Antimicrobial Resistance in Biofilms: Executive summary

Antimicrobial resistnace (AMR) refers to the ability if microbes to resist actions of the chemicals used to control them. Often this is used to refer to the antibiotic resistance of bacteria (as in this report); but in the broader sense can refer to the resistance of other organisms, such as fungi, to other groups of chemicals, for example biocides. AMR is a serious, global public health concern, with the ability to render antimicrobials ineffective, and make currently routine treatments (for example, chemotherapy, organ transplant) highly dangerous. The agrifood chain is known to be a source of AMR, due to selection pressure exerted through the use of antimicrobials.

Biofilms are formed when bacteria secrete extracellular polymeric molecules, which stick bacterial cells together and allow them to adhere to environmental surfaces. Biofilms allow the persistence of bacteria in food processing environments, and may be of concern from an AMR point of view for a number of reasons. As well as protecting bacteria from physical cleaning actions, they can also protect bacteria from the actions of biocides. This may lead to bacteria being exposed to lower levels of biocides, and therefore being able to evolve resistance. There is some evidence that biocide resistance can lead to the co-selection of antibiotic resistance, for example due to biocide- and antibiotic-resistance genes being present on the same mobile genetic element (e.g. plasmid). Biofilms also can reduce the physical distance of bacteria, which may enhance the transfer of AMR genes (ARGs) between them by horizontal gene transfer. Secondary meat processing sites were selected by FSA as a target, due to a lack of previous work in this area.

This project set out to assess the potential contribution of biofilms to the burden of ARGs in secondary meat products by applying molecular techniques to biofilms sampled from food processing facilities. Initially a literature assessment took place to inform the sampling strategy. The objective of the assessment was to determine i) whether particular meat food types were associated with higher AMR/ARG prevalence (to focus sampling on factories producing those products), and ii) whether particular equipment or surface types were prone to biofilm formation (to focus sampling within factories on those location types). Making extensive use of the results of a previous FSA project (FS301059), it was found that poultry may be associated with higher AMR detections, but overall there was not enough data to support a focus on poultry. For the assessment of sites within factories, a wide range of surfaces (various plastics, steel, glass etc.) were found to support biofilm growth. Sites that were moist, hard to clean, in contact with meat and meat exudates, and possibly with worn or scratched surfaces were found to be likely sites of biofilm growth. Based on the results of the literature assessment, it was decided to focus on factories producing products that covered the greatest consumption, i.e. those occurring most frequently in the UK diet, (while acknowledging that willingness of factories to participate would be the ultimate decider of which types of meat-production facility could be sampled). Four factories were recruited to provide samples, producing the following; chicken products; chicken and pork products; bacon; sausages and burgers (containing variously beef, pork, chicken and lamb). Not all meat types were necessarily produced at all times, or on all lines, and the association of samples and meat types in this report is based on information provided by the factories.

A sampling Standard Operating Procedure (SOP) was developed, including a critical step of rinsing surfaces with sterile, molecular biology grade water prior to sampling to remove planktonic bacteria. The bacteria which remained adhered to surfaces are defined as being part of a biofilm (by nature of their adherence), regardless of the mass or durability of that biofilm. A list of potential sampling sites was developed based on the results of the literature review, as well as

discussions with factory technical managers. This list was shared with each factory, along with a copy of the SOP and a kit containing the necessary sampling reagents. Factories undertook their own sampling (due to pandemic restriction), and swabs were returned to Fera for analysis. A total of 146 swab samples were returned, from across the four factories. On receipt at Fera swabs underwent DNA extraction, and DNA was subsequently analysed by several methods. All samples underwent high-throughput non-targeted sequencing on an illumina NovaSeq 6000, to produce an average of 95.8 million raw sequence reads per sample. A subset of 21 samples with the highest concentration of DNA were sequenced on an Oxford Nanopore PromethION sequencer, to assess the ability of long DNA sequence reads to improve metagenomic assemblies, and detection of ARGs co-located on the same DNA fragment.

For samples where sufficient DNA remained after sequencing (n=118) qPCR was performed on three target genes. These were two ARGs (*tet(B)* and *sul1*) and the bacterial 16S rRNA gene. The utility of qPCR for scaling the results of the metagenomic sequencing (which are necessarily always proportional, rather than absolute values) was investigated.

Of the 146 samples that were sequenced, two were judged to have failed sequencing, producing less than 0.05% of the average number of sequences per sample. Among the 144 samples which produced sufficient sequence for analysis, enough sequence data was obtained for these sequences to be assembled computationally into longer, contiguous stretches of DNA on which ARGs could be identified. ARGs were identified by using the RGI tool to compare to the CARD database. As such, we here define an ARG as any gene that is annotated as such in CARD. Across all samples, 144 ARGs were identified, and 96 samples were positive for at least one ARG. Generally, the distribution of ARG frequencies across factories, for example, how many different ARGs are found in samples from each factory, are broadly similar. There is a relatively long tail of high-ARG samples from the plant processing pork and chicken (the four samples with the most ARGs are all from this plant) but the small number of participating plants and the strong correlation of plant and meat type make it impossible to draw firm conclusions about this.

On inspection of the numbers of reads and taxa obtained from the extraction controls, it became clear that a large amount of sequence was observed in some controls, with some taxa being present across samples and controls. This is likely due to a known phenomenon of DNA being present in sampling and DNA extraction kits (the ‘kitome’), exacerbated by the low yields of DNA obtained in most samples, and the great depth of sequencing undertaken here. Taxa which occurred in controls were discounted from samples, and ARGs underwent stringent filtering of hits (based on identity and length of sequence match). After filtering, no ARGs were observed in the controls. The low levels of DNA obtained from most samples may speak to the general cleanliness of the factories studied.

When looking at the ARGs that are found at relatively high incidence within samples (i.e. constitute a large proportion of the sequences within samples), we see ARGs that make sense from a biofilm perspective. The top ARG is *rsmA,* a regulatory gene with a wide variety of functions (including biofilm regulation) which is annotated as an ARG because of its involvement in regulating the releasing of biological products from the bacteria, which can potentially lead to an AMR phenotype. *rsmA* is found in Pseudomonas species, which are known for their ability to form biofilms (although in this instance it is difficult to be certain whether we detected *rsmA* or its homolog *csrA*, which is found in other taxa). Other genes include a range of *qac* genes which are associated with resistance to quaternary ammonium compound biocides, which again is expected to occur for food factory biofilms. Of the antibiotic resistance genes observed, ARGs potentially involved in resistance to tetracycline are observed at high incidence (*tet(H)* and *tet(K))*, though not *tet(B)* which had been selected for qPCR analysis (along with *sul1)* prior to these results being available.

The results of the qPCR analyses were mixed. *tet(B)*was found at very low levels, below the presumed limit of quantification, and it is difficult to differentiate this from background noise. *sul1*

was found more frequently, but it appears that there may be some non-specific amplification of the assay used. This being the case, we believe only eight to ten samples are likely truly positive for *sul1* by qPCR. Of these, only three were positive for *sul1 i*n the sequence data. As well as comparing presence/absence by the two methods, we attempted to use the qPCR data to calibrate the metagenomic data, to allow direct comparisons of the numbers of sequences attributed to ARGs among samples. Comparing the results obtained from this for *sul1* and 16S showed that the two assays did not agree, with quantification by *sul1* being higher than quantification by 16S by five to ten times. However, as we believe the *sul1* assay may be overestimating copy number, and there are only three samples for which a direct comparison can be made, the conclusions that can be drawn from this are limited. When looking at the 16S data across all tested samples, we see a general correlation between quantification by 16S and relative quantification in the sequence data.

Using the ARG data generated here to estimate the contribution of biofilms to the ARG burden of secondary processed meat products is challenging, as there are no readily available, comparable metagenomic sequencing sets to compare to. Instead, we attempted comparisons of our data to two other datasets, a study using array-based detection of ARGs in poultry, and the EU harmonised survey of retail meats in the UK. In comparison to the results obtained from poultry we find that overall the ARGs studied were found in a smaller proportion of samples taken from biofilms than were seen in samples taken from chicken. Whether this is due to genuinely lower presence or technical differences between the studies remains a question. Comparing our study to the EU harmonised survey is even more problematic, as the vast majority of the results from the retail meat survey take the form of phenotypic data, and inferring phenotypic resistance from metagenomic data is not advisable. Therefore, we constrain our results to a summary of the EU harmonised survey (to provide context), and a statement about the degree to which the Escherichia coli phenotypic results from the survey samples overlap with potential (though by no means certain) E. coli phenotypes predicted from metagenomic analysis.

Overall, we have provided data on the ARGs identified in biofilm samples obtained from factories producing a range of secondary processed meat products, from factories which process the four major meat types in the UK (chicken, pork, beef, lamb). Inferring the contribution of these to the ARG burden of food products would require additional sampling. We investigated the utility of combining different types of molecular data (short and long sequences, metagenomic and qPCR data). The long-read data appears to improve our ability to identify ARGs located on the same piece of DNA. The qPCR data is challenging to integrate due to the behaviour of the different assays but shows promise for future investigation.