Supplemental Material

Fertilizing with Animal Manure Disseminates Antibiotic Resistance Genes to the Farm Environment

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Materials and Methods

DNA isolation, handling and quality control

DNA was isolated from the soil and manure samples with the PowerLyzer PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA USA) according to the manufacturer's protocol. A soil kit negative control was produced by performing one of the isolations without a sample to check for cross-contamination during the procedure. The DNA was stored at -20°C in aliquots before the qPCR assays. A single aliquot per sample was used for both the DNA measurement and screening phases to reduce the amount of freeze-thaw cycles of the samples. The DNA concentrations of the samples were measured at 260 nm with the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) and the purity was confirmed by comparing the 260 nm / 280 nm ratios of the samples.

Selection of ARGs and Inhibition test

β-lactams, sulfonamides and tetracyclines are the three most commonly used antibiotics in Finnish animal production. Thus, we chose genes encoding resistance for antibiotics of each these antibiotic classes. Among β-lactamases, we focused to carbapenemases.

The amount of qPCR inhibition in the samples was evaluated by spiking them with a known amount of a standard, which was a PCR product of Rattus norvegicus Glyceraldehyde 3-phosphate dehydrogenase (GADPH) mRNA cDNA, donated by Dr. Jere Lindén, Faculty of Veterinary medicine, University of Helsinki, Finland. The spiking standard was amplified with PCR using the GAPDH-F (5'-ATC CCG CTA ACA TCA AAT GG-3') and GAPDH-R (5'-GTG GTT CAC ACC CAT CAC AA-3') primers. The PCR conditions were as follows: denaturation at 98°C for 30 s,
followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 64°C for 10 s and extension at 72°C for 15 s, after which final extension was performed at 72°C for 10 min before cooling to 4°C until the samples were preserved at 4°C before further experiments. The amount of template was unknown, since the volume was too low to measure. The size of the product was verified by electrophoresis. To check for non-specific amplification or possible contamination with the PCR product, pooled sample was produced by combining 1 µL from all of the biological replicate samples originating from each of the farms. The approximate amounts of template DNA in the reactions were T1: 16.4 ng, T2: 43.1 ng, T3: 24.3 ng and T4: 24.6 ng. The pooled farm samples were used as templates in PCR amplification with the GAPDH-F and GAPDH-R primers, with identical conditions as used in the ARG amplifications in annealing temperature of 64°C.

The inhibition test qPCR reactions were performed in a volume of 13 µL in 1 x DyNAmo SYBR Green qPCR Kit Master Mix (Thermo Fisher Scientific), which included the buffer, enzyme and dNTPs, with concentrations of 0.5 µM forward and reverse primers. 104 copies of the spike and 1 µL of the template (3 -10⁹ ng DNA) were included in the final volume. A standard series was constructed with 10¹–10⁸ copies of the spike. The negative control wells were not spiked.

Quantification standards for qPCR

The quantification standard for the 16S rRNA gene was a 1542 bp fragment from *E. coli* inserted in pUC57, which was obtained from Genscript USA Inc. (Piscataway, NJ. USA). The standard used for the bla<sub>OXA-58</sub> gene was a 152 bp insert in pUC19 and the standard for tet<sub>M</sub> gene was a1336 bp insert in pDRIVE. To linearize them, the 16S rRNA, bla<sub>OXA-58</sub> and tet<sub>M</sub>-plasmids were respectively cut with the restriction enzymes BamHI, BamHI and SmaI (Thermo Fisher Scientific) according to the manufacturer's protocol. New primers, sul1-R388-31627f (5'-GGA AGA ATG ACG CCT AAC CG-3')
and sul1-R388-33725r (5'-GGT TGA TGA TGG AAG TGG AGG-3'), were designed using the sequence for the E. coli r388 plasmid (Genbank: BR000038.1) to amplify a 2119 bp fragment encompassing the sul1 gene, which was to be used as a standard for this ARG. It was amplified by conventional PCR using the concentrations and conditions described previously, with an annealing temperature of 66°C and template DNA amount of approximately 0.6 ng. Linearization of the standards was verified by electrophoresis and the standards were purified with a Qiaquick PCR purification kit (Qiagen, Hilden, Germany). The DNA concentrations of the standards was measured at 260 nm with a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and the standard copy numbers per 1 µL were calculated using the estimated molar mass for the DNA bp (650 Da/1 bp).

The standard series were done with 10¹ – 10⁸ copies (8 data points) of the respective standard, except for two of the 16S rRNA runs, which had a range of 10² – 10⁹ copies (8 data points). In all of the runs were included 6 No Template Controls (NTC) and in addition soil kit negative controls were also analyzed. The specificity of the ARG assays was confirmed by purifying the PCR products from a single farm sample per gene with Qiaquick PCR purification kit (Qiagen, Hilden, Germany), sequencing them and comparing the sequences with NCBI BLAST.

Raw qPCR data analysis

The ARG and 16S rRNA gene data was preliminarily analyzed with the CFX Manager Version 3.0.1224.1015 (Bio-Rad Laboratories). High-resolution melting curve analysis was used to screen the amplicons. The E and R² values were verified to be within the acceptable limits of 90 % < E < 110 % and R² > 0.990. To reach these criteria, one outlier per data point in the standard could be removed from the triplicate, or the data point had to be removed completely and a minimum of 3
data points had to be valid to make up the standard curve. The lowest acceptable standard data point was defined as the one that was at least 3.3 cycles from the lowest Quantification cycle (Cq) of the 6 No Template Controls (NTCs) in the run. Limit of detection (LOD) was defined as the highest Cq-value of the accepted standard with the lowest copy number (10 copies). If the NTCs had only a primer-dimer peak as seen from the melting curve, the lowest otherwise acceptable standard point could be used for that run. In addition to the NTCs, also all of the other technical replicates melting curves were analyzed manually to see if the right product was amplified. The primary product peak in the melting curve was defined as the lone, sharp peak as seen in the standard samples. The primer-dimer peak was defined as the peak seen in the NTCs (if there was a primer-dimer). The technical replicates were then discarded on the basis of following conditions: 1) If the primary product peak was missing; 2) If the melting curve of a technical displayed a major secondary peak (not the primer-dimer peak) in addition to the primary product peak; 3) If the primary product peak was not sharp, but spread over a temperature range; 4) If the primary product peak was not much larger (about 2/3) than the primer-dimer peak. The data from the CFX Manager was exported for further analysis as a *.csv file including the well number, sample identifier, Cq value and the copy number as read from the standard curve.

Statistical methods: Linear mixed models

To compare the changes in the amounts of the resistance genes across samples, the copy numbers were first normalized to the copy number of the 16S rRNA genes. For the 16S rRNA gene the weighted averages for each of the biological replicates was calculated from the log10-transformed copy numbers by using the standard deviations of the qPCR runs as weights. Raw copy numbers of ARGs for the Linear Mixed Models (LMM) were handled by calculating the weighted averages, as described with 16s rRNA gene. For the LMMs, normalization of ARG copy numbers
was done by dividing the weighted average copy number of a biological replicate with the corresponding 16S rRNA gene copy number.

All of the biological samples taken, except farm T3 from the manure application models for \textit{bla}_{OXA-58}, were included in the LMMs even if a specific ARG could not be detected in them. The farm T3 was excluded, since \textit{bla}_{OXA-58} genes could not be detected in the manure samples of that farm. If a biological replicate was missing from a single sample, it was input for normalization as a copy number of 9, which was 1 copy under the lowest LOD for all of the ARGs. The missing replicates' copy numbers were then normalized as described for the other samples. The LMMs were fitted to the log10-transformed normalized copy numbers. The final results were transformed back to normalized copy numbers and reported as fold changes relative to the control samples taken before manure application, or fresh manure samples in case of the manure model.

Extraction of antibiotics and analysis

The extractions were performed from 3 g of manure in sterile 50 mL Falcon tubes by shaking overnight with 30 mL methanol on a linear shaker in ambient temperature and a speed of 250 rpm. Samples were protected from light during extraction. After extraction the samples were centrifuged (1800 × g) and filtered through 589/3 filter paper (Whatman, GE Healthcare, Buckinghamshire, UK) into a sterile polyethylene bottle. The extracts were stored in -20°C before analysis.

For analysis 50 ng of internal standards were added to 1 mL of the extract, which was subsequently evaporated to dryness, and re-dissolved in 1 mL of water. Analysis was carried out using an Agilent 1260 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA) equipped with a Jetspray ESI source and coupled to an Agilent 1290 series HPLC equipped with a binary pump, a vacuum degasser and a thermostatted column oven held at 30 °C. A Zorbax Eclipse C18 (2.1 x 50 mm, 1.8 μm) column was used for chromatographic separation. The mass
spectrometer was run in multiple reaction monitoring (MRM) mode, monitoring two transitions for each compound. All compounds were analyzed in positive ionization mode (ESI+). A gradient elution was carried out using acetonitrile (B) and water (A), both containing 0.1 % formic acid, as eluents. The gradient started at 4% (B) and was raised to 90% (B) over 3.5 min, held there for half a minute before returning to 4% (B) over the next 30 sec. The column was then given one and a half minutes for equilibration before the next injection. The injection volume was 20 µL and the flowrate was 0.55 mL/min.

For the identification and quantification in samples two MRM transitions as well as individual internal standards were used. Deuterium labeled internal standards were available for benzylpenicillin and cloxacillin, while deuterium labeled 4-epi-tetracycline was used as an internal standard for oxytetracycline and deuterium labeled azithromycine was used as internal standard for tylosin.