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Assemblage of Bacteria Communities and Resistome Enrichment by Dairy Flurries Along the Rhizosphere–Bulk Soil Continuum on Dairy Farms

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Abstract: The use of dairy slurries as organic fertilizer amendments is a common practice in agriculture as a cost-saving measure, as well as a residue management strategy. However, concerns related to the increase in antibiotic resistance in the environment under the scope of the One Health strategy are increasing. In this study, we aimed to assess resistome enrichment driven by dairy slurry application in four southern Chile dairy farms. Slurry pits, rhizospheres of *Lolium perenne* amended with those slurries, and bulk soils were sampled. Thirteen antibiotic-resistance genes (ARGs, *tet*A, *tet*G, *tet*M, *tet*Q, *tet*W, *tet*X, *sul*1, *sul*2, *bla*CTX$_{M}$, *bla*$_{OXA-1}$, *bla*TEM, *erm*B, and *dfr*A1) for five antibiotic classes (tetracyclines, sulfonamides, beta-lactams, macrolides, and trimethoprim–sulfamethoxazole), two related integrases (*intl*1 and *intl*2), and total bacteria (16S rRNA) abundance was measured by quantitative PCR (qPCR). Then, the abundance profiles of two enzyme-inactivated ARGs (*tet*X and *bla*TEM) were determined. The differences between the bacterial communities inhabiting the different sample types were explored with 16S rRNA metabarcoding. In general, all measured ARGs were detected in slurries. A decreasing trend in ARG copy numbers was observed with increasing soil depth, with the exception of *tet*X, whose abundance increased in the bulk soil at specific farms. The *tet*X and *bla*TEM communities revealed no differences in the relative abundance of variants in any of the samples. Finally, taxonomic and structural differences were found among all sample types. Thus, the enrichment of the sampled farm soil resistomes was driven by the application of the raw slurries as fertilizer.

Keywords: environmental resistomes; dairy slurries; antibiotic resistance; rhizosphere

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1. Introduction

Organic amendments have been historically applied as a sustainable alternative to improve the fertility and quality of soils in farming-intensive agricultural systems [1]. However, organic fertilization, such as manure or animal farm slurries, leads to the accumulation of antibiotic residues in soils, as well as the dissemination of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) [2,3]. In soils, antibiotic molecules

occur naturally as a mechanism to exert selective pressure upon susceptible populations [4], allowing them to develop different resistance mechanisms (enzymatic degradation, target modification, active efflux, inactivation, etc.) to overcome exposure to one or multiple antibiotic molecules [5]. Alternatively, ARB can convey ARGs to susceptible bacteria through mobile genetic elements (MGEs), such as conjugative plasmids, transposons, or integrons. The collection of all ARGs present in an organism or environment is known as a resistome [6]. In soils exposed to repeated applications of animal slurries, these ARGs are increased, posing a threat to global health [3]. Exploring the abundance and prevalence of antibiotic resistance and its mechanisms of dissemination via slurry application is pivotal for addressing public concerns and safe management of farm ecosystems [5]. Despite the relevance of soil resistomes and ARG transmissibility, studies that evaluate the prevalence, abundance, and dissemination of ARGs in Chilean dairy farm systems are still scarce.

With respect to the input of antibiotics into the environment, dairy farms are large contributors to antibiotic residue and ARG pollution in soil and water bodies [7]. Cattle excrete between 50% and 100% of administered antibiotics [8]; thus, dairy slurries containing multiple antibiotic traces are habitats for ARB and hotspots for ARG transfer to environmental bacteria. The persistence of tetracycline residues has been detected from 144 to more than 1000 days in soils amended with dairy slurries [9,10]. Similar trends have been observed for macrolide, beta-lactam, and sulfonamide classes [11]. These molecules can inhibit the development of susceptible bacterial communities [12], or contribute to the enrichment of their resistomes [13]. Six ARGs related to tetracycline resistance, macrolide resistance, sulfonamide resistance, and beta-lactam resistance and one related integrase (*tet*A, *tet*W, *tet*X, *erm*B, *sul*1, and *bla*CTX-M_1, and *intl*1, respectively) gene copy number have been correlated with dairy slurries applied as organic amendments to soil [13]. Among the abiotic factors that drive this phenomenon, soil sorption properties, the iron oxide:phosphate ratio, physicochemical parameters, and the humic acid content are described as key factors [14–16]. For plant-influenced soil niches, bacteria–bacteria interactions and cooccurring bacterial networks can promote widespread dissemination and high transmission of ARGs among root microbiomes [17]. However, rhizobox experiments have shown that plant selection of rhizosphere components can inhibit sulfonamide ARGs and class-1 integrons in wetlands [18]. Therefore, the input of exogenous ARGs in conjunction with the natural pressure of antibiotics in soils may stimulate the selection and persistence of specific resistomes in the rhizosphere–bulk soil continuum.

In this study, we aimed to determine the effects of raw slurry inputs on the community and resistome compositions of the rhizosphere–bulk soil continuum in southern Chile dairy farms. The abundance of thirteen ARGs for beta-lactam (*bla*TEM, *bla*OXA-1, *bla*CTX-M), macrolide (*erm*B), tetracycline (*tet*A, *tet*G, *tet*M, *tet*Q, *tet*X, *tet*W), sulfonamide (*sul*1, *sul*2), and trimethoprim–sulfamethoxazole (*dfr*A1) was measured from slurry pits, the rhizosphere of *L. perenne* amended with those slurries and bulk soils. The abundance of total bacteria (16S rRNA) and two integrases (*intl*1, *intl*2) were also quantified. Moreover, two enzymatically inactivated ARG communities were explored by sequencing.

2. Materials and Methods

2.1. Sampling Site Description

In June 2021, samples of fresh dairy slurries and *Lolium perenne* L. rhizosphere as well as bulk soils were collected from four different dairy farms: Remehue (RM), Fresia (FR), Esmeralda (ES), and San German (SG) (Figure 1a). According to farm registers, all sampled grasslands were chemically fertilized (NPK) and amended with slurries from their respective slurry pits every year. Slurry samples were taken using a 3 m plastic rod with a 1 L flask attached to one end, as described by Salazar (2012) [19] (Figure 1b).

To obtain homogeneous samples, slurry pits were mechanically mixed prior to sample collection in 1 L pots (four pots per well, considering cardinal points (NSEW) as biological replicates). In parallel, the slurry-amended *L. perenne* plants and bulk soil (~20 cm depth) were sampled, and ~1 kg of each was collected using a clean spade to remove intact roots (Figure 1c). The plant and bulk soils were placed within sterile Whirl-Pak bags (Sigma–Aldrich, Inc., Saint Louis, MO, USA) and stored in coolers at 4 °C. After collection, the samples were immediately transported to the Universidad de La Frontera, Temuco, Chile. In the laboratory, slurry samples were homogenized. Samples were composed of a proportional mixture of the four replicates of each well, while the rhizosphere was separated, and the bulk soil was processed separately. The samples were labeled according to their original matrix (e.g., RMS = RM—slurry; RMR = RM—rhizosphere; RMB = RM—bulk soil). Fractions of each replicate were stored at −80 °C for (i) physicochemical analyses and (ii) extraction of total DNA.

Figure 1. (**a**) Location of Chilean Los Lagos region dairy farm sampling sites, (**b**) dairy slurry well sampling, and (**c**) slurry-amended *L. perenne* rhizosphere and bulk soil sampling. Satellite image obtained from Google Earth platform (https://earth.google.com/web; accessed on 20 July 2022).

2.2. Physicochemical Analyses

The physicochemical properties (N, P, K, salts [Ca^{2+}, Mg^{+}], pH) of slurry and soil samples were determined by Laboratorio de Riles (slurries) and Laboratorio de Suelos (soils), Universidad de La Frontera. The pH was measured in 1:2.5 soil:deionized water

suspensions. The total phosphorus (P) content of the slurries was determined by the standard set by the Chile Ministry of Public Infrastructure [20]. The extractable phosphorus (PO_4) was measured from a Na bicarbonate (0.5 M) extract and analyzed by the molybdate blue method [21]. The total nitrogen (N) content was determined by the Kjeldahl method, and nitrate (NO_3^-), ammonia (NH_4) and organic matter (OM) content was determined as described by Sparks (1996) [22]. Exchangeable cations (K, Ca, Mg, and Na) were extracted with a solution of CH_3COONH_4 (1 M; pH 7.0), and determined flame atomic adsorption spectrophotometry (FAAS) [23].

2.3. Total DNA Extraction

For total DNA extraction, ~0.25 g of each sample was processed under a bead-beating protocol following the instructions of a Qiagen PowerSoil Pro DNA extraction kit (Qiagen, Hilden, Germany). The DNA concentrations and quality were determined on a Qubit^4 fluorometer (ThermoFisher, Waltham, MA, USA). Aliquots were separated and stored at −20 °C, while DNA stocks were stored at −80 °C.

2.4. Quantification of ARGs

The total copy number abundance of the thirteen ARGs was determined using the extracted DNA as a template. Quantification was performed via dye-based qPCR targeting *bla*TEM, *bla*OXA-1, *bla*CTX-M, *tet*A, *tet*G, *tet*M, *tet*Q, *tet*W, *tet*X, *sul*1, *sul*2, *erm*B, and *dfr*A1 ARGs. The abundance of two related integrases (*intl*1 and *intl*2) and the total bacterial community based on the 16S rRNA gene was also measured. All PCRs were set to a final volume of 10 µL via a HOTFire FIREpol Evagreen qPCR Supermix (Solis Biodyne, Estonia). One microliter of DNA was used as a template for all reactions, which were carried out on a QuantStudio3^{TM} (ThermoFisher, Waltham, MA, USA). The PCR conditions, amplicon length, and primer sequences are described in Table S1. Standards for each ARG were synthesized in dsDNA format from known sequences (Table S2, Figures S1 and S2), as described by Xu et al. (2019) [24]. All standard curves were diluted (1:10) from 1 ng/L^{-1} to 1 × 10^{-9} ng/L^{-1} for each gene. The quantified ARG abundance was normalized to the total bacterial community abundance (provided by the 16S rRNA gene data).

Both total and relative ARG abundance were compared via one-way ANOVA ($p \leq 0.05$) and Tukey's HSD test between sample types (slurry, rhizosphere or bulk soil), as well as within sampled farms. To determine slurry-driven soil resistome enrichment, two "enzyme-inactivated" protein-associated ARGs (*tet*X and *bla*TEM) were selected for ARG community composition assays based on their different abundance patterns in the rhizosphere–bulk soil continuum.

2.5. Enzyme-Inactivated ARG Community Composition

To estimate soil resistomes enrichment via slurry application, two enzymatically inactivated ARGs were selected on the basis of their increasing (*tet*X) or decreasing (*bla*TEM) qPCR abundance with increasing soil depth. Both ARGs were amplified via end-point PCR under the same conditions and concentrations selected for qPCR (Table S1) via Kapa HiFi HotStart Readymix (Roche Inc., Basel, Switzerland). Amplification was verified on 1.5% agarose–TAE gels. Libraries were prepared with a Ligation Sequencing Kit (SQK-LSK009), and multiplexed with Native Barcoding kits (EXP-NBD104 and EXP-NBD114) (Nanopore Technologies Inc., Oxford, UK). Pooled libraries were verified on an Agilent Tapestation 4150 (Agilent Technologies, Santa Clara, CA, USA) for size and integrity parameters. Then, ~100–200 fmol of each library was loaded on SpotON FLO-MIN111D (10.4.1) flow cells and sequenced on minION Mk1C for 72 h.

2.6. Total Bacterial Community Sequencing

The total bacterial community assemblage (structure and composition) of all the samples was explored via Illumina MiSeq sequencing (Illumina Inc., San Diego, CA, USA). The V3–V4 16S rRNA libraries were constructed using the 341F–805R primer set coupled with Illumina adapter sequences (Table 1), following the protocol described in Yarimizu et al. 2021 [25]. In summary, the libraries were PCR-indexed with Nextera XT v2 (Illumina Inc., USA), verified at TapeStation 4150 (Agilent Technologies Inc., USA), diluted to 8 pM, denatured, loaded into an Illumina MiSeq Sequencing Kit v3, and paired-end (2 × 300 bp)-sequenced on an Illumina MiSeq platform (Illumina Inc., USA).

Table 1. Physicochemical parameters of analyzed slurry and soil samples.

Parameter [a]				
Soils	**RM**	**FR**	**ES**	**SG**
pH	6.1	6.0	5.9	6.2
OM (%)	11	13	27	25
NH_4 (mg kg^{-1})	231	114	576	156
PO_4 (mg kg^{-1})	35	48	50	86
Na (mg kg^{-1})	159	69	17,802	219
K (mg kg^{-1})	493	450	2244	1142
Ca (mg kg^{-1})	1343	2874	2952	4978
Mg (mg kg^{-1})	376	259	897	820
Slurries	**RM**	**FR**	**ES**	**SG**
pH	7.3	7.7	8.1	7.6
OM (%)	17	28	27	29
Total N (mg kg^{-1})	39,676	22,627	1916	56,052
NO_{3-} (mg kg^{-1})	252	399	267	390
NH_4 (mg kg^{-1})	682	169	113	2359
Total P (mg kg^{-1})	483	1022	349	1508
PO_4 (mg kg^{-1})	381	235	195	672
K (mg kg^{-1})	3991	6993	5853	9309
Ca (mg kg^{-1})	13,836	34,740	15,780	57,058
Mg (mg kg^{-1})	1774	2986	3548	4063

[a] Average values obtained from two sample replicates.

2.7. Enzyme-Inactivated ARG Community Data Analysis

The selected ARG community files were basecalled via "guppy" (Nanopore Technologies Inc., UK) and concatenated into unique ".fastq" files per sample. The quality of the reads was checked using "NanoQC" [26]. Considering the sequencing kit used, the reads were checked for concatemer formation, primers and adapters were removed, and amplicons shorter than the target amplicon length (Table S4) were removed with "NanoFilt" [26]. The sequences were then aligned to the reference sequences of each gene (*tet*X and *bla*TEM; NCBI accession numbers KF905572.1 and KT867019.1, respectively) on "minimap2" (Nanopore Technologies Inc.), and then aligned with the Comprehensive Antibiotic Resistance Database (CARD) with RGI under a protein homology model [27]. Only "Strict" hit alignments were considered in the results. The relative abundance of each gene variant was plotted using "HeatmapR".

2.8. Total Bacterial Community Sequencing Data Analyses

The 16S rRNA community sequences were analyzed with QIIME2 as ASVs using Deblur [28]. Reads with a Q $\geq$ 30 and chimera sequences were removed. Taxonomy assignment was performed with a primer set-specific (341F—805R) SILVA 138.1 naïve

Bayes-trained classifier [29]. The data were then analyzed as "phyloseq" objects in R [30]. To retain only representative ASVs, singletons, phyla with ≤15 reads, chloroplasts, and mitochondria were removed. Only ASVs present in all sequenced replicates (3) of each sample were retained.

To measure the richness and diversity within the sampled communities, as well as the differences among them, we calculated alpha and beta diversity, respectively. Alpha diversity indices (observed ASVs, "Chao 1", "1/Simpson", "Shannon", "Pielou", and "Gini") were determined with the "microbiome" function in R [31]. Statistical differences were calculated via one-way ANOVA and Tukey's HSD test. Beta diversity (weighted UniFrac PCoA and RDA) was calculated with "vegan" [32]. Sample physicochemical parameters (Table 1) and ARG abundance were used as RDA constraints, whereas the influence of sampling site or sample type was measured by PERMANOVA. Beta diversity and taxonomy data were plotted with "microViz" [33]. Finally, microbial indicator taxa were identified ($p \leq 0.01$) via linear discriminant analysis effect size (LEfSe) through "microbiomeMarker" [34].

3. Results

3.1. Physicochemical Parameters

The physicochemical parameters revealed spatial differences between slurries and soil samples and among dairy farms (Table 1). For the soil samples, the main similarity between farms was attributed to the pH, which ranged from 5.9–6.1. Despite this, the organic matter percentage (OM%) was ~2-fold greater in the ES and SG farms than in the RM and FR farms. A higher nitrogen (NH_4) content was detected in the ES farms (576 mg kg^{-1}) than in the RM, SG and FR farms (231, 156 and 114 mg kg^{-1}, respectively). For plant-available phosphorus (PO_4), the observed values were equal between the FR and ES farms (~50 mg kg^{-1}), whereas the SG and RM farms contained 86 and 35 mg kg^{-1} PO_4, respectively. Moreover, the Na^+ content in the ES farms was ~100-fold greater than that in the other three farms (from 69–219 mg kg^{-1}). The K^+, Ca^+ and Mg^+ concentrations were ~2–4-fold and ~3-fold higher, respectively, in the ES and SG farms.

In slurries, the observed pH values ranged between 7.3–8.1, with the highest value occurring on the ES farm. Similar OM percentages were obtained for the FR, ES and SG farms (~28%), whereas the RM farm presented 17%. In particular, for total N, ES contained 1916 mg kg^{-1} N, whereas the SG, RM and FR farms contained ~10× greater amounts (56,052, 39,676, and 22,627 mg kg^{-1}, respectively). A small fraction of that N was plant-assimilable N (either NO_{3-} or NH_4). Among them, NO_{3-} was higher in the FR and SG samples (~390 mg kg^{-1}), while the NH_4 concentrations were ~4–16-fold higher in the RM and SG samples. Similarly, the total P content was greater in the SG and FR farms (1508 and 1022 mg kg^{-1}, respectively) than in the RM and ES farms (483 and 349 mg kg^{-1}, respectively). Compared with total N, a greater proportion of total P was found as PO_4 in the SG and RM samples (672 and 381 mg kg^{-1}, respectively) than in the FR and ES farms. Similar to P and N, the salt content differed among all slurries, with SG slurries containing the highest amounts of K, Ca^+ and Mg^+ (Table 1).

3.2. Antibiotic-Resistance Gene Abundance

In general, decreased gene abundances were observed between rhizosphere and bulk soils compared with those in the slurry, independent of the spatial separation of the sampled farms (Figure 2, Table S4). The total bacteria (16S rRNA gene) were more abundant in the slurry samples (from 3.2×10^9 to 8.7×10^9 copies g $sample^{-1}$) than in the rhizosphere and bulk soils, ranging from 3.1×10^8 to 6.3×10^8 and from 3.3×10^7 to 1.4×10^8 copies g $sample^{-1}$, respectively (Figure 2a, Table S4). Compared with those in rhizosphere and

bulk soils, significantly ($p \leq 0.05$) greater abundances of ARG-related integrases (*intI*1 and *intI*2) in slurry samples (from 2.8 $\times 10^2$ to 7.1 $\times$ 10^4 copies g sample^{-1}) were detected (from 1.1 $\times$ 10^1 to 1.3 $\times$ 10^2 and from 2.8 $\times$ 10^1 to 1.1 $\times$ 10^2 copies g sample^{-1}, respectively) (Figure 2a, Table S4).

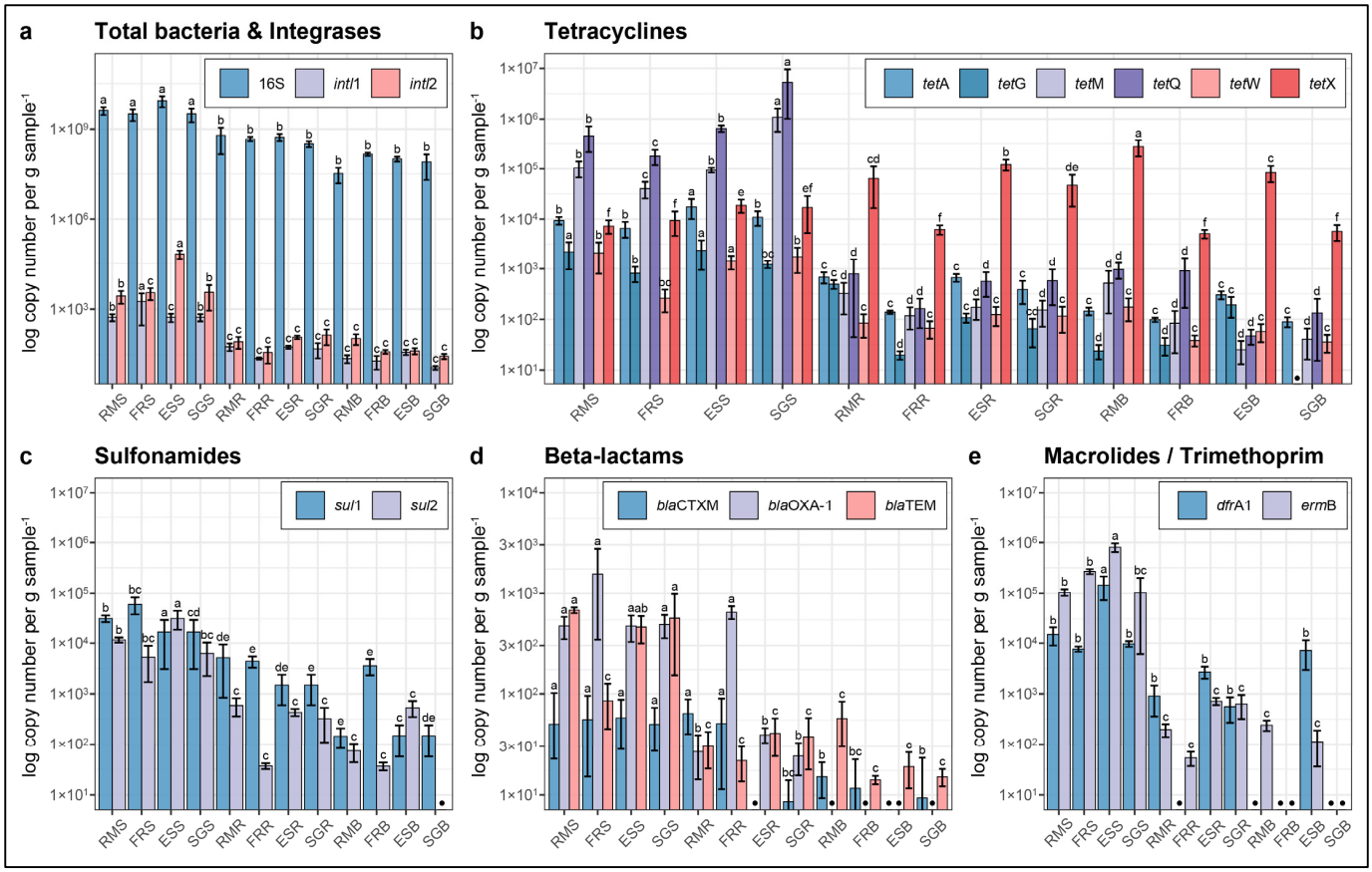

Figure 2. Absolute abundance of ARGs related to (**a**) tetracyclines, (**b**) sulfonamides, (**c**) beta-lactams, (**d**) macrolides or sulfamethoxazole–trimethoprim resistance, and (**e**), integrases. Sample names ending with S, R, and B denote slurry, rhizosphere, and bulk soil, respectively. The error bars represent the means ± standard deviations (SDs). The characters above the bars represent Tukey's HSD for each specific gene. At the axis level, the "•" symbol indicates no amplification detected by qPCR. The detailed values and statistics are available in Table S4.

With respect to ARGs, twelve of the thirteen genes assessed in this study were detected in all farms and sample types, with the exception of *bla*OXA-1 in all farm bulk soils (Figure 2, Table S4). Among the tetracycline-associated ARGs, the *tet*Q (from 1.8 $\times 10^5$ to 5.3 $\times$ 10^6 copies g sample^{-1}) and *tet*M (from 4.0 $\times$ 10^4 to 1.5 $\times$ 10^6 copies g sample^{-1}) genes were the two most abundant ARGs in slurries (Figure 2b, Table S4). These genes were followed by the *tet*A, *tet*G, *tet*W and *tet*X genes, with 10- to 1000-fold lower abundances (from 2.8 $\times$ 10^2 to 1.8 $\times$ 10^4 copies g sample^{-1}) than the *tet*M and *tet*Q genes across the sampled slurry pits. In the soil niches, the abundances of the *tet*M and *tet*Q genes decreased in the rhizosphere (from 8.0 $\times$ 10^2 to 1.2 $\times$ 10^2 copies g sample^{-1}) and bulk soils (from 9.7 $\times$ 10^2 to 2.5 $\times$ 10^1 copies g sample^{-1}) on all the sampled farms. Additionally, the *tet*A, *tet*G and *tet*W genes were not significantly different between the farm or soil niches (~10^1 to ~10^2 copies g sample^{-1}), particularly the *tet*G gene, which decreased under the detection limit for the SG bulk soil samples. In contrast, significant increases in *tet*X abundances were detected in the RM and ES rhizospheres (6.4 $\times$ 10^4 and 1.2 $\times$ 10^5 copies g sample^{-1}, respectively), as well as in the RM and ES farm bulk soils (2.8 $\times$ 10^5 and 8.4 $\times$ 10^4 copies

g sample^{-1}, respectively), compared with those detected in the slurry samples (7.3×10^3 and 1.9×10^4 copies g sample^{-1}, respectively). For the FR farm, the abundance of *tet*X was similar between the rhizosphere and bulk soils, as was the case for the slurries (from 5.1×10^3 to 9.4×10^3 copies g sample^{-1}).

Both assayed sulfonamide ARGs (*sul*1 and *sul*2) tended to decrease in rhizosphere and bulk soils (Figure 2c, Table S4). A significant ($p \leq 0.05$) decrease in *sul*1 abundance was observed in the rhizosphere (from 5.1×10^3 to 2.8×10^2 copies g sample^{-1}) and bulk soils (from 3.6×10^3 to 1.1×10^2 copies g sample^{-1}) compared with that in the slurry samples (from 1.7×10^4 to 6.1×10^4 copies g sample^{-1}). However, FR presented no differences between rhizosphere and bulk soil abundances for *sul*1 (~10^3 copies g sample^{-1}). Despite the lower *sul*2 gene abundance in the rhizosphere (from 3.7×10^1 to 5.9×10^2 copies g sample^{-1}) and bulk soils (from 1.1×10^2 to 3.6×10^3 copies g sample^{-1}), no significant differences were detected for the soil niches compared with the slurries (from 5.3×10^3 to 3.2×10^4 copies g sample^{-1}). Similar to *tet*G, the *sul*2 gene was not detected in the SG bulk soil samples.

In terms of beta-lactams, the observed ARG abundances in slurries were lower than those of the other antibiotic classes in this study (Figure 2d, Table S4). The *bla*CTX$_M$ gene revealed no differences ($p \leq 0.05$) among all slurries (~10^1 copies per g^{-1} sample). In the rhizosphere, no significant differences were detected for RM or FR on slurries (~10^1 copies per g^{-1} sample). The ES and SG rhizospheres and all the bulk soil samples presented abundances close to the detection limit. In parallel, *bla*OXA-1 was 10-fold more abundant than *bla*CTX$_M$ (~10^2 copies per g^{-1} sample), decreased in the rhizosphere (from 6.7×10^1 to 4.4×10^1 copies per g^{-1} sample) and was absent in the bulk soils of all farms. However, the FR rhizosphere did not differ from that of the slurry samples (~10^1 copies per g^{-1} sample). The abundance of *bla*TEM in slurries was 10-fold greater than that in soils (~10^2 copies per g^{-1} sample), with no difference between rhizosphere and bulk soils (~10^1 copies per g^{-1} sample).

On the other hand, larger communities were observed for macrolide- and trimethoprim-associated resistance in slurries (*erm*B and *dfr*A1 genes, respectively). Significant decreases in *dfr*A1 abundance ($p \leq 0.05$) were detected in slurries (7.0×10^4 and 8.1×10^5 copies per g^{-1} sample, respectively) with respect to the sampled soil niches (from ~10^2 to ~10^3 copies g sample^{-1}), although this gene was not detected in the FR rhizosphere. In the bulk soils, this gene was detected in only the ES farm (7.2×10^3 copies g sample^{-1}). Similarly, for *erm*B, a 1000-fold decrease was detected between slurries and the rhizosphere on all farms (from ~10^5 to ~10^2 copies g sample^{-1}, respectively), and no differences were detected between rhizosphere and bulk soil samples for RM and ES. On the bulk soil of the FR and SG farms, *erm*B was not detected.

The total abundance of ARGs related to total bacteria (16S rRNA) confirmed the observed compartmentalization between slurries and soil samples (rhizosphere or bulk soil) (Figure 3, Table S5). Therefore, four clusters based on the ARG:16S ratio were determined: Cluster 1 included *sul*2, *tet*A, *intl*2, *dfr*A1, and *sul*1; Cluster 2 (*tet*Q, *tet*M, and *erm*B); Cluster 3 included only *tet*X; and Cluster 4 (*bla*TEM, *tet*W, *intl*1, *bla*OXA-1, *tet*G and *bla*CTX$_M$). The soil niche and farm spatial separation did not influence the ARG:16S rRNA ratio in the sampled region, rhizosphere or bulk samples, which was significantly similar to the findings of Clusters 2 and 3 (Figure 3, Table S5). In general, slurries presented relatively high ratios of *tet*Q, *tet*M and *erm*B, whereas the soil sample communities were characterized by *tet*X, as represented by the Cluster 3 data. Therefore, although the RMB samples contained the largest *tet*X community in this study (≥2-fold compared with all the soil samples), they also harbored *tet*Q and *tet*M communities comparable to those observed in slurries.

Similarly, this sample also presented high ARG:16 s ratios for Clusters 1 and 4, revealing that it was the richest sample in general.

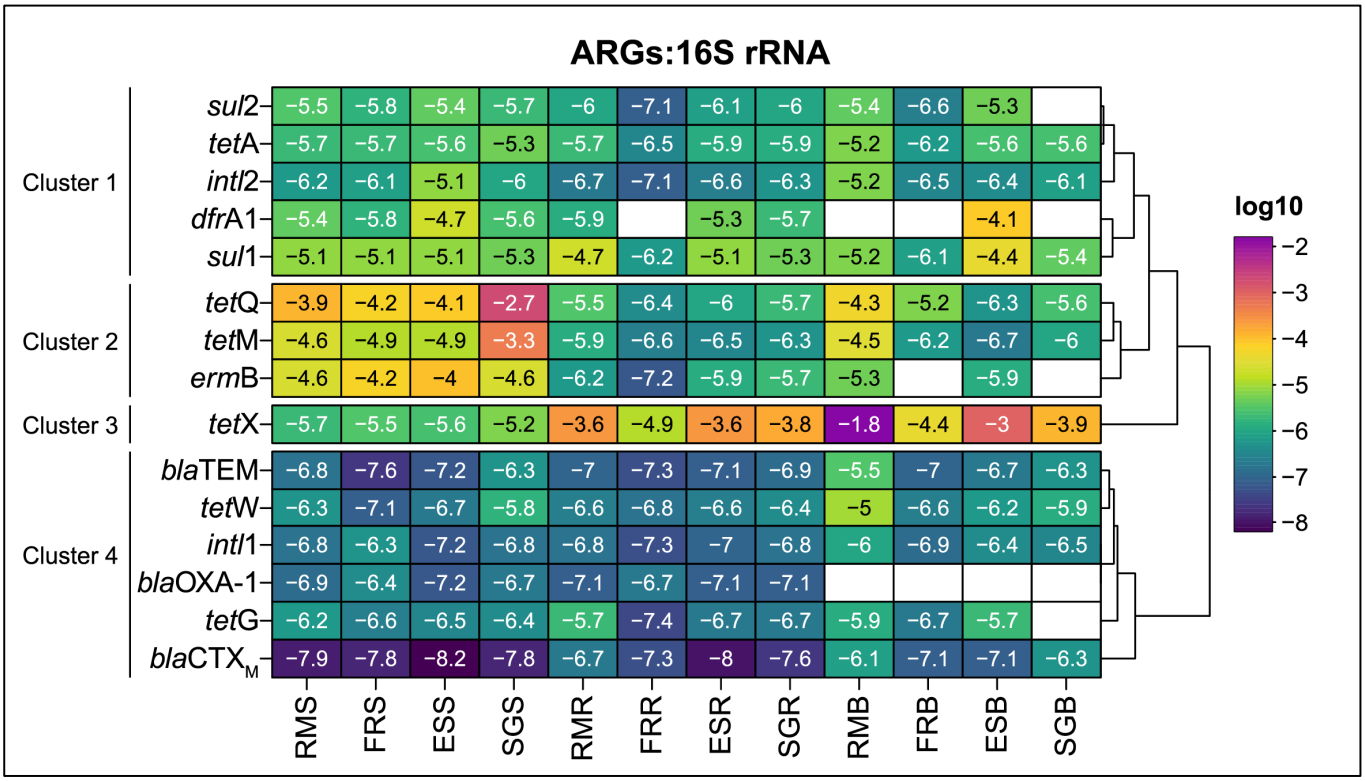

Figure 3. Relative ARGs:16S rRNA abundance ($\log_{10}$) ratio. Cluster ordination determined by Euclidian distance. Empty cells represent absence of ARG in that specific sample. Sample names ending with S, R, and B denote slurry, rhizosphere, and bulk soil, respectively. Detailed values (±SD and ANOVA) are available in Table S5.

3.3. Variant Distribution of Enzyme-Inactivated ARG Communities

The *tetX* and *bla*TEM amplicon sequences revealed similarities across all the sampled farms and sample types assessed in this study (Figure 4). In general, the distribution of both ARGs revealed either an enrichment of soils by slurry application or a stable resistome where both ARGs have been persistent over time. Independent of the absolute abundance of *tetX* measured by qPCR (Figure 4a), the relative abundances of the *tet*(X1), *tet*(X3), *tet*(X4), *tet*(X5) and *tet*(X6) variants were equal across all the samples, with the exception of the FR rhizosphere (Figure 4a). The *tet*(X3) variant represented the largest fraction of this ARG in all the samples, ranging from 46.6–51.6% total *tetX* relative abundance, whereas 26.7–33.9% and 17.7–24.6% were assigned to *tet*(X4) and *tet*(X5), respectively. Fewer than 1% of the sequences were aligned and characterized as either *tet*(X6) or *tet*(X1).

For *bla*TEM, more differences were detected, although only ~75–84% of the total *bla*TEM variant abundance could be explained (Figure 4b). The TEM-229 and TEM-116 variants were detected and clustered as the most significant in all the samples, ranging from 14.6–22.9% and from 11.3–21.5% total *bla*TEM abundance, respectively. Both variants were differentially abundant, independent of the sampled farm or sample type. For example, TEM-116 was more abundant than TEM-229 in all the RM farm samples, with an inverse trend in the SG farm samples. at SG farms. The TEM-162, TEM-102, TEM-197, TEM-157, TEM-171, and TEM-181 presented ≤10% relative abundance, except for TEM-171 in the ES and FR bulk soils; TEM-162 in the FR rhizosphere; and TEM-181 in the FR bulk soil as well as the SG rhizosphere. The remaining variants detected in this study included TEM-208,

TEM-185, TEM-139, TEM-108, TEM-1, TEM-78, TEM-205, and TEM-39, all of which had ≤5% assignations.

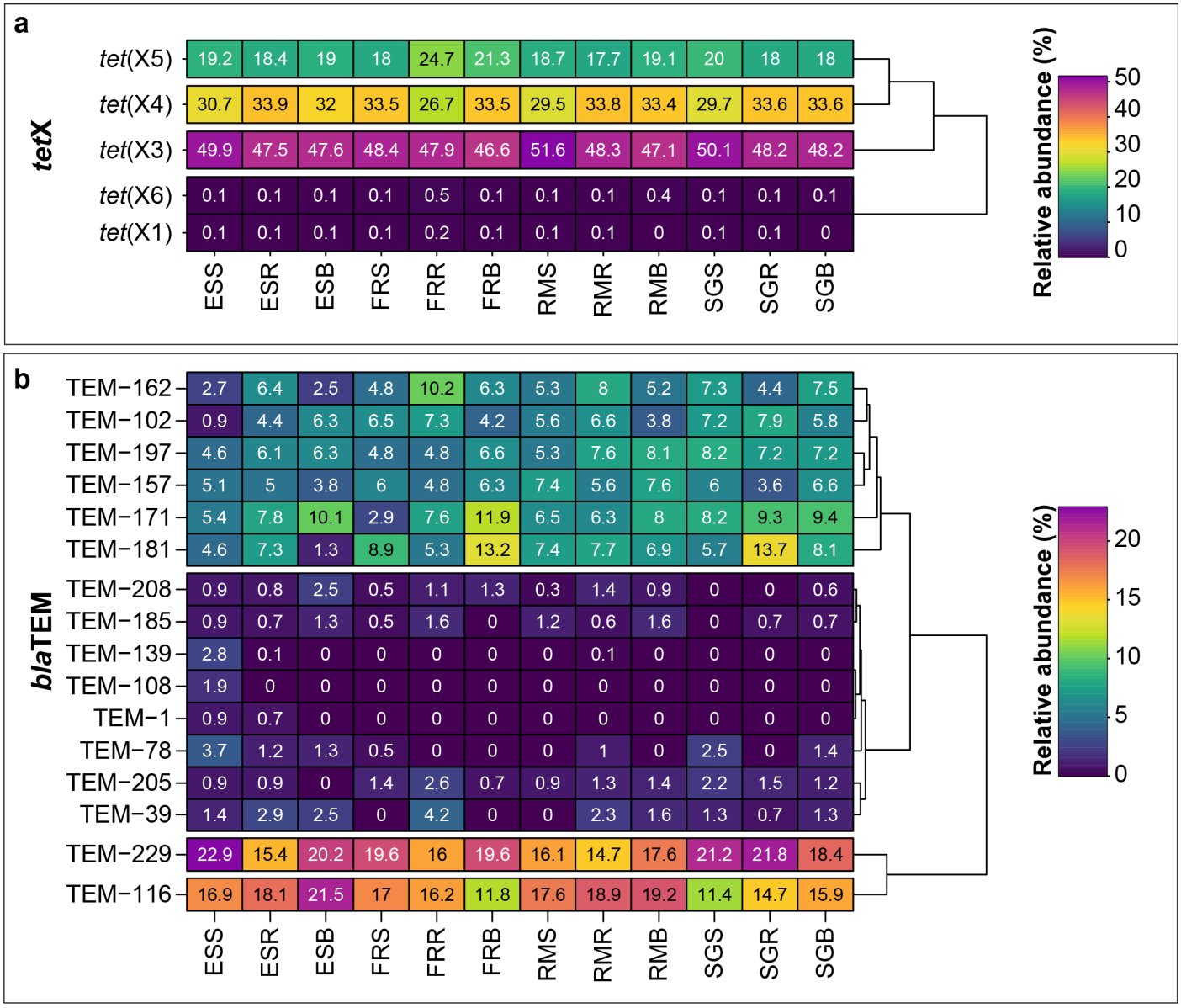

Figure 4. Relative abundance heatmap of tetX (**a**) and blaTEM (**b**) gene variants detected in the slurries, rhizosphere, and bulk soil of sampled dairy farms. Sample names ending with S, R, and B denote slurry, rhizosphere, and bulk soil, respectively. The ARG variants are clustered by Euclidean distances, and samples are arbitrarily ordered. Values inside cells represent mean relative abundance percentage (%).

For *bla*TEM, more differences were detected, although only ~75–84% of the total *bla*TEM variant abundance could be explained (Figure 4b). The TEM-229 and TEM-116 variants were detected and clustered as the most significant in all the samples, ranging from 14.6–22.9% and from 11.3–21.5% total *bla*TEM abundance, respectively. Both variants were differentially abundant, independent of the sampled farm or sample type. For example, TEM-116 was more abundant than TEM-229 in all the RM farm samples, with an inverse trend in the SG farm samples. The TEM-162, TEM-102, TEM-197, TEM-157, TEM-171, and TEM-181 presented ≤10% relative abundance, except for TEM-171 in the ES and FR bulk soils, TEM-162 in the FR rhizosphere, and TEM-181 in the FR bulk soil, as well as

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3.4. Total Bacterial Community Structure and Composition

With respect to the bacterial community of the slurries and soil samples, alpha diversity analysis revealed greater richness and diversity in the soil communities (rhizosphere or bulk soil) than in their slurry counterparts (Table 2). The slopes revealed values of Chao-1 ranging from 819.6–923.9, whereas the values in the rhizosphere ranged from 822.3–1417 and from 916.7–1266.6, with the exception of 666.7 in the FRR samples. The Shannon and Simpson indices ranged from 45.8–203.3 and from 5.1–6.0, respectively, in the slurry, whereas the values in the rhizosphere ranged from 31.6–333.6 and from 4.9–6.6, whereas those in the bulk varied from 187.6–290.2 and from 6.1–6.4, respectively. In addition, evenness (Pielou) was lower in the slurry samples (ranging from 0.8–0.9) than in the soil samples but inversely related to dominance (~0.9). Similar to those of slurries, the evenness and dominance indices were similar in the rhizosphere and bulk soils. The ES farm rhizosphere and bulk communities were identified as the most diverse ($p \leq 0.05$) in this study.

Table 2. Total 16S rRNA community alpha diversity indices.

Sample Type	Observed ASVs	Chao 1	Diversity (1/Simpson)	Diversity (Shannon)	Evenness (Pielou)	Dominance (Gini)
Slurry						
RMS	839.3 ± 8.0 Ab	844.1 ± 7.2 Ab	63.4 ± 4.8 Cc	5.35 ± 0.05 Cc	0.80 ± 0.01 Cc	0.96 ± 0.00 ABa
FRS	883.0 ± 168.1 Aab	890.8 ± 171.3 Aab	203.3 ± 2.3 A	6.02 ± 0.07 Ab	0.89 ± 0.02 Aa	0.94 ± 0.01 Cb
ESS	813.3 ± 34.3 Ab	819.5 ± 35.9 Ab	45.7 ± 0.8 Db	5.09 ± 0.03 Db	0.76 ± 0.00 Db	0.97 ± 0.00 Aa
SGS	919.6 ± 9.0 Ac	923.8 ± 8.2 Ac	80.1 ± 8.5 Bc	5.71 ± 0.04 Bc	0.8 ± 0.00 Bb	0.9 ± 0.00 BCa
Rhizosphere						
RMR	810.6 ± 36.0 Cb	822.3 ± 37.5 Cb	111.8 ± 4.6 Cb	5.65 ± 0.03 Cb	0.8 ± 0.00 Cb	0.96 ± 0.00 Aa
FRR	659.0 ± 10.5 Cb	666.7 ± 11.1 Db	31.6 ± 4.6 Dc	4.84 ± 0.08 Dc	0.75 ± 0.01 Cb	0.97 ± 0.00 Aa
ESR	1196.6 ± 65.9 Ba	1212.0 ± 66.1 Ba	283.1 ± 27.5 Ba	6.39 ± 0.10Ba	0.90 ± 0.01 Aa	0.91 ± 0.01 Bb
SGR	1398.0 ± 87.7 Aa	1417.0 ± 87.7 Aa	333.5 ± 15.2 Aa	6.60 ± 0.05 Aa	0.91 ± 0.00 Aa	0.89 ± 0.00 Cc
Bulk soil						
RMB	911.3 ± 28.0 Ca	916.7 ± 27.7 Ca	187.5 ± 2.4 Ca	6.10 ± 0.03 Ba	0.91 ± 0.00 Aa	0.93 ± 0.00 Aa
FRB	1084.0 ± 29.0 Ba	1096.6 ± 35.0 Ba	230.9 ± 5.9 Ba	6.2 ± 0.01 Ba	0.89 ± 0.00 Aa	0.93 ± 0.00 ABc
ESB	1250.6 ± 68.7 Aa	1266.6 ± 67.7 Aa	290.1 ± 26.3 Aa	6.44 ± 0.10 Aa	0.90 ± 0.10 Aa	0.91 ± 0.01 Cb
SGB	1240.3 ± 37.4 Ab	1254.5 ± 33.1 Ab	289.5 ± 8.7 Ab	6.44 ± 0.02 Ab	0.90 ± 0.00 Aa	0.91 ± 0.00 BCb

Values after ± symbol represent mean standard deviation (SD). Uppercase characters after each value represent statistical differentiation within sampling sites for that specific niche on Tukey HSD test ($p \leq 0.05$). Lowercase character after each value represents statistical differentiation among all samples on Tukey HSD test ($p \leq 0.05$).

Beta diversity revealed noticeable differences in the bacterial community structure between both sample types and sampling sites, as revealed by MDS analysis (Figure 5a). In this analysis, the MDS1 and MDS2 axes explained 72.8% of the variability in the bacterial communities ($p \leq 0.05$); in particular, the sample type was more relevant than the sampled farm (Figure 5a). This was confirmed via PERMANOVA, with $R^2 = 0.64$ and 0.11, for sample type vs. sampled farm ($p \leq 0.05$) (Table S3). When total physicochemical parameters were used as constraints for redundancy analysis (RDA), the variance explained was reduced to 61.8% and 20.5% in the slurries and soil samples, respectively (Figure 5b,c). However, the bacterial structures present in the slurries and soil samples included OM (0.97 and 0.42%), pH (0.72 and 0.30) and NH_4 (0.54 and 0.30 mg kg^{-1}). When all samples and ARG abundances were considered, the RDA revealed that they explained 66.7% of the community variance, with a particular relationship to the slurry samples. These findings suggest that both chemical parameters and ARG abundance are not relevant for these bacterial community structures. However, the RDA confirmed the observed significant relationship of the *tet*X gene with the soil communities (specifically with the bulk soil), whereas the remaining significant genes (*bla*TEM, *bla*OXA-1, *bla*CTX_M, *tet*A, *tet*G, *tet*M, *tet*Q, *sul*1, and *sul*2) were related to the slurry communities (Figure 5d).

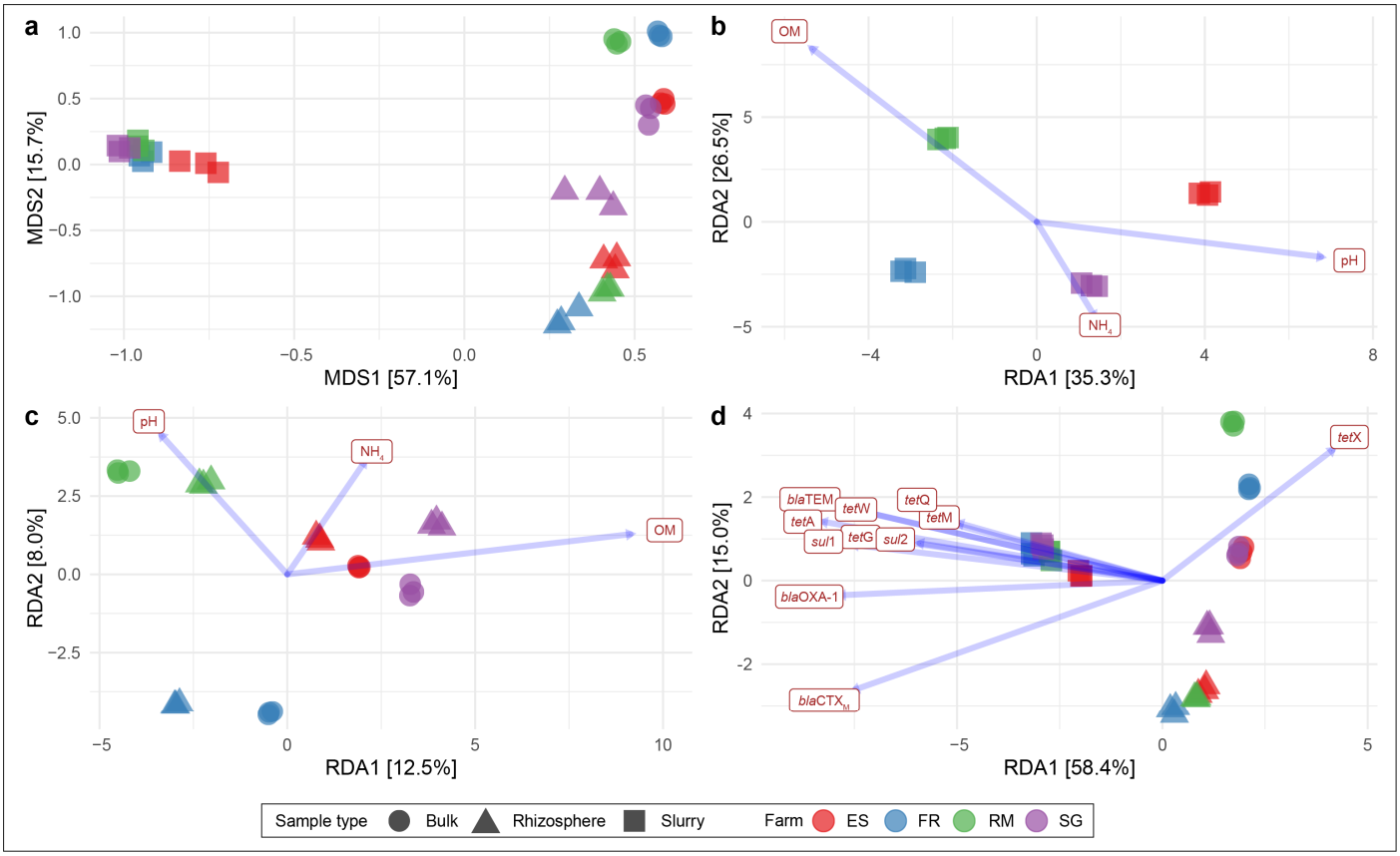

Figure 5. Bacterial community structures represented by beta diversity: (**a**) all samples' PCoA calculated with "unifrac" distances, (**b**) slurry, and (**c**) soil samples RDA with physicochemical parameters as constraints (Table 1); and (**d**), all samples RDA with ARG abundance as constraints. Significant ($p \leq 0.05$) constraints are plotted in figure. Axis values represent explained variance percentages for each plot ($p \leq 0.05$).

Taxonomic analysis of the composition of bacterial communities revealed similar patterns of phyla between slurries and soil samples but different relative abundances according to sample type (Figure 6a,b). On average, the dominant phyla in slurries were identified as Bacillota (a.k.a. Firmicutes) (from 32.8–51.6%), Bacteroidota (from 13.7–37.7%), Pseudomon-

adota (a.k.a. Proteobacteria) (from 6.3–29.5%) and Actinomycetota (a.k.a. Actinobacteria) (from 2.6–13.4%). A notably greater abundance of Proteobacteria (32.2%) was detected in the ES farm than in all the slurry samples. Similarly, Bacteriodota and Actinobacteria were more abundant in slurry samples from RM (13.4%) and FRS (37.7%), while those from SG farms were 29.7% and 8.5% more abundant, respectively. In contrast, Proteobacteria was the most abundant taxon in the rhizosphere samples, ranging from 30.6–45.6%, independent of farm. This phylum was followed by Actinobacteria (from 11.6–41.0%), Bacteroidota (from 14.6–29.9%), and Firmicutes (from 2.8–9.8%). For Actinobacteria and Bacteroidota, an inverse relationship was detected between RM and FR and ES farms and SG farms. In the bulk soils, Actinobacteria (from 21–35.2%), Proteobacteria (from 18.1–24.2%), and Acidobacteria (from 9.2–20.5%) were the most dominant phyla, followed by Bacteroidota (from 4.0–13.5%) and Firmicutes (from 1.0–9.4%) (Figure 6a). In contrast to those observed in the rhizosphere, Acidobacteria were ~2-fold more abundant in RM and FR. In addition, WPS-2 was detected in only the RM farm, representing ~10% of the total bacterial community.

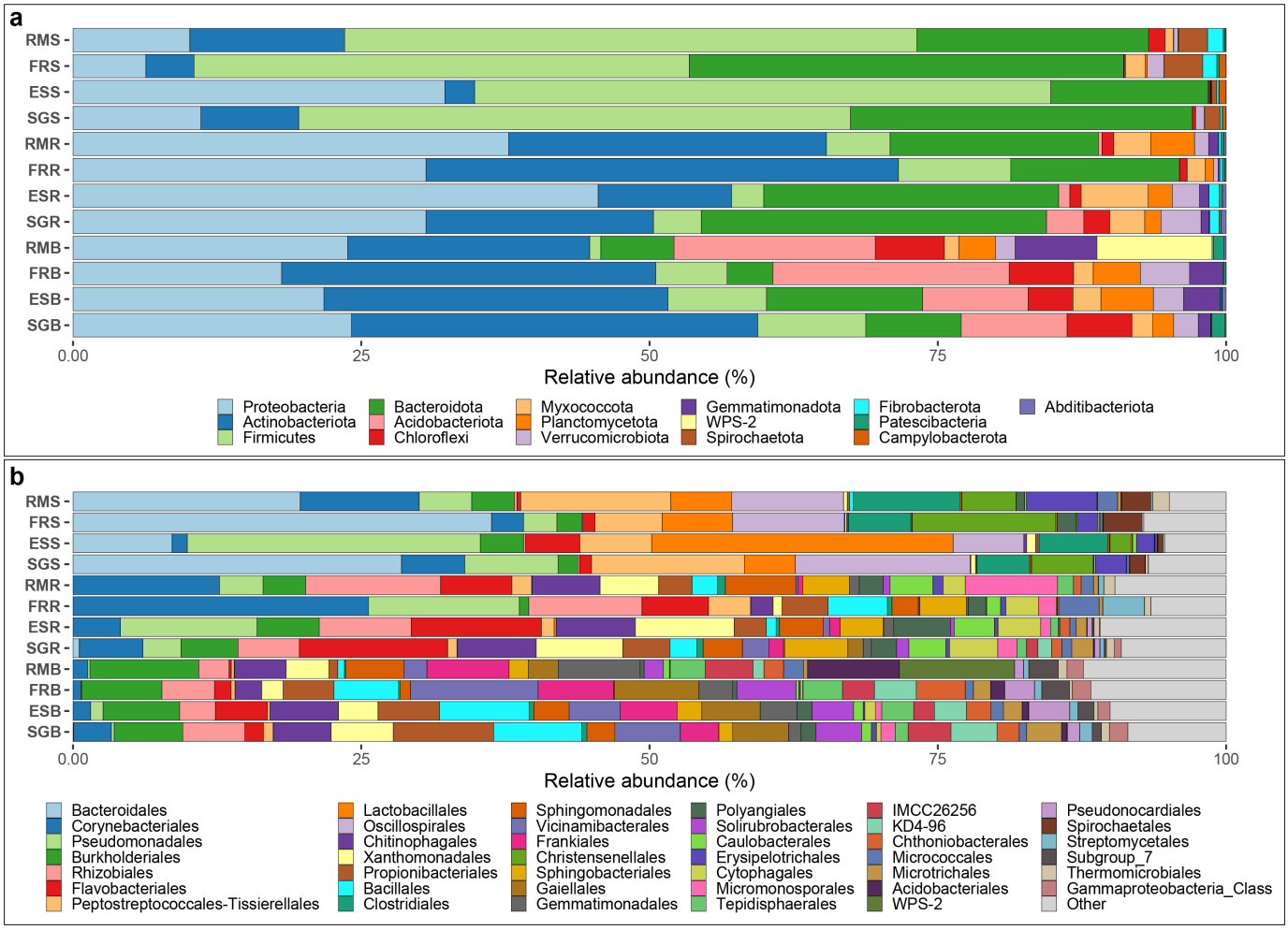

Figure 6. Taxonomic assignments of relative abundance at the (**a**) phylum and (**b**) order levels. "Other" represents taxa under 0.05% relative abundance toward total ASV assignments. Sample names ending with S, R, and B denote slurry, rhizosphere, and bulk soil, respectively. More detailed abundance values are available in Supplementary File S1.

Despite sharing the main phyla, the application of slurries did not enrich the soil bacterial communities with Firmicutes enteric-associated taxa (Figure 6b). These phyla were dominated by the *Lactobacillales* (from 4.4–26.2%), Oscillospirales (from 6.1–15.2%) and Peptostreptococcales–Tissierellales (from 5.8–13.3%) orders. In the rhizosphere, how-

ever, it was represented mainly by Bacillales (from 0.9–5.1%), and Peptostreptococcales-Tissierellales bulk soils, only Bacillales (0.6–7.8%) were detected. Despite the higher abundance of Bacteroidota observed in slurries, bacteria from this phylum did not colonize the rhizosphere or the bulk soils in this study. In slurries, this phylum was represented mainly by the Bacteroidales order (from 8.6–36.3%), and soil samples were absent. In rhizosphere and bulk soils, it was diversely represented by Flavobacteriales (from 0.2–12.9%), Chitinophagales (from 1.9–6.9%) and Sphingobacteriales (from 1.2–5.5%). Among Actinobacteria, Corynebacteriales was the most dominant order, ranging from 1.3–10.3% in slurries and from 4.1–25.6% in the rhizosphere, whereas in bulk soils its abundance decreased from 3.3% to 0.7%. Finally, the slurry samples were also dominated by members of the order *Pseudomonadales* (2.9–25.4%); however, their relative abundance decreased from 13.1% to 3.3% and 1.1% to 0.1% in the rhizosphere and bulk soils, respectively (Figure 5b). A general increase in *Rhizobiales* (2.6% to 11.7%), *Xanthomonadales* (0.8% to 8.6%), *Sphingomonadales* (0.9% to 6.1%) and *Burkholderiales* (0.8% to 9.5%) was observed for both sample types (Figure 6b).

All the previously stated taxonomic differences were statistically confirmed at the genus level via linear discrimination analysis effect size (LEfSe) (Table 3). For all sample types, differentially abundant taxa presented LDA scores ≥ 4. In slurries, the main discriminant taxa were associated with Firmicutes, represented by *Romboutsia*, *Christensenellaceae* ASVs, *Turicibacter*, *Proteiniclasticum*, and *Clostridium*. *Acinetobacter* (Proteobacteria) and *Corynebacterium* (Actinobacteria) also differed in abundance. Rhizospheres were characterized by differential abundance of *Devosia*, *Pseudomonas*, and *Pedobacter* for Proteobacteria and *Mycobacterium* and *Micromonospora* for Actinobacteria. Similar to the diversity indices and taxonomic distributions, the bulk soils presented greater diversity of differentially abundant taxa, as observed at the phylum, order, and genus levels. Among those, thirteen ASVs were significant (Gaiellales_order, IMCC26256, *Acidothermus*, 67-14, Vicinimabacteriales_Order, Acidobacteriales_Order, *Xanthobacteraceae*_Family, SC-I-84, *Bacillus*, KD4-96, WD2101_soil_group, Candidatus_*Udaeobacter* and *Gemmatimonas*).

Table 3. Differential abundance microbial taxa indicators per sample type.

Phylum	Order	Genus *	LDA Score	*p* Value
Slurry				
Firmicutes	Clostridiales	Christensenellaceae_R-7_group *	4.42	5.17×10^{-7}
	Clostridiales	Clostridium_sensu_stricto_1 *	4.21	3.39×10^{-6}
	Clostridiales	*Proteiniclasticum*	4.12	5.30×10^{-7}
	Clostridiales	*Romboutsia*	4.36	3.22×10^{-6}
	Erysipelotrichales	*Turicibacter*	4.09	2.57×10^{-6}
Proteobacteria	Pseudomonadales	*Acinetobacter*	4.59	3.03×10^{-6}
Actinobacteria	Corynebacteriales	*Corynebacterium*	4.31	1.60×10^{-5}
Rhizosphere				
Proteobacteria	Hyphomicrobiales	*Devosia*	4.30	1.02×10^{-6}
	Sphingobacteriales	*Pedobacter*	4.14	5.15×10^{-6}
	Pseudomonadales	*Pseudomonas*	4.25	2.12×10^{-6}
Actinobacteria	Actinomycetales	*Micromonospora*	4.20	7.86×10^{-6}
	Actinomycetales	*Mycobacterium*	4.25	2.06×10^{-6}

Table 3. *Cont.*

Phylum	Order	Genus *	LDA Score	*p* Value
Bulk soil				
Actinobacteria	Gaiellales	Gaiellales_Order *	4.34	8.66×10^{-7}
	Actinobacteriales	IMCC26256	4.24	5.30×10^{-7}
	Acidothermales	*Acidothermus*	4.22	6.19×10^{-7}
	Solirubrobacterales	67-14	4.08	8.81×10^{-7}
Acidobacteria	Vicinamibacterales	Vicinamibacterales_Order *	4.39	4.92×10^{-7}
	Acidobacteriales	Acidobacteriales_Order *	4.08	4.92×10^{-7}
Proteobacteria	Hyphomicrobiales	Xanthobacteraceae_Family *	4.17	1.10×10^{-7}
	Burkholderiales	SC-I-84	4.07	3.24×10^{-7}
Firmicutes	Bacillales	*Bacillus*	4.38	6.62×10^{-6}
Chloroflexi	KD4-96	KD4-96 **	4.22	1.98×10^{-6}
Planctomycetota	Tepidisphaerales	WD2101_soil_group *	4.12	3.68×10^{-7}
Verrucomicrobiota	Chthoniobacterales	Candidatus_Udaeobacter *	4.07	5.30×10^{-7}
Gemmatimonadota	Gemmatimonadales	*Gemmatimonas*	4.02	5.36×10^{-7}

* Taxa represents a single ASV, but only classified to this level according to EMPO-trained 16S SILVA database 139.1. ** Taxa described only at phylum level in literature.

4. Discussion

A total of thirteen ARGs were detected in slurries, rhizosphere and bulk soil samples from four dairy farms in the Los Lagos region, Chile. Only *bla*OXA-1 was detected in all the bulk soils, whereas *tet*G and *dfr*A1 were not detected in the SG farm soil samples (Figure 2; Table S4). Since approximately 2010, recurrent studies have investigated farm antibiotic residues and ARB and ARG transfer into soil worldwide [13,35–37]. In Chile, however, studies aiming to determine the impact of environmental resistomes and antimicrobials have focused mainly on their impact on marine ecosystems as a consequence of salmon production, where high antibiotic inputs are needed [38]. This is concerning at the local level, as cattle and poultry production also demand large volumes of these drugs [39]. Few reports have explored ARGs in domestic and wild animal feces and Antarctic soils as contamination indicators [40–42]. In general. Our results revealed that the slurries harbored the greatest number of ARG copies per g^{-1} sample at all farms, with decreasing absolute abundances related to soil depth (slurry > rhizosphere > bulk soil), as observed by McKinney et al. (2018) [13]. This decrease, however, was not linear for every ARG, as the results revealed no significant differences between rhizosphere and bulk soils (e.g., *tet*A, *tet*Q, *tet*M, *erm*B, *bla*TEM) or between ARG-related integrases (*intl*1, *intl*2) (Figure 2; Table S4). For certain ARGs, similar numbers to the slurry abundances were observed (*bla*OXA-1 in the FR farm rhizosphere and *sul*1 plus *dfr*A1 in the ES farm rhizosphere and bulk soil) (Figure 2, Table S4). Despite these findings, the increase in ARGs derived from manure- or dairy slurry-fertilized farms is considered a vector for the transfer of ARGs into food production systems [43]. For example, the application of swine manure and dairy slurries to tomato, carrot, radish, cucumber, pepper, and lettuce increased the number of ARGs detectable in both the soil and plant microbiomes at harvest [44]. In terms of relative abundance, some ARGs assessed in this study were similar to those reported in other studies. Under 'animal-derived' organic fertilization, Qing et al. (2022) [45] reported ~10^{-4} copies of *tet*X:16S rRNA in soils, similar to our results. In contrast, their results also revealed a spike in the relative abundances of *tet*M and *intl*1 (~10^{-4} and 10^{-2}, respectively), which we did not observe. In comparison with other soil types, only RM farm bulk soils were similar to slurries in terms of ARG relative abundance (harboring comparatively large proportions of *tet*X, *tet*Q, *tet*M, and *tet*W; Figure 3). For other ARGs (such as *bla*), relatively low abundance has also been reported in environmental samples, with a value of 10^{-6}

in arctic and agricultural soils [46]. In addition, our observed relative abundance of *sul*1 and *sul*2 is similar to that reported in soils after 40 years of manure application to soils (~10^{-4}–~10^{-5}) [47]. Hence, the copy numbers observed in our study are consistent with ARG abundance naturally occurring in the environment.

On the other hand, tetracycline ARGs were noticeably different between the slurries and the analyzed soils. *tet*M and *tet*Q were more abundant in slurries, whereas *tet*X abundance was notably greater in soils (Figures 2b and 3). We observed that the total *tet*X abundance in the rhizosphere and bulk soils was equal to or greater than that in their slurry-associated samples (Figure 2b; Table S4). This revealed a differentiation in the dominant resistance mechanism present in both sample types, as *tet*M and *tet*Q are described as ribosomal inactivation proteins, whereas *tet*X has been categorized as an enzymatic inactivation protein [48]. Thus, selection has occurred with respect to which resistance mechanisms are transferred to the soil communities [47]. Additionally, this incremental trend in soil *tet*X abundance vs. the applied slurries was not observed for the other ARGs measured in this study.

Considering their ARG abundances (increasing and decreasing in abundance with increasing soil depth), we assessed two enzymatic-inactivated ARGs: *tet*X and *bla*TEM. Both revealed a homogeneous variant distribution across all the samples (Figure 4). The three dominant variants of *tet*X [*tet*(X3), *tet*(X4) and *tet*(X5)] represented up to ~99% of the total *tet*X. These three variants have been described as derived from *Acinetobacter* spp. (common ARG-carrying bacteria present in slurries and clinical environments), and *Enterobacteriaceae* isolates from animal farms (both in our slurries and rhizosphere samples; Figure S4) are plasmid transferable and confer resistance to tigecycline, omadacycline, and eravacycline, which are all last-generation tetracyclines [49]. These variants have already been described as being present in the food chain in China and are strongly related to insertion sequences in lettuce [50]. For the *bla*TEM variant distribution, TEM-229 and TEM-116 were the most relatively abundant variants, accounting for ~40% of the total *bla*TEM abundance, followed by TEM-162, TEM-102, TEM-197, TEM-157, TEM-171, and TEM-181. In this regard, TEM-116 was described as derived from TEM-7, and modifications were also present in TEM-139 (scarcely observed only in ESS and ESR samples from this study; Figure 4b) [51]. Likewise, TEM-229 has evolved from TEM-116 and is able to degrade last-generation beta-lactams belonging to the carbapenem and cephalosporine families [52]. Both variants, considered 'extended-spectrum beta-lactamases (ESBLs)', have been found in urban wastewaters, are reportedly plasmid transferred, and are present in both environmental and clinical contexts [52,53]. In our opinion, as all sampled farms presented a similar distribution of these ARG variants for both genes independent of sample type, we consider that these soil resistomes have been enriched or modulated by the application of dairy slurries.

All sample types presented differences in bacterial community diversity indices (Table 2, Figures 5 and 6). The alpha diversity indices revealed an increase in diversity with increasing soil depth, which was consistent with the findings of other studies. Slurry diversity and richness are dominated by selection in the cattle rumen [54]. Similarly, manure fertilization reduces soil bacterial diversity [6]. On the other hand, plants also select their rhizobacterial constituents, thus reducing diversity, as they recruit bacteria that supply nutritional components [55]. We found this compartmentalization among the three sample types. However, when only the soil samples were considered for RDA, the rhizospheres were more related to their respective bulk soils than to the other rhizospheres (Figure 5b,c). In similar ARG studies, compartmentalization was observed by Rovira et al. (2019) [56], where PCoA and ANOSIM confirmed the same differentiation between manure, soil, and wastewater. This phenomenon has been defined as a trend by nature and involves bacterial communities and resistomes of swine [57], and poultry farms [58]. We identified only pH,

OM and NH_4 as significant variables for both the slurry and the soil samples via RDA. In the soil sample RDA, the variance explained was reduced when slurry data were removed from the analysis, suggesting that the significant constraints were insufficient to explain the assessed community structures (Figure 5c), which was also reported by Zhu et al. (2013) [57]. On the other hand, the use of ARGs as RDA constraints revealed a significant association with slurries, which, in our opinion, was related to the larger copy numbers measured in those slurries, as the only abundant ARG in soils (*tet*X) was significantly related to soil samples. Similarly, (and similar to physicochemical parameters), ARGs did not significantly increase the variance explained by the community structure (Figure 5a,d), which we consider to be regulated by the sample nature (Table S3).

In terms of bacterial composition, differences were observed, dominated by sample type and followed by farm type (Figure 6). The dominant phyla in all the samples were identified as Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidota, which are common in soils and slurries. For slurries, the relative abundances of these four phyla revealed that the samples collected were fresh slurries, as observed by Wang et al. (2018) [59] and Zalewska et al. (2024) [60]. With longer residence times in slurry pits, changes in the phyla distribution are reported, as we observed larger proportions of Proteobacteria in ES farm slurries. At deeper taxonomic levels, differences were clearly established, as all three sample types revealed specific community members (such as *Lactobacillales* in slurries; *Pseudomonadales* and *Flavobacteriales* in the rhizosphere; and *Frankiales* or *Vicinimabacteriales* in bulk soils; Figure 6b), as evidenced in Sukhum et al. (2021) [61].

In particular, for Bacteroidota, *Bacteroidetes* spp. (the main representative taxa of this phylum in the RM FR and SG slurries; Figure S4) presents a high risk of ARG transfer to other bacteria. This intestinal genus has been described as multiresistant, with a special emphasis on carbapenems [62,63]. However, we did not detect its presence in the rhizosphere samples. In contrast, large proportions of *Flavobacterium* (~25%) were detected. For those, multidrug efflux pumps confer resistance to molecules associated with beta-lactams, aminoglycosides, and phenicols [64]. For Firmicutes, the *Romboutsia* genus (habitants in soil, sediment, and the intestinal tract of mammals) was found in all the samples and constitutes a potential source of ARG transfer mediated by plasmids and integrons [59]. *Clostridium* spp. (observed in large proportions in our slurry samples and known for their ubiquity in the rumen) also represent a resistome enrichment risk [65] because of their ability to adapt quickly to antibiotic pressure and their clinical relevance. Similarly, *Turicibacter* (present in all samples) has been reported to quickly reduce ciprofloxacin concentrations in soil environments [66], a potential biotechnological alternative to reduce antibiotic contamination; however, this is a biosafety risk due to the transfer of ARGs to other species.

For Proteobacteria, *Acinetobacter* spp. were abundant in all slurry and rhizosphere samples (Figure S4). This genus, also considered a notorious ARB in clinical contexts, is a component of rumen communities and a source of *tet*X dissemination ([50]. However, *Acinetobacter* is also considered a common inhabitant of soils and a colonizer of earthworm feces [67].

Despite the presented data, there is no information available in a public Chilean survey examining the environmental ARGs available to report and contrast our results. Further research is needed to assess and compare ARG profiles in other animal production systems, such as swine and poultry, which are prevalent in Chile. This is crucial, as several clinically relevant bacterial genera were identified in this study.

5. Conclusions

In this study, we observed a modulation or enrichment of farm resistomes driven by the application of dairy slurries as fertilizer, considering the established differences

by sample type and sampled farms. Despite the observed taxonomical and structural differences, this modulation was confirmed by the ubiquitous distribution of both *tet*X and *bla*TEM variants across all our samples on farms considerably separated by distance. Under our scope, the high abundance of *tet*X in rhizosphere and bulk soils must be carefully monitored, as this ARG constitutes a risk for clinical contexts at several latitudes. This study constitutes the first Chilean approximation comprising the impact of the application of raw dairy slurries as fertilizer. Considering the aforementioned, we consider that local monitoring plans should be established with consecutive surveys that deeply assess the impact of practices such as dairy slurry application to prairies to properly fulfill the targets established by the One Health strategy in Chile.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15020397/s1, Table S1: Primer sets, sequences and conditions used in this study; Table S2: qPCR standard curve sequences synthesized in this study; Table S3: Variance explanation of the sampled microbial communities; Table S4: Total log ARGs copy abundances observed in this study; Table S5: Relative abundance between ARGs and total bacteria (log ARG:16S rRNA copies per g^{-1} sample) observed in this study; Figure S1: qPCR standards specificity amplification. First and last wells loaded with 1000 bp ladder; Figure S2: Linearized standard curves for ARGs assayed in this study. X axis represents log value, whereas Y axis corresponds to obtained Ct values; Figure S3: Taxonomic assignation at top genus level for (a) Firmicutes, (b) Bacteroidota, (c) Actinobacteria, and (d) Proteobacteria; Figure S4: Principal component analysis (PCA) for (a) Firmicutes, (b), Bacteroidota, (c) Actinobacteria, and (d) Proteobacteria; Supplementary File S1: Relative abundance percentage for all 16S rRNA communities explored in this study.

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