 4. However, differences in ARG relative abundance between effluent from the two farms were statistically significant, with a higher ARG relative abundance

in effluent from Dairy 1. Effluent samples harbored the most diverse range of ARGs, some with a recognized public health risk. Soil samples had the highest ARG relative abundance (excluding the single waste milk sample); however, the ARGs in soil were not classified with a recognized risk to public or animal health, highlighting that genetic context and risk (disease-associated and mobility) as well as relative abundance should be considered when analyzing resistomes using shotgun metagenomic sequencing. However, caution should be used when comparing between studies using shotgun metagenomic sequencing methods to ensure that the ARG identification, normalization methods and sequencing depths are comparable.

MATERIALS AND METHODS

Study population, sample collection and processing

A comparison of farm management practices and AMU on the two farms during the study period has been previously discussed; most of the antibiotics used on Dairy 1 were penicillins, and both penicillins and first-generation cephalosporins were used on Dairy 4. Antibiotics classified as red-tier by the NZVA were infrequently used, and the sample level prevalence of EBSL-producing *E. coli* was low (53). The two dairy farms are located <5 kilometers apart, both operate a closed dairy farm system and are pasture-based systems with the use of supplementary feed when required. The farms have a spring calving system, and Dairy 1 milks once a day, whereas Dairy 4 milks twice a day. Dairy 4 also has a freestall barn (200 cow capacity). The samples were collected as described previously (53) and consisted of feces ($n = 30$), soil ($n = 30$), farm dairy effluent ($n = 28$), milk ($n = 24$), and waste milk ($n = 1$). Composite soil cores and composite cow feces from cow pats were collected from a recently grazed paddock. Farm dairy effluent is a by-product of dairy cattle being in the farm dairy, feed pads, or yards and consists of cattle feces and urine diluted with wash-down water (83). Two effluent collection sites were used on Dairy 1. For the first site, settled effluent was collected from the sump prior to filtration, but from July 2019 onward, effluent more dilute watery in composition, was collected from within a drain in the cowshed. Bulk tank milk is the collection of raw milk from multiple cows, which is stored before being collected for milk processing and would be pasteurized prior to human consumption. There are strict storage and hygiene conditions for bulk tank milk in NZ. Waste milk is any form of unsaleable milk produced on-farm. The composition of waste milk varies and may consist of (i) milk from dairy cows receiving antimicrobial treatment (either systemic or intramammary) that has a withholding period, (ii) milk from cows receiving non-antimicrobial drugs, (iii) colostrum from cows shortly after calving, and (iv) milk from ill cows or milk with a somatic cell count exceeding the saleable limit (33, 70). The milk sample collected in October 2019 from Dairy 1 was waste milk (sample DF0167), rather than bulk tank milk. This sample collection issue was discovered post-sequencing, and therefore, 24 bulk tank milk and one waste milk sample were included in the study.

For each sampling visit, feces and soil samples were homogenized and stored in 0.25 g aliquots at -80°C . To concentrate the microbial DNA and reduce the protein and fat content, approximately 400 mL of milk was centrifuged at $10,000 \times g$ (Sorvall LYNX 4000 Superspeed Centrifuge, ThermoFisher Scientific, Waltham, Massachusetts, United States) for 45 min at 4°C . The fat and supernatant were discarded, and the pellet re-suspended in the remainder of the supernatant and centrifuged at $10,000 \times g$ for 10 min at 4°C . The supernatant was discarded, and the pellet was washed twice in 5 mL PBS (0.01M, pH 7.3) (ThermoFisher Scientific, Waltham, Massachusetts, United States) at $10,000 \times g$ for 10 min at 4°C . The re-suspended pellet was transferred and stored in duplicate at -80°C . To concentrate the effluent (based on the method described in [20]), approximately 400 mL of effluent was centrifuged at $10,000 \times g$ (Sorvall LYNX 4000 Superspeed Centrifuge, ThermoFisher Scientific, Waltham, Massachusetts, United States) for 20 min at 4°C . Later, 95% of the supernatant was decanted, and the pellet was re-suspended in the remainder of the supernatant and stored at -80°C .

DNA extraction

Genomic DNA was extracted using the Presto Stool DNA Extraction Kit (Geneaid Biotech Ltd, New Taipei City, Taiwan) according to the manufacturer's instructions with minor modifications. Briefly, 0.25 g feces and soil and 200 μ L of milk and effluent were used for the DNA extraction. For lysis, the sample was vortexed at maximum speed for 7 min (Vortex Mixer, Labnet International, New Jersey, USA) and then centrifuged at 8,000 $\times g$ for 2 min. Next, 500 μ L of supernatant was transferred to a new tube, and 5 μ L RNase A (100 mg/mL; QIAGEN, Hilden, Germany) was added and incubated for 10 min at 37°C. To elute the DNA, 30 μ L of elution buffer was added to the center of the column and left to stand for 2 min. The sample was then centrifuged, and the elution step was repeated using the buffer containing the eluted DNA and stored at -20°C. All centrifuge steps were carried out at room temperature. The DNA concentration was quantified using a Qubit 4.0 fluorometer (ThermoFisher Scientific Inc., USA) and $A_{260/280}$ and $A_{260/230}$ ratios were determined using the Nanodrop microvolume spectrophotometer (Nanodrop 2000c, ThermoFisher Scientific Inc., USA). DNA integrity and size were visualized on a 0.8% [wt/vol] agarose gel using a high molecular weight *Hind*III λ ladder (ThermoFisher Scientific Inc., USA).

Shotgun metagenomic sequencing

The metagenomic sequencing run was performed on an Illumina Novaseq S4 platform with 2 \times 150 paired-end reads (\geq 40 million paired-end reads or approximately 13 Gb per sample). The libraries were prepared using the NEBNext DNA Library Preparation Kit (New England Biolabs, Inc, Ipswich, Massachusetts, USA) and sequenced by Novogene, Singapore. The following controls ($n = 10$) were included in this study: (i) blank reagent control for each DNA extraction kit batch ($n = 2$), (ii) phosphate buffered saline (PBS, 0.01M, pH7.3) ($n = 2$) used for preparation of the milk samples, and (iii) ZymoBIOMICS Microbial Community DNA Standard ($n = 3$) (84) and ZymoBIOMICS Microbial Community DNA Standard II (log distribution) ($n = 3$) (85).

Bioinformatic analysis

The shotgun metagenomic sequencing analysis was based on a previously described workflow (54), and the key steps of the workflow are outlined in Fig. S5. Briefly, TrimGalore v0.6.6 (86) was used for raw read trimming and quality assessment, using Cutadapt v1.18 (87) and FastQC v0.11.9 (88), respectively. A Phred quality score threshold of 20 was used. Host contamination (*Bos taurus*) and human reads (*Homo sapiens*) were removed by aligning the reads to the bovine genome UMD3.1.1 (accession number: [AAFC00000000.3](https://www.ncbi.nlm.nih.gov/nuccore/AAFC00000000.3)) and human genome GRCh38.p14 (accession number: [GCF_000001405.40](https://www.ncbi.nlm.nih.gov/nuccore/GCF_000001405.40)) respectively, using BMTagger in bmttools v3.101 (89). For the ARG normalization calculations, the number of 16S rRNA genes per sample was identified using METAXA2 v2.2 (90). The processed sequencing reads were taxonomically classified using Kraken2 v2.1.1 (91). Bacterial classifications used in this paper are those given by Kraken2 with the standard database v20200919 and do not reflect the recent name changes at the higher levels of bacterial taxonomy (92). The relative abundance of taxa within the samples was estimated at various classification levels using Bracken v2.6.0 (93) and phyla present in \leq 5% total relative abundance were grouped together as "Other" for subsequent analyses. The processed reads were assembled into contigs using MEGAHIT v1.2.9 (94) with default parameters. Unless otherwise stated, all tools were used with default parameters.

Resistance gene analysis

The resistome in the sequencing reads was analyzed following the AMR++ pipeline (<https://www.meglab.org/amrplusplus/>) with minor modifications. Briefly, the antimicrobial, biocide, and heavy metal resistance genes were identified by mapping the processed sequencing reads to the MEGARes database v2.0 (46) using BWA v0.7.17 with

default settings (95). The SAM formatted alignment files were analyzed using ResistomeAnalyzer v2018.09.06 (<https://github.com/cdeanj/resistomeanalyzer>) to generate the sample resistome for each level of the database hierarchy (gene, group, mechanism, and class). For the gene-level analysis, a gene fraction (proportion of nucleotides in the reference sequence that were aligned to by at least one sequence read) threshold of 80% was used to reduce false positive hits. Rarefaction analysis was performed per sample to determine whether the sequencing depth used in this study was sufficient to detect the antimicrobial, biocide, and heavy metal resistance genes present. The SAM formatted alignment file was used as input for RarefactionAnalyzer v2018.09.06 (<https://github.com/cdeanj/rarefactionanalyzer>), with sub-sampling of sequencing reads at 5% increments and a gene fraction threshold of 80%. To allow for more accurate comparisons, ARG gene-level data were normalized to avoid bias associated with ARG size and the microbial load per sample and is well-utilized in this field allowing for a comparison of results from this study with other current literature. ARG relative abundance was expressed as “ARG copies per copy of 16S rRNA gene,” and normalization calculations were performed as previously described (96).

ARGs, excluding those arising from point mutations, were identified in contigs with Abricate v1.0.1 (<https://github.com/tseemann/abricate>) (97) using the MEGARes database v2.0 (46). Contigs containing ARGs were taxonomically classified with the Contig Annotation Tool (CAT) v5.2.1 (98) using NCBI taxonomy files and the NCBI nr database (generated 2021–01-07) with default settings.

Statistical analysis and data visualization

Statistical analysis was performed in R v4.0.2 (99) and RStudio v1.3.959 (100). Data visualization was conducted in R using the packages “ggplot2” (101), “tidyverse” (102), and “dplyr” (103). As many of the resistance classes were detected at low levels, only antibiotic, metal, or biocide classes found in >10% of all feces, effluent, and soil samples ($n = 88$) were included, consisting of 23 out of 37 resistance classes. “glm2” (104) was used to fit binomial generalized linear models on the presence or absence of each resistance class (from the count matrix) at the sample level (response variable) to test associations with “farm” and “season” (fixed effect). The model was run separately to test the fixed effects (“farm” or “season”) with each response variable (resistance classes). For season, “emmeans” (105) was used, and the pairwise comparisons were adjusted using the Tukey method. The overall normalized relative abundance of ARGs per sample type and farm was compared using a *t*-test in “ggpubr” (106). Differences in microbiome profiles, at the taxonomic order level, based on Bray-Curtis distance measures were analyzed using non-metric multidimensional scaling (NMDS) in the “vegan” package (107). PERMANOVA analysis was performed using the *adonis2* function in “vegan” (107), with the microbiome data matrix from kraken2 for each sample type (excluding milk and waste milk) as the response variable, compared with “farm” and “season” (explanatory variables) with the permutations constrained within blocks defined by “visit,” which is useful for accounting for repeated measures.

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ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental material (AEM01390-24-s0001.pdf). Tables S2 to S5; Figures S1 to S5.

Table S1 (AEM01390-24-s0002.xlsx). Sample metadata, including BioProject and BioSample accession numbers.

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