

Antimicrobial Resistance in Biofilms: Methodology

2.1 Sampling Strategy

2.1.1 Literature review

We conducted a scientific literature review in this project to provide information for two purposes:

1. To inform on whether there were heightened AMR risks associated with particular meats or meat products, which might indicate that these meats should be a focus for sampling.
2. To identify locations or equipment within meat manufacturing facilities on which biofilms are prone to form, which would inform our choice of swabbing locations within individual factories.

If any quantitative information about AMR burden in different meats or meat products was found, then it could possibly have been used to contextualise the results of this project.

Given the time constraints this was not intended to be a systematic literature review, but an overview of the available literature to contribute to the practical outcomes described above. The project made extensive use of the search terms developed and results obtained by FSA project FS301059 [10].

2.1.2 Factor selection

Food manufacturers who operated appropriate secondary meat processing factories were approached, either directly (if a relevant contact already existed) or through industry contacts. If the manufacturer was willing an initial, remote meeting was held to describe the project and explain the benefits and potential drawbacks of participation. Factories would receive a summary report of their results, and could be pseudonymised in the final project report. However, the sequence data from their samples would be made publicly available. Manufacturers were then free to be involved, or not, on a purely voluntary basis. Not all manufacturers approached agreed to take part, and to preserve the identities of the participating factories limited further information is provided.

Based on the results of the literature review, no meat types were ruled out of scope or selected for enhanced sampling based on known AMR risks. Factories were therefore selected based on the products manufactured, to obtain a variety of meat types and to attempt to sample the most widely consumed meat types. Four factories were sampled (Table 1.).

Table 1: Description of the products manufactured in each of the four factories sampled

Factory	Products
Factory A	Ready to cook chick products
Factory B	Bacon and gammon
Factory C	Sausages and burgers, containing pork, chicken, beef and lamb
Factory D	Pork sausages, chicken sausages and burgers

2.1.3 Identification of sampling sites within factories

Based on the results of the literature review and a structured meeting with technical experts from the factories, a list of potential sampling sites was compiled for each factory. These were to focus on sites of previous microbiological detection, hard-to-clean locations, and food-contact sites.

2.2 Sampling Methodology Development

Biofilm cultures were grown in 25cm x 25cm lidded plastic bioassay dishes (Sigma-Aldrich) by adding 1g soil collected from Fera grounds, to 100ml warm tap water containing 5g Nutrient Broth powder (Oxoid – ThermoFisher Scientific). This was to help dissolve the nutrient broth, and also to encourage growth of bacteria. The trays were incubated at 37°C for 72 hours to develop biofilms. The liquid was then poured off, and the trays were rinsed with molecular biology grade water (MBGW) (HyClone, Fisher-Scientific) to remove non-adhered particles and bacteria. Several methods were tested for the various steps of the sampling process:

- commercially available swabs (3M Sponge-stick sponge dry swabs – SLS, pipe foam swabs Technical Services Consultants) were tested with two swabbing conditions -(dry swab, PBS-wetted swab (Phosphate Buffered Saline PBS pH7.4 SigmaAldrich))
- swabs were stored at a variety of time/temperature combinations to simulate transport from the factory back to Fera and subsequent storage before extraction. These were: storage at 4°C for 1 hour and 6 hours followed by immediate extraction; storage at 4°C for 1 hour and 6 hours, then storage at -20°C overnight before extraction; storage at 4°C for 1 hour and 6 hours, then storage at -20°C for 1 week before extraction; storage at 4°C for 6 hours, then storage at -40°C and -80°C for 48 hours and 8 days before extraction.
- swab DNA extraction was tested using different disruption methods as follows: -
- massaging (by adding the swab to a small ziplock bag and covering with 5ml PBS and massaging the swab in the PBS for 30 seconds before removing the PBS to a 15ml Falcon tube prior to pelleting bacteria by centrifugation at 4,500 xg for 15 minutes)
- shaking with ball-bearings (swab was transferred to a 50ml Falcon tube to which 5ml PBS and 5 x 5mm diameter stainless steel ball bearings (Qiagen) were added, then shaking by hand for 30 seconds before removing the PBS to a 15ml Falcon tube prior to pelleting bacteria by centrifugation at 4,500 xg for 15 minutes)
- sonication (swab was transferred to a 15ml Falcon tube and covered with 5ml PBS, followed by sonication for 20 seconds and 40 seconds before removing the PBS to a second Falcon tube prior to pelleting bacteria by centrifugation at 4,500 xg for 15 minutes. Following centrifugation, the bacterial pellet was extracted as set out in 2.4.1 below.

To determine the success of the extractions, a standard 16S amplicon polymerase chain reaction (PCR) was set up, followed by gel electrophoresis to visualise the presence of amplicons. PCR was performed using 16Sv4 primers in order to amplify bacterial DNA. PCR reactions comprised 0.3mM dNTPs, 0.3µM each of forward and reverse primer, and 0.6 units Phusion® High Fidelity DNA Polymerase (New England BioLabs) in 1 x HF buffer and 1µl DNA extract as template in a total volume of 25µl. A positive control sample NGSgBlock (synthetic oligonucleotide encompassing primer binding sites for 16S and ITS primers) at 0.005ng/µl, and a PCR negative control comprising 1µl molecular biology grade water were also amplified alongside the samples for quality control purposes. Primers for 16Sv4 [11-14] were:

Nex_16S_515F

(TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA)

Nex_16S_806R

(GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT)

Samples were amplified with the following 'touch down' thermocycling conditions on a BioRad C1000 thermal cycler:

Initial denaturation at 98°C for 2 minutes, followed by 22 cycles of denaturation at 98°C for 20 seconds, primer annealing at 65°C for 45 seconds decreasing 0.5°C per cycle down to 54°C, extension at 72°C for 60 seconds, then a further 8 cycles of 98°C for 20 seconds, 54°C for 45 seconds, 72°C for 60 seconds, followed by a final extension at 72°C for 10 minutes and hold at 4°C. Total number of cycles was 30.

Following thermocycling, amplification success was measured by visualisation of amplicons on agarose gels containing 0.1 µg/ml ethidium bromide (Sigma). Five microlitres of the PCR reaction was added to 1 µl 6X Orange DNA Loading Dye (ThermoFisher) and electrophoresed through a 1% agarose gel in 1X TBE buffer for 1 hour at 140V. Amplicons were visualised on a UV transilluminator and verification of correct amplicon size was by comparison to a DNA size standard ladder (Quick Load DNA Marker Broad Range - New England BioLabs).

The results of the above were used to develop a swabbing Standard Operating Procedure (SOP) (see Appendix 2). This was then shared with technical staff at Newcastle University who had not been involved in the SOP development. The SOP was then used to sample lab-grown biofilms, which were couriered to Fera for DNA extraction. This was undertaken to ensure that the SOP could be followed by new users, and to ensure that transport of biofilms by courier did not affect our ability to extract DNA from the samples. The samples were supposed to be couriered chilled, but this instruction wasn't followed. On arrival, two swabs were extracted immediately, and two were stored at -80°C for three weeks (over the Christmas period) before extraction.

Finally, after feedback from food manufacturing hygiene experts, Biofinder spray (Freedom Hygiene) was identified as a potential aid for sampling in the facilities. Biofinder is a spray formulation that can be used to detect the presence of biofilms, which by their nature, can be visually hard to detect. It contains three ingredients: an orange colourant, a surfactant, and hydrogen peroxide. It is considered to be a safe product to use in food environments. When the Biofinder comes into contact with catalase (present in many important biofilm-forming bacteria), the catalase reacts with the hydrogen peroxide constituent of the spray to generate white microbubbles of oxygen which are easily visible. To ensure the Biofinder did not degrade DNA in biofilms such that it could not be sequenced, and to examine any taxon-specific effects of the Biofinder, biofilms of known composition (two strains of *Pseudomonas fluorescens* and one strain of *Escherichia coli* in equal cell counts as measured by OD600) were grown on glass slides for parallel sampling and DNA extraction with and without Biofinder treatment. DNA extracts were then sequenced on the ONT MinION Flongle sequencer, to identify taxa present. The resulting sequences were taxonomically identified using the Kraken2 software [15].

2.3 Sampling

The sampling SOP developed in section 2.2 was couriered to each participating factory, as well as a kit containing all necessary sampling kit and reagents, and the list of sampling sites (2.1.3). Samples (n=146) were taken according to the SOP by factory staff experienced with microbiological sampling, and then returned to Fera chilled, immediately after sampling, either by collection by Fera staff (three factories) or by courier (one factory). Samples were immediately frozen at -40°C on arrival at Fera, and stored no longer than 3 weeks before extraction. Samples were all taken during May 2021, on one (two factories, one sampled on 23/05/2021 and the other on 26/05/2021) or two days (one factory sampled 07/05/2021 and 13/05/2021, the other on 07/05/2021 and 26/05/2021).

2.4 DNA extraction, HTS Library preparation and sequencing

2.4.1 DNA extraction

Swabs (either 3M Sponge-Stick™ or SLS foam pipe swab) were removed from the freezer and allowed to defrost for approximately 30 minutes before commencing extraction. Phosphate buffered saline (PBS) pH 7.4 (15ml) was added to the swab, either in the swab bag for 3M Sponge-Stick™ swabs, or in a ziplock bag to which the pipe swab had been transferred from its plastic tube. The swabs were massaged in the PBS for 1 minute. The PBS was then poured into a 15ml centrifuge tube and centrifuged at 4,500 x g for 20 minutes. The supernatant was poured off, and the pellet was resuspended in 180µl lysis buffer (20 mg/ml lysozyme in 1 x TE buffer pH 8.0 with 1.2% v/v Triton X-100). The Qiagen DNA extraction procedure for Gram positive bacteria was then followed, using the Qiagen DNeasy Blood and Tissue kit, following the manufacturer's protocol as follows:

Samples (180µl) were transferred to 1.5ml microcentrifuge tubes and incubated at 37°C and 400rpm in a thermomixer for a minimum of 30 minutes up to 90 minutes. Following incubation, 25µl proteinase K and 200µl buffer AL were added, mixed thoroughly by vortexing, and incubated at 56°C and 550rpm in a thermomixer for a further minimum of 30 minutes. Ethanol (96-100%, 200µl) was added to the sample and again mixed thoroughly by vortexing. The mixture was pipetted onto a DNeasy mini spin column and centrifuged at 13,000 x g for 1 minute (or longer if the sample was particularly fibrous from swab matrix carryover). The columns were then washed by the addition of 500µl of Buffer AW1 and centrifugation at 13,000 x g for 1 minute, followed by 500µl Buffer AW2 and centrifugation at 13,000 x g for 3 minutes. The flow-through was discarded and the columns centrifuged at 13,000 x g for 1 minute to ensure there was no ethanol carryover. The columns were placed in clean 1.5ml microcentrifuge tubes and the DNA was eluted by the addition of 60µl of Solution AE to the centre of the membrane. Following incubation at room temperature for 5 minutes, the columns were centrifuged at 13,000 x g for 1 minute. The eluate was stored at -40°C. For each batch of samples processed, an extraction blank was included which comprised either 15ml PBS with no swab, or 15ml PBS added to an unopened swab (EB6).

2.4.2 Illumina sequencing

All sample DNA extracts were quantified either using a Qubit dsDNA HS Assay Kit (Invitrogen) and Qubit fluorometer (Invitrogen), or a Quant-iT™ Picogreen™ dsDNA Assay Kit (Invitrogen) and a Fluoroskan Ascent plate reader (Thermo Scientific). The samples then underwent Illumina DNA Prep library preparation following the Illumina protocol 1000000025416 v09 June 2020. As well as the biofilm samples, fourteen control samples were also subject to metagenome sequencing: 3 positives (labelled "pos1" to "pos3" and comprised of a synthetic oligonucleotide molecule), 3 index-negative samples ("indexneg1" etc comprising molecular grade water as template for the index PCR reaction) and 8 extraction blanks ("EB1" etc comprising either an empty tube taken through the extraction process (n=7), or an unopened swab for EB6 (n=1)).

Briefly, the DNA undergoes fragmentation and addition of Nextera tags in a single enzymatic step. Unique dual index adaptors were added via a PCR reaction, followed by a double-sided bead purification of the libraries to remove any very small or very large fragments. The libraries were quantified as above. In addition, a selection of high and low quantifying libraries plus the extraction blanks and index PCR negative controls were analysed on the Agilent TapeStation using HS D1000 tapes, size ladder and sample buffer.

Following TapeStation analysis, it was checked that the index PCR negatives, and extraction blanks were below 10% of the mean sample values for DNA concentration (ng/µl).

Three further critical points were checked from the TapeStation traces:

- ensured the libraries have peaks between 350 – 800bp
- ensured the absence of smaller sized peaks

- ensured the absence of peak presence at the libraries size in the index negative

Once the quality of the libraries had been assessed, the libraries were pooled in equimolar amounts to create a 4.45nM library pool in a 735µl total volume.

Following confirmation of the quality and concentration of the library, the prepared sequencing library was couriered on ice to Newcastle University. Clustering QC was carried out on an Illumina MiSeq using Reagent Kit V2 Nano (Illumina). The library was then prepared for sequencing according to the NovaSeq 6000 Sequencing System Guide (Illumina Document # 1000000019358 v14 Material # 20023471 September 2020) using one S2 300 cycle Flowcell and one S4 300 cycle Flowcell. Sequence data in fastq format was made available to Fera via download over an SFTP server.

2.4.3 Oxford Nanopore Technologies Sequencing

The 21 samples with the highest quantity of DNA were selected for long-read sequencing on the PromethION. Samples were prepared using the PCR barcoding (96) genomic DNA sequencing kit (SQK-LSK109; Oxford Nanopore Technologies) with the PCR Barcoding Expansion Pack 1-96 EXP-PBC096 (Oxford Nanopore Technologies) according to the manufacturer's protocol. Briefly, the double-stranded DNA fragments were initially end-repaired and dA-tailed before being ligated to barcode adaptors. Barcodes were then added via a PCR reaction. The barcoded libraries were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen) and Qubit fluorometer (Invitrogen) before being combined in equimolar amounts to form a single pool. Sequencing adapters were then ligated onto the pooled DNA. The DNA pool profile was analysed using the Agilent Genomic kit through the Agilent TapeStation system (Agilent) according to the manufacturer's protocol in order to assess the average library size in base pairs (1500bp). The library was also again quantified using the Qubit dsDNA HS assay in order to determine, along with the library size, the concentration of library as a range of 5-50 fmols can be loaded onto the PromethION flow cell.

The prepared library was divided into two in order to run two flow cells. The two library samples (50 fmols per sample) were loaded onto two PromethION flow cells and loaded into the PromethION sequencing device (Oxford Nanopore Technologies). The sequencing run was performed over a maximum of 72 hours.

2.5 Sequence Analysis

2.5.1 Illumina pre-processing

Raw fastq files were downloaded from the Newcastle University SFTP, inspected to confirm that no file corruption had occurred during the process, and transferred to Fera's bioinformatics servers. Paired-end fastq files for each sample from both the S2 and S4 flowcells were then combined.

2.5.1.1 Quality Control and Assembly

Adapter removal and PHRED score quality trimming was performed using BBDuk [16]. A minimum length of 45 and a minimum quality score of 30 were imposed. These values were chosen based on the overall high quality of the data, as well as length restrictions placed on the data by downstream analysis.

The forward (R1) trimmed reads were screened for contamination by non-bacterial sequences with Bbsketch [16], and a number of possible contaminations were observed. Genomes were obtained from GenBank [17] based on these contaminants. The resulting chicken, cow, pig, sheep and human genomes were indexed with BWA-MEM2 [18], before samples were mapped to each genome where evidence for contamination was present, again using BWA-MEM2. The

results of this process can be found in Appendix 10.

The host filtered reads were assembled using the SPAdes assembler [19], running in 'meta' mode. Assemblies were annotated with Prokka [20].

2.5.2 Nanopore pre-preprocessing

Raw fastq files were basecalled and demultiplexed with Guppy (version 4.0.11), and transferred to Fera's bioinformatics servers. Fastq files for each sample from both flowcells were then combined.

2.5.2.1 Quality Control and Assembly

Universal ONT PCR adapters were trimmed and reads which contained adapters in the middle of the sequence were split with Porechop [21]. A random subset of 10,000 reads was taken from each sample and NanoQC [22] was used to inspect the base composition at the start and end of the reads. Based on the observed nucleotide biases, the first 25 and last 25 nucleotides were then removed with NanoFilt [22].

The long-read assembler Flye [23] was used in metagenome mode to assemble the samples. Assemblies were subject to a Blast [24] search, and any contigs which were assigned to the Metazoan taxonomic lineage were removed. After contaminant removal, 6 samples were removed from further analysis due to high numbers of contaminant DNA, with the remaining 15 samples taken forward for further analysis. Assemblies were annotated with Prokka.

2.5.3 Hybrid Assembly

Illumina data was combined with Nanopore data for the 15 samples that had passed the quality control steps outlined in 2.3.2.1. The hybrid assembler OPERA-MS [25] was used to produce assemblies.

2.5.4 ARG Detection

The assembled contig sequences (scaffolds assembled by SPAdes from the Illumina reads) were processed with the RGI software, which uses the CARD database as a reference [26]. This analysis involved running RGI in 'main' mode, specifying '--data wgs', which is the recommended data type for assembled metagenome sequence data. Many of the reference ARG sequences are long, and a reasonably large proportion of contig sequences will be short in comparison, or at least of a length such that they might include only a segment of an ARG rather than the full-length gene. The proportion of relatively short contigs is expected to be higher in samples at the lower end of the read-count distribution. We therefore used a parameter (formally, '--low-quality') which is designed to deal with short contigs. (We later applied a filter which excluded ARG-matches which accounted only for a small proportion of the reference gene).

For other RGI parameters we used default values. This includes those categorised by RGI as either 'perfect' or 'strict' matches, and excludes the 'loose' category; in our previous experience, 'loose' predictions appear to be too liberal for our purposes. However, default behaviour of RGI also includes the upgrading ('nudging') of 'loose' to 'strict' if the percentage sequence identity of the match is very high. Our results therefore include 'nudged' predictions.

2.5.4.1 Filtering the RGI ARG matches

RGI provides metrics for each match of an input sequence and a reference ARG sequence. These metrics include the percentage sequence identity of the matching segment, which is not

necessarily the full length of the ARG, and the length of the match expressed as a percentage of the reference ARG length. We refer to the latter as 'ARG coverage' (strictly speaking, this is the coverage breadth of the ARG). It is possible for this coverage to be > 100%, because the matching part of the contig can be longer than the ARG. This can occur if the contig sequence contains insertions relative to the ARG sequence.

We subsequently examined a subset of the RGI output created by discarding all contig-ARG matches where either the percentage sequence identity was less than or equal to 80%, or the percentage ARG coverage breadth was less than or equal to 80%.

2.5.5 ARG quantification

ARG incidences (frequencies of each ARG sequence match in a sample) do not represent relative quantities of ARGs occurring in each sample. Each ARG is identified from a contig, and each contig can be assembled from different numbers of sequencing reads. Thus, although incidences may be the same in two samples, the number of sequencing reads attributed to each ARG could be very different.

Consideration of the short-read frequencies alone enables only within-sample comparisons, not between-sample comparisons. This is because metagenomic sequence data sets are compositional in nature, i.e. the frequency with which any DNA fragment is sampled by short reads relates to the fragments relative (proportional) frequency in the sample, not its absolute frequency.

Here, we used a quantification method to determine relative frequencies of each ORF in the contigs data. The relative frequencies of any ORF which had a matching ARG sequence was treated as a proxy for the relative frequency of the ARG (this is an approximation because ORF sequences in the contigs data do not necessarily account for a full-length gene).

In brief, the method, KALLISTO [27] uses a back-mapping method (unassembled reads are compared to the assembled sequences, in this case ORFs), to estimate the read frequencies corresponding to the ORFs, and takes account of the length of the assembled sequences to determine relative frequencies of the ORF themselves. Longer DNA sequences in the same sample will give rise to more reads than shorter DNA sequences, all else being equal. The frequency units obtained are "transcripts per million (transcripts)", or TPM. This name arises from the methodology's origin in transcriptomics, but the principle is the same in the metagenomic context (where TPM should be taken to mean 'fragments of genomic DNA containing a gene, per million fragments of any genomic DNA'). For example, 50 TPM for an assessed sequence (in this case an ORF, which may represent an ARG) means that of all biological DNA sequences present in the sample, 50 out of every million were instances of this sequence.

Therefore, the sum of a sample's TPM values for all ARGs represents the total number of biological sequences estimated to be protein-coding ARGs, out of every million sequences. Like the incidence values, the mean TPM values are indicative. The normalisation applied enables within-sample comparison of abundances (regardless of ARG length), not between-sample, due to the proportional nature of the metric. Therefore, mean TPMs must be treated with caution.

We also performed quantification by the same method on data supplemented by the sequences of 16S rRNA amplicons as indicated by *in silico* PCR, in order to compare the resulting TPM values with the qPCR results (see section 2.6).

2.5.5.1 Comparison of relative ARG quantities from metagenomics with qPCR

The above quantification enables within-sample comparisons (between ARGs) but not between sample comparisons, due to the compositional nature (relative abundances). In principle, the

results of quantification of selected genes by qPCR (section 2.6), which aims to provide absolute quantities (copy numbers), enables the calibration of these relative quantities in each sample.

For each sample, we calculated the copy number per TPM. In theory, with unlimited sequencing depth, the copy number per TPM should be the same for all genes in one sample (but may be very different between samples due to the total DNA quantities being different). We compared the data for the genes which we assayed by qPCR, *sul*(I), *tet*(B) and the 16S rRNA gene.

Because the qPCR assays for protein-coding genes are very specifically targeted, the comparison with CARD-RGI predictions (which may involve non-identical matches) is not necessarily appropriate (and the 16S gene will only be reported as an ARG for particular cases). We therefore performed *in silico* PCR by applying ecoPCR [28] using the same primer sequences (see section 2.6.1) to the metagenome assemblies. First, the assembled (scaffolded) data was converted into an ecoPCR database, before an *in silico* PCR was performed for 16S, *tetB* and *sul*(I) genes. In order to replicate the qPCR as closely as possible, the exact same primers were used, with up to 3 mismatches allowed in each primer.

For the 16S *in silico* PCR, the resulting amplicon sequences were assessed by Blastn versus the NCBI nt database [29] to determine which were bacterial and which are likely to be off-target amplifications (typically, eukaryote mitochondrial in this context). Total TPM values of the two types of 16S sequences were obtained separately.

This identified the relevant ORF and 16S rRNA gene sequences, whose TPM quantities were used for this comparison. We also determined which, if any, of the ORFs had resulted in a positive detection by RGI.

2.5.6 Co-location

Antimicrobial resistance genes were identified in the hybrid assemblies and filtered using the CARD-RGI workflow, as outlined in section 2.5.4. ARG co-location was defined as more than one ARG identified on the same contig. With this definition, a custom python script was used to annotate the ARGs which were identified as co-located.

2.5.7 Mobile Genetic Elements (MGEs)

A blast [24] database was created using ACLAME [30] database (version 0.4). ACLAME is a database containing a number of MGEs from sources such as phage, plasmid and transposons, last updated 17/09/2013. Each assembled genome was subject to a blast search against this database, with the resulting matches filtered to only include sequences with a percentage identity $\geq 95\%$, and an alignment length $\geq 95\%$ of the reference sequence.

2.5.8 Metagenomically Assembled Genomes (MAGs)

Two ways that MAGs can be identified include inspecting single genome-sized contigs or using a binning approach to group contigs which likely represent the same organism. In this study, due to the availability of hybrid assembled genomes, we opted to select and identify the taxonomy of single, genome-sized contigs. Maximum contig lengths were inspected for each sample, and any contigs which were shorter than 1.5Mb were excluded. The remaining contigs were taxonomically classified using kraken2 [15].

2.5.9 Taxonomic Identification

Taxonomy was identified using the Metaphlan3 software [31] and the accompanying CHOCOPhan database (version 201901). This software was selected in order to minimise the number of false positives that may be present through the use of other taxonomic annotation

software. In exchange for this decreased false positive rate, it is possible that some low-abundant taxa are not reported. Taxonomy was identified from quality-controlled reads from each sample and consolidated into one file.

2.5.10 Identification of Gram negative Bacteria

ARGs passing the criteria outlined in section 2.5.4.1 were taxonomically classified to the Phylum level using the Kraken2 software [15]. Each bacterial Phylum identified was annotated with the known Gram type, and the total number of each bacteria was recorded per sample.

2.6 Quantification of selected AMR genes using qPCR

2.6.1 Assay selection

We selected assays for two AMR genes to quantify using real-time PCR (qPCR). Those were based on a previous project (FS301050) on AMR genes in meat products that, by extension, may be expected to occur in biofilms from meat processing premises as well, and where assays for these genes were available from previous published AMR gene studies. Assays for genes for tetracycline resistance, *tet(B)* [32] and sulfonamide resistance, *sul(I)* [33] were selected for this analysis. Additionally, we selected an assay for 16S (515F-806R; [34]) to provide an estimate of the overall bacterial load for each sample (Table 2).

Table 2. qPCR assays used for quantification of the AMR genes in biofilms

Name	Sequence
Sul (I) F	CGCACCGGAAACATCGCTGCAC
Sul (I) R	TGAAGTTCGCGCAAGGCTCG
tetB F	AGGCGCATCGCTGGATT
tetB R	CAGCATCCAAAGCGCACTT
tetB Pe	FAM-CTTATTGCTGGCTTTTT-MGB
16S-515F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA
16S-8067R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAA T

2.6.2 Quantification of targets

In order to produce a positive control and PCR calibrant for the assay targets we designed and had synthesised a synthetic double strand DNA fragment, known as a gBlock (Integrated DNA Technologies, Iowa) (Appendix 12). This contained the sequences complementary to the assay primers, and where appropriate probe, for each of assay targets for the ARG genes. An additional synthetic gBlock control was used for the 16S assay. Calibration standards were prepared by 10-fold serial dilution to produce a range from 10⁷ down to 10³ target copies/μl in order to generate calibration curves for each assay.

PCR reactions followed a two-step cycling of 95°C for 15 seconds followed by 60°C for either 1 minute (*sul(I)* assay) or 30 seconds (*tet(B)* assay) for 40 cycles. Reactions contained Power Up™ SYBR™ Green Master Mix (16S and *sul(I)* assays, Applied Biosystems™) or TaqMan™ Universal Master Mix II (*tet(B)* assay, Applied Biosystems™) along with 2.5-3.0μl of sample extract or control (gBlock standards or no template controls). Reactions were performed on QuantStudio™ Flex instruments (Applied Biosystems™) and analysed using manufacturers software to generate CT (cycle threshold) values.

2.7 Summarising UK dietary consumption

The most recent UK dietary consumption data were extracted from the National Diet and Nutrition Survey (NDNS) rolling program years 1-11 collected by Public Health England and from the Diet and Nutrition Survey for Infants and Young Children (DNSIYC). The latter covers infants aged 4-18 months and the former has age 18 months and older. Together, these therefore cover the full range of age groups required. The NDNS provides consumption data for over 1100 unique food types, 18325 individuals and over 1.5 million individuals/food combinations for the rolling program years 1-11 (2008/9 – 2018/19) [35]. The DNSIYC study was only for a single year (2011), but as it is the only information available for the youngest age category was included along with the more recent data [36]. For each individual, the data included a sampling weight that accounts for any non-random sampling within the survey. Using these weights when calculating the statistical summaries provides a more robust estimate of the true percentiles of consumption.

The Food Standards Agency recipes database (MRC, 2017) [37] was used to disaggregate each food, so the consumption statistics also account for any item that contained the food type (beef, pork or chicken) as part of a recipe.

2.8 Comparison of genes in the sampled biofilms with existing surveys

Data on AMR *E. coli* phenotype were extracted from recent EU harmonised surveys in retail meats (pork, beef and chicken were sampled). For example, the FSA provided details of UK samples collected during 2017-2019 [EU harmonised survey of antimicrobial resistance on retail meats pork, beef and chicken \(food.gov\)](#). Data from other EU Member States and earlier reports are available from the ECDC website: [Surveillance and disease data: EU summary \(ECDC website\)](#).

For the genes found in the biofilms or in the meat surveys, drug classes to which these are predicted to confer resistance were assigned. Drug classes are automatically assigned to the biofilm sample results as an output of RGI. The drug classes of the antibiotics to which isolates from the meat surveys were tested were obtained from ARO. This allowed for the studies to be directly compared with respect to antibiotic drug classes. An assessment was then made (to identify which of the genes found in our study are consistent with *E. coli*) and either confer resistance by their presence (as single gene system) or are efflux pumps or parts of operon. We compared all of the CARD sequences to a database of almost 19,000 *E. coli* genome sequences using blast, and those which had completely identical genomic matches were thus treated as consistent with *E. coli* for the purpose of this assessment (some will occur in related species as well). The ARO ontology annotations of these CARD sequences were used to indicate the resistance mechanism. There are numerous caveats to this approach, including that the particular genes identified in biofilm samples do not necessarily confer resistance to all antibiotics within a particular class, nor do we know that they are capable of conferring AMR at all (for example, are within live bacterial cells, or part of intact gene clusters). Having to compare these data types is a consequence of the paucity of comparable metagenomic studies available.

2.8.1 Comparing ARG prevalence in samples taken from chicken lines to ARG prevalence in bacteria detected in chicken

The prevalence of ARG in Gram-negative bacteria isolated from UK chicken samples has previously been reported [38]. All samples in the survey contained Gram-negative bacteria; hence, the reported prevalence is also the prevalence of chicken samples that contained each of the tested ARGs. We compared the prevalence of ARGs detected in either the chicken survey or in our biofilm survey (with the number of samples that were found to contain Gram-negative bacteria as the denominator). The significance of differences was assessed using Fisher's Exact

Test (adjusted for multiple comparisons); confidence intervals for differences were gained by pairwise subtraction of random samples from the posterior beta-distributions for the proportions estimated for each ARG. The comparison of these prevalences may be informative if we expect that there is a strong link between the prevalence of meat samples that contain ARGs and the prevalence of biofilm samples that contain ARGs.