What is the Burden of Antimicrobial Resistance Genes in Selected Ready-to-Eat Foods?

Final Report to Food Standards Agency

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Lay Summary

Antimicrobial Resistance (AMR) occurs when bacteria evolve to become resistant to antibiotic substances. This happens, and has always happened, with naturally occurring antibiotics and with antibiotics that are used to treat animals or people. AMR can arise during farming or food production. This provides a potential exposure route to consumers via food. Ready-to-eat foods are particularly important here because they are eaten without further cooking in the home. This means that bacteria present on these food items that have AMR, or that contain DNA (called AMR genes; or ARGs) associated with AMR are more likely to be eaten intact. The aim of this project was to look for ARGs in a range of ready-to-eat foods, to provide information about the ARGs which are present on ready-to-eat foods typically consumed in the UK. However, the presence of ARGs does not necessarily mean that there are antibiotic-resistant bacteria present. Finding an ARG is like finding part of the blueprint for a car: it doesn't necessarily mean a car can be built, or would work if it was built, but it does mean that one of the things necessary to get building is in place.

We identified ARGs on ready-to-eat foods using *metagenomic sequencing*. Metagenomic sequencing works by simultaneously analysing all of the different types of DNA from a sample to identify what genes or bacteria may be present. There are many thousands of known ARGs. It is the ability to test for *everything all at once* that makes metagenomics so useful. Metagenomics will detect AMR genes regardless of the bacteria that they are in. Sometimes we can tell which specific bacteria ARGs were in, but often all we can tell is that a particular type of ARG DNA is present in *something*: we can't necessarily tell whether the bacteria it was in was harmful, harmless or helpful; whether the ARGs actually made the bacteria resistant to antibiotics in practice, or whether the bacteria that contained the ARGs were alive or dead.

Metagenomics is not yet used routinely for detecting ARGs in food. And, like every detection method, it is not perfect. Sometimes ARGs that we know about and that are present a sample will not be detected, particularly if they are present in small amounts. In

other cases, parts of the DNA in bacteria may look enough like a particular ARG to give us a positive result even though the ARG isn't there. Hence, the results contain some false negatives and false positives. There is always trade-off between these two types of detection error. In general, we attempted to prioritise minimising false positives.

The aims of this project are twofold – to estimate the burden of AMR genes in ready-to-eat foods on retail sale in the UK, and to examine how to best use metagenomics to do this. We looked at single-food-type products to do this (tomatoes, milk, ham, not sandwiches, pies or pasties) so that we could identify in which particular types of food we had found AMR genes.

We had the capacity to take and test 1001 ready-to-eat food samples to meet these aims. We first looked at previously published scientific work on AMR in ready-to-eat foods to decide how many samples of each food-type to test. We found no evidence AMR was consistently more or less common in any particular food type. Hence, we assigned sample numbers to individual food types in proportion to their consumption in the UK. The aim here was to get a set of samples that represented the average UK consumption of ready-to-eat foods. These samples were then analysed to see which samples provided the most bacterial DNA.

We selected a subset of 256 samples for metagenomic sequencing to identify ARGs, again based on consumption, but also selecting more foods that gave larger amounts of bacterial DNA, because we thought that these types were most likely to give reliable results about ARG presence. The results were combined with information about the amount of each food in UK diets to estimate the ARG burden from ready-to-eat foods in the UK.

We estimated that the great majority of UK diets contain at least one ARG from RTE food. Some classes of ARGs were much rarer than others. For example, ARGs associated with resistance to the antibiotics colistin and methicillin were found rarely; ARGs associated with resistance to vancomycin, fluoroquinolone or carbapenem antibiotics and some others were found more often. There are several things to consider here. Firstly, despite the very

stringent filtering of results that we applied, it remains possible that a small minority of ARGs identified from short fragments of DNA were false positive detections. Conversely, our efforts to reduce false positive hits (along with other inherent limitations that come from testing samples) mean that it is unlikely that we detected every single ARG present in the samples. So, the results presented here give the ARGs for which the evidence of presence was strongest rather than a perfect list of all ARGs present in the samples that we tested. It is always important to remember that many of the ARGs identified here were for a single part of a complex resistance mechanism. Hence, it is likely the true burden of current active *AMR* provided by the ARGs that we detected is lower than the burden of those *ARGs* in the diet. However, there may be an unquantified AMR burden from ARGs that we failed to detect.

We also tested 24 samples using a more experimental technique that could allow us to identify *where* the ARGs were present for example in which types of bacteria. Only small amounts of DNA sequence data were generated by this approach, which reduced the number of times we could assign locations to ARGs in this study. Nonetheless, the technique did show promise for providing additional information about ARGs. For example, in some cases, the experimental approach was better able to reconstruct entire bacterial genomes (the total DNA sequence present in a bacterium) from the samples.

This study has provided useful data on ARG presence on ready-to-eat foods in the UK, and their consumption in the typical UK diet. Significant insights on the use of metagenomic approaches have been gained, for instance about the problems of identification of ARGs from DNA sequences, especially in a study of this scale, and with such a wide variety of types of food. Recommendations for future improvements and complementary further work have been made which include; wider sampling to estimate the relative contributions of different foodstuffs to the ARG burden of the diet (and in a wider range of diets, for example vegan diets), generation of more sequence data per sample to allow ARGs to be identified with greater confidence, and efforts to quantify ARGs present in samples to allow different measures of burden and assessments of risk to be used.

Executive Summary

Antimicrobial Resistance (AMR) is increasingly recognised as a vitally important, global public health issue. The emergence of AMR in the food chain can be driven by entirely natural processes or it can arise in response to the use of, for example, veterinary or prophylactic antibiotics. Hence, consumers may be exposed to AMR via food. Ready-to-eat (RTE) foods are of particular interest because they are eaten without further cooking in the home. Hence, any AMR genes (ARGs) or AMR bacteria present on RTE food items are likely to be consumed intact.

Metagenomic sequencing is based on non-targeted sequencing of DNA from a sample; the identified sequences can be used to identify ARGs that are present. This has several advantages over more traditional, phenotypic screening for AMR bacteria: principally that we can test simultaneously for the presence of all the ARGs that we know about. ARGs can be detected regardless of the bacterium in which they are present, and if sufficient DNA sequence is generated the bacterium that contains the ARG can also be identified. However, metagenomics is not in routine use for systematically screening foods for ARGs and has very rarely been used in RTE foods in any context.

Therefore, the aims of this project are twofold – to estimate the burden of ARGs in RTE foods on retail sale in the UK, and to evaluate the use of metagenomics to this end. For this study only single-food-type RTEs were in scope (oranges, cheese, smoked salmon; not quiche or salads). This was so that the particular food type in which an ARG had been found could be identified. Hence, the sampling plan provided samples that were representative of in-scope RTE food rather than RTE food as a whole.

To achieve these aims a literature review was first undertaken to identify available quantitative information about AMR prevalence in RTE foods. Data from the National Diet and Nutrition Survey was used to identify RTE foods that fell into four broad categories (fresh produce, dairy, cooked meats, seafood). Literature relating to AMR in the identified

foods was retrieved, and quantitative data extracted. No evidence was found of consistent differences between food types in the proportion of samples found to contain AMR, though this may have been due to the relatively small number of available studies. Based on the results of this literature review, a sampling strategy was developed to collect 1001 RTE food samples for testing. As no consistent differences were identified among food types across the reviewed studies, the number of samples to be taken of each food type was in proportion to UK consumption. Samples were taken from regions in proportion with population, and from retailers according to market share. The aim here was to provide a set of samples that were representative of the typical consumption of RTE.

All samples underwent DNA extraction and metabarcoding sequencing of Variable Region 4 of the 16S locus, to provide an estimate of the proportion of bacterial DNA in the DNA extracted from the samples. A subset of 256 samples were then selected for non-targeted metagenomic sequencing based on of food type consumption and weighted towards samples from food types that were found to have higher bacterial to host DNA ratios. This weighting was done to increase the power of subsequent sequencing to detect the presence of ARGs.

Sequences from the 256 samples were analysed bioinformatically to identify ARGs. Several approaches were trialled, and limitations associated with these approaches were identified. The diversity of bacteria present and the amount of host DNA obtained from some samples prevented the use of assembled sequences for ARG detection. Among other impacts, this meant that the genomic context of which plasmids or bacteria ARGs were found in could not be identified. In addition, this meant that ARGs were identified on the basis of individual short DNA sequences (using the gold standard ARG database) which reduces the confidence with which the presence of ARGs can be confirmed compared with whole-gene matching. The ARGs identified in each food group were combined with consumption data to estimate the ARG burden from RTE foods in the average diet in the UK.

We estimated that there are ARGs from RTE food present in almost all average diets. The prevalence in diets varies considerably among individual ARGs, with genes encoding certain efflux pumps being found in approximately 97% of average diets, while many types of beta-lactamase gene were found in less than 25% of diets. ARGs associated with components of resistance to a number of antibiotics of interest were examined in further detail. Genes associated with components of colistin and methicillin resistance were very rare, with only two ARGs detected for each antibiotic. However, because of the measure of incidence used, and the fact they were found in relatively highly consumed foods, the incidence of one intrinsic colistin resistance gene was estimated at 46% of diets in the UK general population and one mobile colistin resistance gene was estimated at 7% of diets. Genes encoding components of methicillin resistance were estimated to be present in 17-37% of average diets, depending on the gene in question. Genes associated with components of resistance to fluoroquinolones or carbapenems, and potential extended spectrum beta-lactamases were estimated to be present in all average UK diets. However, it is possible that the proportion of diets containing some ARGs may have been over- or underestimated because of the difficulty of identifying the genes present based on short DNA fragments. In addition, the presence of ARGs does not map directly to the presence of AMR bacteria. While this may seem counterintuitive, there are many reasons why this is the case. For example, an ARG might encode one component of a larger gene complex, of which every element is required to generate phenotypic antimicrobial resistance. Or the ARGs may only be expressed under certain environmental conditions. Furthermore, ARGs are not a modern phenomenon. While anthropogenic sources can greatly increase selection pressure for ARG evolution, some ARG families (for example β-lactamases) have existed for millions of years.

Only low amounts of DNA sequence data were generated by the long-read sequencing approach. This was probably caused by low amounts of input DNA. Nonetheless, the technique did show promise for the identification of ARGs co-located on the same section of genome and demonstrated the ability to identify whole bacterial genome sequences from metagenomic data. For example, a whole genome likely to belong to *Acinetobacter*

albensis could be assembled from one of the long-read sequenced samples. Development of this procedure may provide the key to more direct measurement of the presence of AMR bacteria.

This study has provided useful data on AMR gene presence on ready-to-eat foods in the UK, and their consumption. Significant insights on the use of metagenomic approaches have been gained, especially about challenges such as identification of AMR genes from DNA sequences in diverse food types. Recommendations for future improvements and complementary further work have been made. These include wider sampling, to estimate the relative contributions of different foodstuffs to the AMR gene burden of the diet (and in a wider range of diets, for example vegan diets), generation of more sequence data per sample, to allow AMR genes to be identified with greater confidence, and efforts to quantify ARGs present, to allow additional measures of burden and assessments of risk to be used.

Glossary

Term	Definition
Reads	Individual nucleotide sequences generated from input DNA or RNA in the sequencing process. High Throughput Sequencing platforms can produce thousands or even millions of reads per sample on a single run.
Short-read	Referring to sequence data generated from sequencers such as Illumina's NovaSeq. Typically, data generated by these sequencers are between 75-300 nucleotides long and this process is referred to as short-read sequencing.
Long-read	Referring to sequence data generated from sequencers such as Oxford Nanopore's PromethION. Typically, data generated by these sequencers can be thousands of nucleotides long and this process is referred to as long-read sequencing.
Phred Quality	A measure of quality automatically assigned to nucleotides in a read. A Phred quality score of 10 indicates a 10% chance of an incorrect base, a score of 20 = 1% chance of an incorrect base, a score of 30 = 0.1% chance of an incorrect base etc.
Contigs	A set of overlapping sequences which together represent a consensus region of DNA. Long contigs can be generated from shorter reads in the Assembly process.
Assembly	A collection of contigs generated from a sample which together aim to best represent the original genomic content of that sample. The process of Assembly also refers to the generation of contigs from reads.

Term	Definition
NG50	The size of the contig which, along with larger contigs, represents 50% of the sequence of a genome. This can be used as a measure of assembly completeness, when the genome size of the particular organism being sequenced is known.
N50	The size of the contig which, along with larger contigs, represents 50% of the sequence of an assembly. This can be used as a measure of assembly completeness.
GC Content	The percentage of nucleotides in a genome, or section of a genome, that are made up of either guanine ‘G’ or cytosine ‘C’.
Coverage	Often referring to the ‘depth of coverage’. This is the number of times that a nucleotide has been sequenced for a given assembly/genome. Genomes are often referred to as having a coverage of for example 50x, which means that an average of 50 nucleotides were sequenced for each nucleotide position across the genome.
Host DNA	The DNA from the host organism that will also be present when extracting DNA for sequencing microbial communities. For example, apple DNA would be expected from samples extracted from an apple and cow DNA would be expected from samples extracted from beef or milk.
Metabarcoding	The targeted amplification and sequencing of a specific gene or gene region in order to identify different organisms present within a sample. The region should be conserved enough to be present and amplifiable in most organisms within a broad group (for example bacteria) and sequenced easily, yet variable enough to be able to identify different taxa (for example genera or species) within that broad group.

Term	Definition
V4 Region	A region of the 16S ribosomal RNA gene which is variable enough to be used as the specific region for metabarcoding studies of bacteria and archaea.
Metagenomics	The non-targeted sequencing of total DNA from a sample. The amount of DNA sequence generated will depend on factors such as the yield of DNA extracted, and the output of the sequencing technology used. Metagenomic sequencing will capture DNA from multiple organisms, such as bacteria, fungi, plants, animals etc. Depending on factors such as the amount of DNA sequence generated and the complexity of the communities being sequenced, metagenomic sequencing allows for the assembly of large fragments of DNA, and the possibility of assembling entire genes, chromosomes and genomes.
Mobile Genetic Elements (MGEs)	Regions of DNA which are capable of moving around either within or between genomes. These DNA regions could replicate on their own within the cell (for example, plasmids) or be integrated into the chromosome(s) of the organism in question (for example, Integrative and Conjugative Elements).

1. Introduction

1.1 Background to the study

Antimicrobial Resistance (AMR) is increasingly recognised as a vitally important, global public health concern (O'Neill, 2014), potentially causing untreatable infectious diseases and making recent medical advances (for example chemotherapy, organ transplant) unusable. This is especially important when considering the emergence of resistance to so called critically important antimicrobials (CIAs) (for example(Liu et al., 2016)), which can be the last line of defence against bacteria already resistant to frontline antibiotics. The use of antimicrobials in the agrifood chain is known to lead to the evolution of AMR, which may be transmitted to human pathogens or the human commensal microbiota (Hudson et al., 2017, van Bunnik and Woolhouse, 2017).

An evidence gap exists about the extent to which consumption of foodstuffs contributes to antimicrobial resistance in the human microbiome, especially for ready-to-eat (RTE) products. These products are of particular interest, as they are consumed without further cooking in the home, implying that any AMR bacteria (or intact AMR genes (ARGs)) present could contribute to AMR in the microbiome of the consumer.

RTE products also span a range of production techniques which may differ in the extent to which they promote the evolution of AMR, based on differing antimicrobial inputs during production. Cooked, RTE meats and dairy products are both animal-based foods, and the animals involved may have been treated with antibiotics during the primary production process. Non-RTE meats including red meat (APHA, 2020) and poultry (APHA, 2019) are known to harbour AMR bacteria, and it is not currently known how prevalent AMR bacteria are on RTE meats on retail sale in the UK. Dairy animals can be exposed to antibiotics to treat diseases such as mastitis either therapeutically or prophylactically (for example via dry cow therapy (Saini et al., 2013)), and this may lead to the evolution of AMR. Fresh produce, including RTE fruits and vegetables, may also have been directly treated with or indirectly exposed to antibiotics which can lead to the evolution of AMR on crops (Haynes et al., 2020). Other RTE food types, such as seafood, also have considerably antimicrobial

inputs during production, for example in aquaculture (Schar et al., 2020). Beyond primary production, slaughter/harvest and secondary processing of foods may contain sources of antimicrobial compounds which may drive AMR evolution. For example, there is some evidence that biocides may lead to co-evolution of resistance to antibiotics (Li et al., 2019, Khan et al., 2016), although the picture is still mixed (Lin et al., 2016, Murray et al., 2019).

Metagenomic sequencing involves non-targeted sequencing of DNA from a sample, and can be used to identify ARGs that are present. This has several advantages over more traditional, phenotypic screening for AMR bacteria. For example, ARGs can be detected regardless of the bacterium they are present in (including pathogenic and commensal species). If sufficient DNA sequence is generated, sequences can be assembled into longer contiguous lengths of DNA sequence (contigs). This enables ARGs to be placed in their genomic context, by identifying the plasmid or bacterium they are found in. However, metagenomics is not in routine use for screening foods for ARGs and has very rarely been used in RTE foods in any context.

The multiple and varied routes by which AMR may evolve in RTE foods, the enhanced risk from the fact that they are not cooked at home, and the knowledge gaps on ARG presence in RTE foods at retail sale all led to the commissioning of the project reported here.

1.2 Aims and objectives

This project was designed to begin to address that evidence gap described above, and as a pilot project to better understand the advantages and limitations of a metagenomic approach to ARG detection in RTE foods. We sampled 1000 products from a variety of RTE food categories (cooked meats; dairy products; fresh produce; RTE seafood was excluded from the scope by the FSA because it is consumed in small quantities compared with the other categories), weighted by consumption data and any available evidence on AMR prevalence. Samples were first assessed for suitability for further investigation using a 16S metabarcoding screen, which will amplify both bacterial DNA, and organellar (mitochondrial or chloroplast) in samples with high levels of foodstuff (or ‘host’ DNA). A subset of 256 samples were then sequenced metagenomically to identify the presence of

AMR genes, regardless of the bacteria (pathogen or commensal) which contain them. This data was combined with food consumption data from the National Diet and Nutrition Survey to estimate the burden of AMR in UK diets. This will provide important insights into the fitness for purpose of existing AMR targets for surveillance, and contribute to FSA's mission to ensure food is safe to eat. In recent years, metagenomic approaches have provided a wealth of AMR data from other areas of food production, but very few studies have been performed on the metagenome of RTE foods in any context. To achieve these objectives, the project undertook the following steps.

1. Short literature review on AMR in selected ready-to-eat foods

Review of available information on AMR in appropriate ready-to-eat (RTE) foods, and extraction of quantitative data on AMR gene/bacteria prevalence.

2. Define sampling strategy

Used data from the National Diet and Nutrition Survey (NDNS) data and data from the literature review to finalise the samples to be taken from specific RTE food types.

3. Collection of 1000 samples of ready-to-eat foods

Sampled RTE products according to the sampling strategy, and courier to Fera

4. 16S Metabarcoding of 1000 samples

Extracted DNA from products as they arrive, and store DNA at -30°C. Then performed 16S metabarcoding sequencing and analysis on DNA extracts from 1000 samples, to identify bacteria present.

5. Shotgun metagenomic sequencing of 256 samples

Using data from 16S metabarcoding, identified samples which could go forward for metagenomic sequencing based on the presence of sufficient bacterial DNA sequence. Then using stratification from the sampling plan selected 256 samples for metagenomic sequencing on the Illumina NovaSeq. Of these samples, 24 were also selected for long-read, PromethION sequencing.

6. Identification of AMR genes in shotgun metagenome data

Short read data and long read DNA sequence data were analysed to identify AMR genes (ARGs). Only ARG data derived from the short-read, Illumina data was used for subsequent modelling steps. Long-read data from the PromethION was considered more experimental and was used to assess the applicability of the technology.

7. Exposure modelling of AMR gene intake in average diets

Data from NDNS was combined with the results of the ARG identification to estimate dietary exposure to AMR genes.

A further remit of the project, not directly connected to AMR, is in the food-safety context. This is a check of the sequence data for evidence of foodborne pathogens of concern (*"priority taxa"*), i.e. those which might flag the need for laboratory-based confirmation, which could then necessitate statutory action by the Food Standards Agency if positive. This would only affect food samples from batches whose use-by date had not expired. Naturally, all samples were purchased and received at the laboratory prior to the expiry date, but many food classes such as most fruit, vegetables and meats have a short or very short consumption window (for example, meat which should be consumed within one month of domestic freezing by the consumer), and would therefore has passed the expiry

date by the time our sequence analysis results were available. There are some notable exceptions among necessarily frozen foods such as ice cream, whose expiry date may be many months post-purchase.

The sequence data alone cannot be considered sufficient to confirm presence (or indeed absence) of these pathogens, and the 16S metabarcoding data in particular has significant limitations in terms of taxonomic resolution. In general, it is possible to find such sequence-based evidence which may be completely consistent with, but not demonstrative of the presence of taxa of concern. The degree to which such evidence may be narrowed down to the level of genus, or in some cases even species, does differ from one taxonomic group to another. Some genera within the Enterobacteriaceae are especially difficult to distinguish.

Nonetheless, one undertaking is the analysis of the 1,000 samples' 16S metabarcoding data sets to check for evidence of any of the priority taxa in a list of foodborne pathogens provided by the FSA. We also analysed the selected 256 samples' metagenomics sequence data set with the same aim.

2. Methodology

2.1 Sampling Strategy

2.1.1 Literature Review

2.1.1.1 Background to Literature Review

The purpose of this assessment was to provide information to guide the sampling of ready-to-eat foods (1000 samples) for a study on AMR presence. Hence the main quantities of interest are the proportion of samples of different types of food that may contain AMR bacteria and the extent to which this may vary within and between foods.

2.1.1.2 Review Methodology

Initially, the National Diet and Nutrition Survey (NDNS) data rolling program (years 1-8, 2008/9-2015/16) was used to identify RTE foods in the four broad food groups of interest (fresh produce, dairy, cooked meats, seafood). These survey data are based on the returns given by participants, and as such can include freeform text. Food types were assessed, and where appropriate, food types were amalgamated. Food types were also assessed for their ready to eat status (for example, have they been cooked, or do they require cooking). For example, the following food types were all amalgamated under the name ‘Whole milk’:

- Milk whole summer pasteurised
- Milk whole pasteurised winter
- Milk whole sterilised
- Milk whole UHT
- Milk whole channel island pasteurised summer
- Milk whole channel island pasteurized winter
- Whole milk after boiling

- Milk whole unpasteurised
- Whole milk dried

Of these sample types, two (Whole milk after boiling and Whole milk dried) were not classed as RTE as they had either been cooked or required further processing after purchase. After discussion with FSA, food types which were canned or UHT were also excluded from scope, as they should not contain viable bacteria, and were unlikely to contain detectable bacterial DNA.

Food types were also classed as either informative or non-informative, based on whether they contained a single ingredient which would allow the source of an ARG to be isolated to that ingredient, or multiple ingredients. Non-informative products were deemed out of scope, as any ARGs identified in them could not be traced to their original ingredients. All food items in the NDNS were assessed in this manner. This assessment and classification were performed by different team members, independently, and the results compared. Any inconsistencies in assessment were then discussed, and a consensus reached. Out of 4720 food type: 1166 were assessed to be RTE; 475 of RTE products were assessed as in scope of which 90% of consumption within each of the four food groups consisted of 58 products: ham not smoked, corned beef, chicken slices unsmoked, frankfurter, turkey slices unsmoked, salami, roast beef slices, ham smoked, chorizo, roast pork slices, cucumbers, strawberries, apple juice pasteurised, white grapes, melon, carrots, lettuce, black grapes, pineapple, plums, white onions, nectarines, orange juice freshly squeezed, kiwi fruit, mangoes, peaches, avocado, raspberries, cherry tomatoes, red peppers, raisins, iceberg lettuce, blueberries, semi skimmed milk, whole milk, skimmed milk, cheddar cheese, soya milk sweetened, salted butter, vanilla ice cream, unsweetened yogurt, reduced fat spread, probiotic yogurt drink, one percent milk, low fat unsweetened yogurt, fat spread, spreadable butter, soya milk unsweetened, ham not smoked, corned beef, chicken slices unsmoked, frankfurter, turkey slices unsmoked, salami, roast beef slices, ham smoked, chorizo, roast pork slices.

The list of foodstuffs that were deemed RTE and informative and represented at least 90% of the consumption within each of the four product groups was then used to design a search term to return literature for the review. The search term was then iteratively improved to reduce false positive returns. The final search term used was;

("AMR" OR "antimicrobial resistan*" OR "anti-microbial resistan*" OR "antimicrobial resistance gene" OR "anti-microbial resistance gene" OR "antibiotic-resistan*" OR "antibiotic resistan*")

AND

(food* OR "ready to eat" OR "ready-to-eat") AND ("smoked salmon" OR "smoked mackerel" OR crabstick* OR ham OR "corned beef" OR "chicken slice*" OR frankfurter* OR "turkey slice*" OR salami OR "roast beef slice*" OR chorizo* OR banana* OR apple* OR "orange juice pasteurised" OR "pasteurised orange juice" OR tomato* OR pear OR pears OR orange* OR "small citrus" OR cucumber* OR strawberry OR strawberries OR "apple juice pasteurised" OR "pasteurised apple juice" OR "white grape*" OR melon* OR carrot* OR lettuce OR "black grape*" OR pineapple* OR plum OR plums OR "white onion*" OR nectarine* OR "orange juice freshly squeezed" OR "freshly squeezed orange juice" OR "kiwi fruit" OR mango* OR peach* OR avocado* OR raspberry OR raspberries OR "cherry tomato*" OR "red pepper*" OR raisin OR raisins OR "iceberg lettuce*" OR blueberries OR blueberry OR watermelon* OR grapefruit* OR "semi skimmed milk" OR "semi-skimmed milk" OR "whole milk" OR "skimmed milk" OR "cheddar cheese" OR "soya milk sweetened" OR "sweetened soya milk" OR "salted butter" OR "vanilla ice cream" OR "unsweetened yogurt" OR "unsweetened yoghurt" OR "reduced fat spread" OR "probiotic yogurt drink" OR "probiotic yoghurt drink" OR "one percent milk" OR "low fat unsweetened yogurt" OR "low fat unsweetened yoghurt" OR "fat spread" OR "spreadable butter" OR "soya milk unsweetened" OR "unsweetened soya milk"))

NOT

("smoked chicken slices" OR "smoked turkey slices" OR orange-ii OR "mull. arg." OR "muell. arg." OR "orange-" OR "-orange" OR "sea cucumber" OR "pv. tomato" OR "Eagle's F12 Ham" OR "cactus-pear" OR "prickly pear")

This term was searched against several scientific and grey literature databases. The dates in parentheses indicate the time period that that the databases cover, but in this instance the search was limited to the last five years, to return the most recent, relevant results, and to ensure a manageable number of references were returned;

- Web of ScienceTM Core Collection (1981-present)
- BIOSIS Citation IndexSM (1985-present)
- CABI : CAB Abstracts® and Global Health® (1973-present)
- Current Contents Connect® (1998-present)
- FSTA® - the food science resource (1969-present)
- KCI-Korean Journal Database (1980-present)
- MEDLINE® (1950-present)
- Russian Science Citation Index (2005-present)
- SciELO Citation Index (1997-present)
- Zoological Record® (1993-present)
- Base (2014-present)
- Copac (2014-present)
- EFSA (2014-present)
- Google Scholar (2014-present)
- Microsoft Academic (2014-present)
- National Archives (2014-present)
- PubMed (2014-present)
- WorldWide Science (2014-present)

2.1.2 Sampling Strategy

Based on the results of the literature survey, the sampling strategy was to be based on the total consumption of food types within four broad categories (dairy, fresh produce, cooked meats, seafood). These broad categories were proposed in the response to tender, as they encompassed the diversity of different RTE food types on retail sale in the UK. The 1000 samples to be taken were to be divided evenly between these broad food categories (250 samples of each type), and then samples were to be taken within each food category based on consumption within each category. However, when consumption of these different broad categories was compared, it was observed that consumption varied widely between them. Dairy accounted for approximately 55% of consumption, produce for 42%, cooked meat for 3% and seafood less than 0.5%. Based on this the FSA decided to refocus sequencing effort on high consumption food items, and to pick food types from all RTE foods according to consumption. This improves the ability to estimate the consumption of AMR genes in the average diet, while reducing the ability to say as much about AMR content of particular food types, or different diets.

The sampling strategy was then amended to sample the most consumed food types such that the sampled food types represented 90% of the consumption of in-scope RTE foods (quantity reported consumed of sampled in-scope RTE foods in the diet survey was 90% of the quantity of all in-scope RTE foods). The number of samples of each food type was in proportion to the amount of the food type in the average diet, but with a minimum of five samples per food type. This resulted in 52 different food types being sampled, comprising 33 type of produce, 17 types of dairy, and two types of cooked meat. While fewer dairy types were sampled than produce types, more than half of the samples taken were dairy samples. The number of samples of each food type taken from each UK region was assigned in proportion to the region's population and agreed with FSA. 90 sets of duplicate samples were taken: a duplicate consisted of two samples of the same product, from the same lot, bought from the same location at the same time; samples to be duplicated were selected at random (by product type, region, and retail outlet). Hence the sampling plan called for 910 distinct samples (defined by product type, lot, region, retail outlet and date) and 90 samples which were duplicates from among the 910 samples. Lastly the sample

plan was based on samples being taken from a total of 60 different retailers to control cost.

Table 1 gives a summary of the final sampling plan; Table 2 gives additional detail about product types.

Table 1: Summary of sample plan

Food Group	Total Samples	Unique Samples	Products
Dairy	546	492	17
Meat	20	20	2
Produce	434	398	33

Table 2: Sampling plan with products

Produce:

Product	Samples	Unique samples
Bananas	74	66
Apples	56	54
Orange juice pasteurised	31	30
Tomatoes	29	27
Pears	21	18
Small citrus	19	16
Oranges	18	17
Apple juice pasteurised	16	14
Strawberries	16	15
Cucumbers	14	14
White grapes	10	10
Carrots	7	7
Cherry tomatoes	7	5
Lettuce	7	7
Melon	7	7
Olives in brine	7	5
Plums	7	5
Avocado	6	5
Black grapes	6	6
Blueberries	6	5
Iceberg lettuce	6	5
Kiwi fruit	6	5
Mangoes	6	5
Raspberries	6	5

Product	Samples	Unique samples
White onions	6	5
Grapefruit	5	5
Nectarines	5	5
Orange juice freshly squeezed	5	5
Peaches	5	5
Pineapple	5	5
Raisins	5	5
Red peppers	5	5
Watermelon	5	5

Dairy:

Product	Samples	Unique samples
Semi skimmed milk	257	234
Whole milk	159	137
Skimmed milk	28	26
Cheddar cheese	24	22
Reduced fat spread	7	6
Salted butter	7	7
Soya milk sweetened	7	7
Unsweetened yoghurt	7	6
Vanilla ice cream	7	7
Double cream	6	5
Lactose free semi skimmed milk	6	5

Product	Samples	Unique samples
Spreadable butter	6	5
Fat spread	5	5
Low fat unsweetened yoghurt	5	5
One percent milk	5	5
Probiotic yoghurt drink	5	5
Soya milk unsweetened	5	5

Meat:

Product	Samples	Unique samples
Ham not smoked	15	15
Corned beef	5	5

2.2 Sampling

Samples were collected by HallMark Veterinary & Compliance Services based on the sampling strategy, and from the eight largest supermarket retailers. Samples were taken according to market share. For the purposes of sample handling, samples were divided into two broad categories – dairy and produce (for logistical reasons, cheese and cooked meats were sampled with the produce samples). Dairy samples (538 in number, not including cheese samples) were collected during the first half of the collection period which ran from 17th June 2019 to 27th August 2019. Produce samples (504 in number, including cheese and cooked meats) were collected in the second half of the collection period which ran from 2nd September 2019 to 28th October 2019. These totals include samples rejected by either HallMark or Fera, which were subsequently resampled.

FSA provided a letter to be given to the retailers by HallMark, explaining the purpose of the sampling.

2.3 DNA Extraction

2.3.1 Dairy Samples

Samples were extracted on the day of receipt where possible. When this was not possible due to a large number of samples arriving, half of the samples were frozen for processing on the next day on which samples were not due to arrive.

For dairy samples, two x 1.8ml aliquots were taken from each sample, one of which was frozen at -40°C as a reserve sample. The samples were extracted using the Qiagen DNeasy® PowerFood® Microbial Kit according to the Milk Extraction Protocol as follows:

Samples were centrifuged at 13,000 *x g* for 5 minutes and the fat layer was removed with a 10µl loop plus 0.8ml of the supernatant. The samples were then centrifuged for a second time at 13,000 *x g* for 5 minutes and any remaining fat was again removed with a 10µl loop plus 0.5ml of the supernatant. The resulting pellet was resuspended in the remaining supernatant using a pipette with wide bore tip. The suspension was then transferred to a clean 2ml microcentrifuge tube and again centrifuged at 13,000 *x g* for 5 minutes. All the remaining supernatant was removed. This tube transfer step aimed to reduce the amount of residual fat contamination present in the original sample tube when the fat layer was removed. The resulting pellet was resuspended (by pipetting) in 450µl Solution MBL (from the Qiagen kit). The samples were incubated at 75°C in a thermomixer for 5 minutes at 550rpm. The samples were then transferred to PowerBead tubes and incubated for a further 10 minutes at 75°C and 550 rpm on the thermomixer. The tubes were then mixed for 15 minutes at maximum speed by vortexing horizontally on a Vortex Genie 2 fitted with a 12 x 2ml tube holder adaptor. The tubes were then centrifuged at 13,000 *x g* for 1 minute at room temperature and the supernatant was transferred to a clean 1.5ml microfuge tube containing 100µl Solution IRS. The tubes were vortexed to mix and incubated on ice for 5 minutes. The tubes were again centrifuged at 13,000 *x g* for 1 minute at room temperature. The entire volume of supernatant was transferred to a clean 1.5ml microcentrifuge tube containing 900µl of Solution MR and mixed by vortexing. The supernatant (650µl) was loaded onto an MB Spin Column and centrifuged at 13,000 *x g* for 1 minute. The flow

through was discarded and this step repeated until all the supernatant had been processed. The columns were then washed by the addition of 650µl of Solution PW and centrifugation at 13,000 *x g* for 1 minute, followed by 650µl ethanol (96-100%) and centrifugation at 13,000 *x g* for 1 minute. The flow-through was discarded and the columns centrifuged at 13,000 *x g* for 2 minutes 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 100µl of Solution EB 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 in the case of dairy samples was 450µl Solution MBL added to a clean tube with no bacterial pellet and processed in the same manner as the dairy samples.

2.3.2 Produce Samples

All produce samples, including cooked meats but excluding cheese (both of which were included in the produce samples collection set for logistical reasons), were processed on the day of receipt. Cheese samples were stored at -40°C as they required a different extraction protocol.

Samples were processed according to their sample type – see the list of processing conditions below below. Briefly, samples that were usually consumed whole were rinsed to remove external bacteria and reduce contamination with host/matrix DNA. Samples that were usually consumed peeled were peeled, and the consumed flesh was then rinsed, as per guidance from FSA.

Initial sample processing conditions for produce sample types:

Rinse outside of whole fruit/slice of meat or 25g if sample weighs less for example, blueberries (25ml rinse buffer):

- Apples
- Pears
- Nectarines

- Peaches
- Plums
- Strawberries
- Blueberries
- Raspberries
- Cherry tomatoes/tomatoes
- Cucumber – use 50ml rinse buffer
- Lettuce
- Red Pepper
- White/Black grapes
- Ham
- Corned Beed
- Raisins
- Olives

Peel and rinse whole interior (25ml rinse buffer):

- Banana
- Orange
- Small citrus (for example, satsuma/mandarin/clementine)
- White onion
- Grapefruit – use 50ml rinse buffer
- Carrot

Cut into with scalpel and rinse 25g interior flesh:

- Melon
- Watermelon
- Pineapple
- Kiwi
- Mango
- Avocado

Juice - centrifuge sample directly:

- Orange Juice 1ml
- Apple Juice 15ml

Solid samples were placed into ziplock bags and 25ml/50ml rinse buffer (1x TE with 1.2% (v/v) Triton X-100 and 3% (v/v) antifoam) was added. The bag was shaken gently (soft fruits/meats), or the fruit was rubbed in the buffer for approximately 30 seconds. The buffer was transferred to a 50ml Falcon tube and centrifuged at 10,000 *x g* for 10 minutes to obtain a pellet

Liquid samples were centrifuged directly to obtain a pellet. For orange juice, which tended to generate more sediment than apple juice, 1ml was centrifuged at 10,000 *x g* for 10 minutes. For apple juice, 15ml was centrifuged at 3,430 *g* for 10 minutes (lower speed due to larger volume requiring a centrifuge and rotor with lower maximum speed).

All samples from this point underwent the same extraction procedure for gram positive bacteria using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol as follows:

The supernatant was removed and 180µl lysis buffer (20 mg/ml lysozyme in 1 x TE buffer pH 8.0 with 1.2% v/v Triton X-100) was added to the resulting pellet. In cases where a large pellet was obtained, more lysis buffer was added and volumes of reagents in

subsequent steps were adjusted upwards accordingly. Samples were incubated at 37°C and 400rpm in a thermomixer for a minimum of 30 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). 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 100µl 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 1ml rinse buffer processed in the same manner as orange juice.

2.3.3 Cheese Samples

The cheese samples were stored at -40°C to enable them to be processed as one batch of 24 samples. The samples were thawed thoroughly and 1g weighed into a 15ml Falcon tube. Initial processing for DNA extraction occurred following (Arcuri et al., 2013). Briefly, 5ml 2% w/v sodium citrate solution (in water) was added to the tube, vortexed for 1 minute, shaken using a MoBio vortex adapter for 10 minutes, followed by further vortexing for 2 minutes. Samples were then centrifuged at circa 5,500 *x g* for 10 minutes, the supernatant removed, and 1ml 1 x TE buffer (pH 8) added. Tubes were then vortexed again for 1 minute, the samples transferred to a 1.5ml microcentrifuge tube and centrifuged at 13,000 *x g* for 10 minutes, following which the supernatant was removed. The pellet was processed for DNA extraction using the QIAGEN DNeasy Blood & Tissue kit following the manufacturer's protocol for gram positive bacteria as for the rinsate samples above, with slight variations. Briefly, this entailed addition of 250µl lysis buffer (as above) to the pellet,

followed by incubation at 37°C and 550 rpm on a thermomixer for 90 minutes. Proteinase K (35µl) and buffer AL (280µl) were added, samples mixed by vortexing, and incubated at 56°C and 550 rpm on a thermomixer for 90 minutes. Ethanol (96-100%, 280 µl) was added to the sample and again vortexed. The sample mixture was added to a DNeasy mini spin column and processed as for the rinsate samples with the slight modification of a second elution step with an additional 100 µl buffer AE giving a total volume of eluate of 200 µl. The column eluate was stored at -40°C until PCR amplification was performed. An extraction blank comprising 5ml 2% w/v sodium citrate solution was processed in the same manner as the cheese samples.

2.4 16S Metabarcoding

2.4.1 Rationale

Metabarcoding consists of sequencing a particular "marker gene" common to all of the organisms of interest. The DNA sequence of the marker gene must have some highly conserved regions, i.e. be near-identical in all of the taxa in order that a segment of DNA between two such regions can be targeted for amplification by PCR using cognate DNA primer oligonucleotides ("primers"). At the same time, the amplified segment therein (the "barcode") must have a sufficiently variable DNA sequence such that it differs between taxa.

In this case, the gene encodes the ribosomal RNA small subunit, also known as the 16S subunit in bacteria. The targeted segment is within the V4 variable region of the gene, and the amplified segment ("amplicon") between the PCR primers is most commonly 253 nucleotide base-pairs (bp) in length.

2.4.2 PCR Amplification

Prior to PCR, samples were distributed across 6 x 96-well plates for dairy samples and 6 x 96-well plates for produce samples, ensuring at least two samples of the same type would be on the same MiSeq run, and also that samples from a single type were distributed across at least two MiSeq runs, to account for any inter-run variation.

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 (Caporaso et al., 2012, Apprill et al., 2015, Parada et al., 2016, Walters et al., 2016) were:

Nex_16S_515F
(**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGYCAGCMGCCGCGGTAA)

Nex_16S_8067R
(**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACNVGGGTWTCTAAT)

Nextera tag sequences are highlighted in bold. These allow the index tags to be added to the samples during library preparation to allow discrimination of individual samples following sequencing.

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).

Following gel visualisation, 4 critical points were checked:

- Ensure amplicon bands of the appropriate size (450 – 500 bp for 16SV4) are present in the samples.
- Ensure an amplicon band is present in the positive control.
- Ensure there is an absence of bands in the PCR negative control.
- Ensure there is an absence of bands in the extraction blanks.

The proportion of samples with a visible band after PCR amplification was relatively low (55% dairy samples generated visible amplicons, 65% of produce samples generated visible amplicons). However, with no a priori information about what proportion of samples should generate high quality amplicons all samples were taken forward for sequencing.

2.4.3 Sequence Library Preparation

Library preparation took place based on the Illumina protocol for 16S Metagenomic Sequencing Library Preparation. Firstly, the remaining 20µl amplicon for each sample underwent a size-selection magnetic bead clean up to remove unincorporated PCR components and any small non-specific products (for example primer-dimers). Briefly, 16µl AMPure XP (Agencourt) magnetic beads were added to 25µl PCR reaction and mixed by pipetting. After incubation for 5 minutes at room temperature, samples were placed on a magnetic stand for 2 minutes to pellet the beads. The supernatant was removed and the beads washed twice with 200 µl freshly-prepared 80% ethanol, incubating for 30 seconds after addition of the ethanol before removal. Following the second wash, the samples remained on the magnetic stand for 5-10 minutes until the beads were dry. Samples were removed from the magnet and either 52.5µl or 25.5µl molecular biology grade water added (smaller volume added to less concentrated amplicons – as judged by gel band intensity). The beads were resuspended by pipetting and incubated at room temperature for 5

minutes. Samples were then replaced on the magnetic stand and the beads allowed to pellet for 2 minutes. The cleared supernatant (50µl or 22µl depending on resuspension volume) was transferred to a clean 96-well plate prior to indexing PCR.

Index PCR was performed using Illumina Nextera XT Index Kit v2 dual index adapters. PCR reactions comprised 0.3 mM dNTPs, 5µl each of N7 and S5 adaptors from the index kit, 1mM $MgCl_2$. and 1 unit Phusion® High Fidelity DNA Polymerase (New England BioLabs) in 1 x HF buffer and 5µl cleaned amplicon as template in a total volume of 50µl. An index negative was included which comprised 5µl molecular biology grade water. Samples were index amplified with the following thermocycling conditions on a BioRad C1000 thermal cycler:

Initial denaturation at 95°C for 3 minutes, followed by 8 cycles of denaturation at 95°C for 30 seconds, adapter annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes and hold at 12°C.

Indexed samples (or 'libraries') then underwent a second magnetic bead clean to remove unincorporated PCR components. The protocol was as for the first-round bead clean with the exception of bead volume and elution volume. For the index PCR bead clean, 56µl AMPure XP magnetic beads were added to the 50µl index PCR reaction, and following bead drying, 27.5µl molecular biology grade water was added to resuspend the beads with a final volume of 25µl supernatant being transferred to a clean 96-well plate following bead pelleting.

The qualities of the libraries were then assessed by quantifying all libraries using either a Qubit™ dsDNA HS Assay (Invitrogen) and measuring library concentration on a Qubit™ fluorometer, or a Quant-iT™ Picogreen™ dsDNA Assay Kit (Invitrogen) and measuring library concentration on a Fluoroskan Ascent plate reader (Thermo Scientific). In addition, a selection of high and low quantifying libraries plus all controls (i.e. PCR positive, PCR negative, extraction blanks, index PCR negative) were run on an Agilent Technologies TapeStation 2200 using HS D1000 tapes, size ladder and sample buffer.

Following quantification, 3 critical points were checked:

- Ensure the extraction blanks and PCR negative controls are below 30% of the mean sample values.
- Ensure the positive control is at least 10 ng/µl.
- Ensure the majority of samples are above 10 ng/µl.

Following TapeStation, 3 further critical points were checked:

- From the traces, ensure the libraries have peaks 300 – 800 bp in size and of the expected shape for amplicons.
- From the traces, ensure the absence of peaks at circa 100 – 150 bp as this could indicate the presence of primer dimers following incomplete removal, and a second bead clean up may be required.
- From the traces, ensure there is minimal peak presence at the amplicon size in the PCR negative, index negative, and extraction blank.

Once the quality of the libraries had been assessed, the libraries from two 96-well plates (192 samples maximum including extraction blanks, positive and PCR negative/index negative controls which can be inspected for indications of contamination, for example high bacterial read counts in negative controls) were pooled in equimolar amounts to create a 20nM library pool in a 1ml total volume.

The pool was quantified using a Qubit™ dsDNA HS Assay to determine the actual concentration, and the average size of the pool was determined by running the pool on the TapeStation. These were then used to dilute the library pool to 4nM in preparation for running on the Illumina MiSeq.

2.4.4 MiSeq 16S Amplicon Sequencing

Libraries were then sequenced on an Illumina MiSeq sequencer, using the MiSeq Reagent Kit V3. A total of 10 pmol sample pool and 10% PhiX was loaded on to the machine for sequencing.

2.5 16S Amplicon Sequence Analysis

2.5.1 Microbial Community Analysis

This analysis served to confirm the presence of amplifiable and identifiable bacterial DNA in all of the samples, while providing qualitative indications about the relative abundance of bacterial versus host DNA. A further purpose was to provide one source of evidence of the presence of any priority taxa (foodborne pathogens of concern which warrant further investigation), since in principle the methodology provides the taxonomic origin of all of the DNA fragments sequenced. This complemented a more targeted approach to detection of these priority taxa (2.5.2).

1001 samples were thus processed using Qiime2 (Bolyen et al., 2018), a decentralised microbiome analysis package, which contains a number of useful tools that are used to analyse High Throughput Sequencing data. A bioinformatics workflow is shown in Figure 1, which outlines the analysis steps that are undertaken.

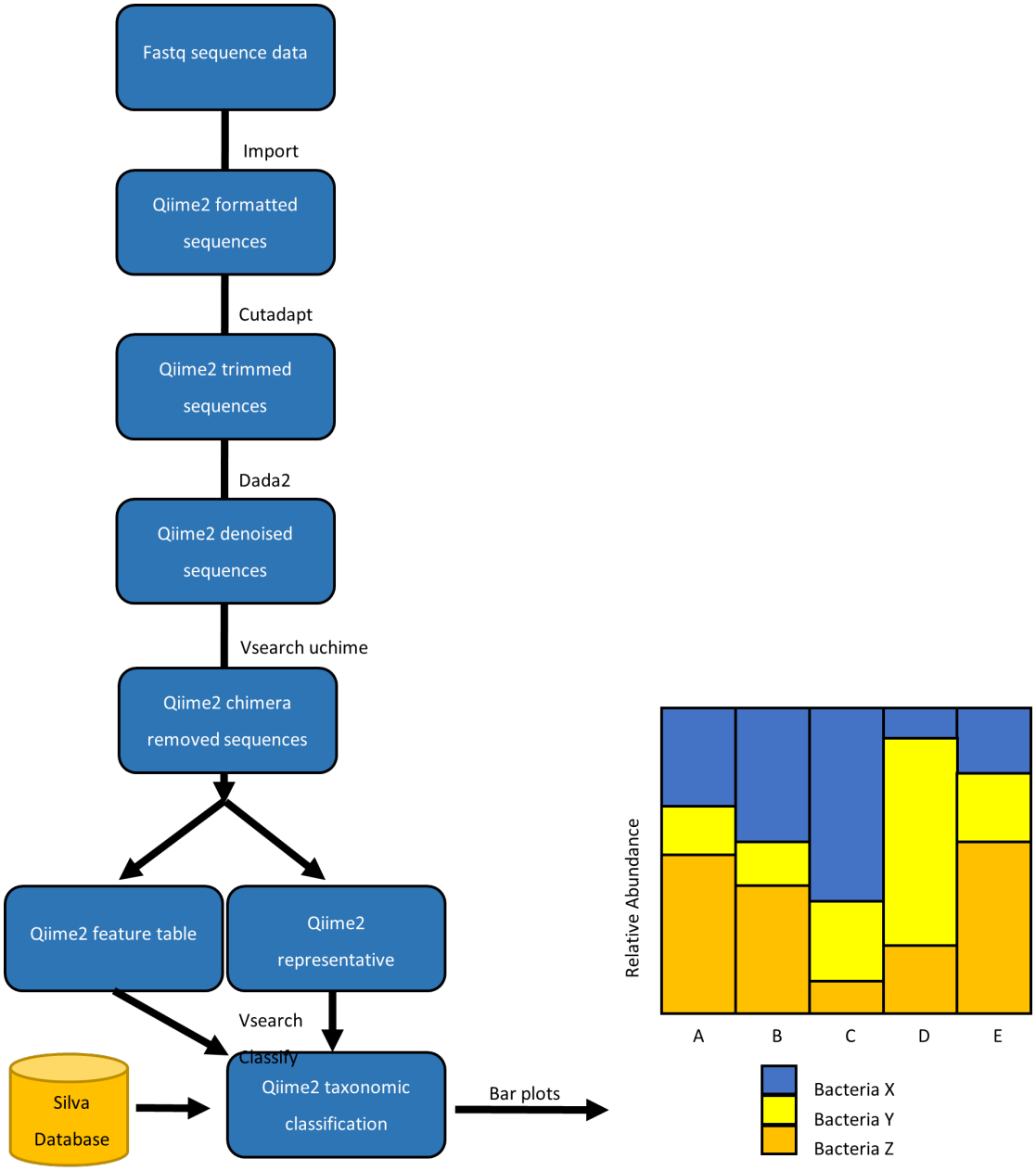


Figure 1. Qiime2 analysis pipeline used to identify taxa in the 1001 ready to eat samples.

Figure 1 describes the pathway through the Qiime2 pipeline, which is described in more detail below. Firstly, paired end fastq files are imported into Qiime2. Sequences are then trimmed by the cutadapt software tool (Martin, 2011), which looks for primers in the sequences and trims them. In addition to this, any sequences which are shorter than 50

nucleotides are removed so as to exclude erroneous sequences from passing through to further analysis. Sequences are then denoised using Dada2 (Callahan et al., 2016), which attempts to correct sequencing errors, where possible, to determine real biological sequence variants (ASVs). Any sequences which were shorter than 160 nucleotides were discarded. This threshold was chosen so that host sequences (such as cow or pig mitochondria) would be included in the final dataset which can be used to infer the relative proportion of bacteria to host DNA. Sequences were also quality filtered and joined, and an initial chimera removal step is performed. Chimeras are then checked for again using the Vsearch tool (Rognes et al., 2016), and a feature table file and representative sequence file is produced. Features are then classified using Vsearch, with the Silva database (Quast et al., 2013) providing taxonomic information. Finally, bar plots are produced, which detail the relative abundance of each taxon per sample (this data can also be view as a table for exact read numbers).

2.5.2 Priority Taxon Detection

In addition to the more conventional analysis described above, a targeted approach to detecting the priority taxa was undertaken. This does not constitute part of the AMR study itself, and was for food-safety considerations (see 1.2 Aims and objectives); it is therefore summarised briefly here, and described in more detail in Appendix 1A.

The reason for supplementing the standard, general approach to determining which bacterial taxa are present (2.5.1) is essentially that no such methods are flawless, and false-negatives may result, especially if the frequency of amplicon sequences corresponding to a taxon of concern are very low. Further, the standard methods will generally classify the amplicon data to bacterial genus level and no further, whereas in some particular cases it may be possible to narrow down the origin taxa to one of a small number of species of interest within the wider genus. We therefore took a parallel, complementary approach, targeted towards the taxa of concern (various foodborne pathogens specified at the genus and species level). Full details are in Appendix 1A.

2.6 Selection of Samples for Metagenomic Sequencing

2.6.1 Selection of Samples for NovaSeq Sequencing

We had the capacity to sequence 256 samples. Hence, a subset of 256 samples were selected for sequencing. The selection was weighted towards sample types that were more likely to provide a larger number of bacterial reads, and hence a larger number of ARG bacterial reads if ARGs were present. i.e. sequencing was directed towards sample types with the greatest power to discriminate between ARG presence and absence in bacteria present.

Based on the results of the 16S metabarcoding analysis, food types were separated into food types with a high proportion of reads of bacterial origin, food types with a low proportion of reads of bacterial origin, and food types showing a continuum of bacterial:host DNA. Food types for metagenomic sequencing were then weighted such that 80% of samples come from the high bacteria and continuum food types, and 20% come from the low bacteria food types. Samples were randomly allocated from within these types. This was done in order to focus on those foods which are most likely to give information about ARG content (note, NOT those which are most likely to have ARGs), and not on those foods where we are unlikely to get information on ARG content (note, NOT those which are unlikely to have ARGs). This is somewhat analogous to limits of detection of tests – directing testing towards sample types where our test has a better limit of detection.

This design still permitted the sequencing of a minority of samples from the foods where we are unlikely to get much information about ARG content (i.e. those with low proportions of bacterial 16S sequences), but it was felt that i) it would be useful to some extent for checking the validity of our upstream decision making, and ii) that this would allow some highly consumed food types to be sequenced that would otherwise be omitted (for example bananas).

Where available, a duplicate sample pair was sequenced for each food type. We also sequenced ten samples that were technical fails for 16S sequencing, to check whether failure of 16S sequencing is a good predictor of failure of metagenomic sequencing.

The number of samples for each food type selected for NovaSeq sequencing are shown in Table 13.

2.6.2 Selection of Samples for PromethION Sequencing

The sequence data generated by the PromethION was not intended to be used for burden estimation, but to gain insights into the additional benefits to be gained from using long-read sequencing technologies. As such there was no requirement to stratify by consumption or select samples randomly. That being the case, 24 samples were picked based on a preliminary ARG assessment using DeepARG on contigs assembled using MEGAHIT (Li et al., 2015) (Data not shown), to obtain samples showing both high and low levels of putative co-localisation, high and low ARG richness, and a variety of sample types.

2.7 Metagenomic Sequencing

2.7.1 NovaSeq Library Preparation

The 256 samples selected for NovaSeq sequencing underwent Illumina Nextera Flex library preparation (now Illumina DNA Prep) following the Illumina protocol (document 1000000025416 v 7 May 2019). Briefly, the DNA undergoes fragmentation and addition of Nextera tags in a single enzymatic step (the Nextera tags being the same sequence as above on the 16S primers). Unique dual index adaptors were added via a PCR reaction, in a similar way to the amplicon index PCR, followed by a double-sided bead purification of the libraries to remove any very small or very large fragments. The libraries were quantified as before using a Quant-iT™ Picogreen™ dsDNA Assay Kit (Invitrogen) and measuring the library concentration on a Fluoroskan Ascent plate reader (Thermo Scientific). In addition, a selection of high and low quantifying libraries plus the index PCR

negative were analysed on the Agilent Tapestation using HS D5000 tapes, size ladder and sample buffer.

Following TapeStation analysis. 2 critical points were checked:

- The index PCR negative was below 10% of the mean sample values
- The majority of samples were above 10ng/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 20nM library pool in a 1ml total volume. The pool was quantified using a Qubit™ dsDNA HS Assay to determine the actual concentration, and the average size of the pool (approximately 370bp) was determined by running the pool on the TapeStation.

2.7.2 NovaSeq Sequencing

Following confirmation of the quality and concentration of the library, the prepared sequence library was couriered on ice to Newcastle University. Clustering QC was carried out on an illumina MiSeq using Reagent Kit V2 Nano. The library was then prepared for sequencing according to the NovaSeq 6000 Sequencing System Guide using two NovaSeq S2 300 cycle (2 x 150bp) Flowcells. Sequence data in fastq format was submitted to Fera via recorded delivered of a portable hard drive.

2.7.3 PromethION Library Preparation

The 24 samples selected for long-read sequencing on the PromethION were prepared using the native barcoding genomic DNA sequencing kit (SQK-LSK109; Oxford Nanopore Technologies) with expansion kits EXP-NBD104 and EXP-NBD114 (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 barcodes. 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 (799bp). 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 5-50fmols can be loaded onto the PromethION flow cell.

2.7.4 PromethION Sequencing

The prepared library was divided into two in order to run two flow cells. The two library samples (6.8 fmols per sample) were loaded onto two PromethION flow cells loaded into the PromethION sequencing device (Oxford Nanopore Technologies). The sequencing run was performed over a maximum of 72 hours.

2.8 Bioinformatic Analysis of Metagenomes

2.8.1 Overview of metagenomics sequence data analysis

The principal aim of the analysis of metagenomic sequences from the food samples is to identify antimicrobial resistance genes (ARGs) present, with presence/absence data for each ARG being used as the input to the estimation of ARG burden (section 2.9). There are a number of prerequisite quality control and other processes to this end.

A further aim was to identify instances of co-location of genes, i.e. where two or more ARGs occur close to each other on the same segment of DNA.

An incidental analysis was to detect sequence-based evidence of the presence of any of a number of foodborne pathogens of concern, in order that any unexpected food-safety concerns would not be missed and could be acted upon by the FSA if necessary.

Several additional aims constitute assessments of the sequencing bioinformatics methodology. In short, to answer questions on whether the DNA extraction, the volume of sequencing (referred to as "depth") and each data analysis method are suitable to determine the required information about ARGs, for each food type.

Overall, the metagenomics analyses used the following sequencing data (section 2.7) as inputs, with the characteristics of each of the below sequence data described in Appendix 3b:

1. The **short-read** sequence data (Illumina NovaSeq), unassembled
2. Longer "**assemblies**" ("**contigs**") obtained by assembling the short reads
3. The **long-read** sequence data (ONT PromethION; for a limited number of samples only)
4. Overlapping long-read PromethION sequences can also be expected to occur, so these too are assembled into contigs.

2.8.1.1 Summary of analysis of the different types of sequence data

The steps involved in the detection of likely ARG sequences in the above sequence data are as follows.

- Basic quality-control, which consists mainly of trimming off any poor-quality bases from the ends of each read, and completely rejecting some reads if appropriate.
- Removal of DNA sequence reads of likely "host" origin. Although the laboratory methodology aims to maximize bacterial DNA and avoid the DNA of the food organism, there is an inevitable tendency for some plant or animal DNA to be present in the sample.
- Analysis of the short reads (input data type 1 above), to identify ARG sequences.
- Assembly of the short reads to create "contigs" (input data type 2)
- Assessment of the quality and consistency of the assembled contigs, by a technique known as "back-mapping" (see below).

- Analysis of the assembled contigs to identify ARG sequences (full-length gene sequences will be detected, while some others may only be partially present on the ends of contigs).

These are described in more detail in the following sections.

2.8.2 Quality Control and Host Genome Filtering

The workflow for quality control and production of sequence data for ARG analysis is shown below (Figure 2). Raw sequence data was trimmed to a minimum quality score of 20 and a minimum length of 50 using the Sickle (Joshi and Fass, 2011) software.

In order to remove sequencing reads which originated from a non-bacterial source, i.e. the food "host genome", the trimmed sequence data was then compared to a relevant host genome, where available, using the bwa (Li, 2013) software, which performs "mapping" of reads to reference sequences. Refer to Appendix 2 (metagenomics_sequencing_by_sample.xlsx) for genomes used for each sample. For example, the cow genome is used for dairy products and corned beef; many fruit and vegetable categories have a publicly available genome sequence for the plant species. Samples which had no suitable host genome readily available were mapped to a related genome instead. Unmapped reads were then extracted using the samtools (Li et al., 2009) software. These reads were then assembled (see below) using MEGAHIT (Li et al., 2015).

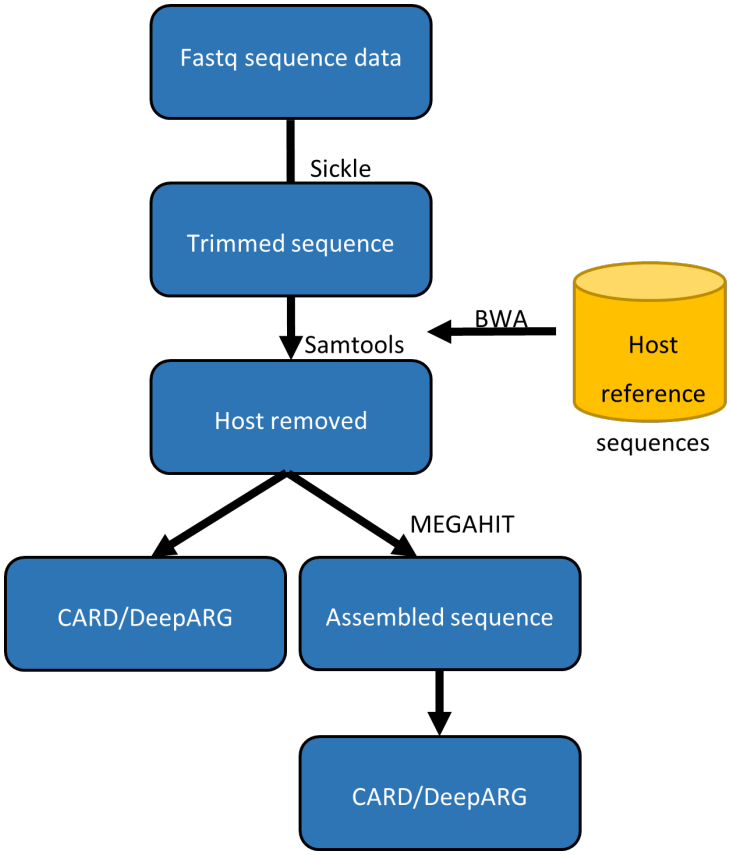


Figure 2. Flowchart outlining the bioinformatics analysis prior to ARG detection analysis.

2.8.3 ARG-detection software and reference databases

As proposed in the original schedule, we used two third-party software tools and associated databases for identification of ARGs from DNA sequence data. Since submitting the proposal, our evaluation of available bioinformatics resources remains that use of more than one tool is recommended, and that the two software tools/databases are still appropriate. These programs are:

- RGI/CARD (Alcock et al., 2020): RGI is the software which uses the curated CARD database.
- DeepARG (Arango-Argoty et al., 2018), which also comes supplied with a dedicated database.

The CARD database is long established, highly cited and has been used as the basis of other ARG-prediction tools and databases - indeed the DeepARG database is partly constructed from it. We regard the CARD database as the standard, but other researchers have built upon it and created resources which potentially contain a broader range of annotated ARG sequences. This was the motivation behind DeepARG, which uses machine-learning methods (specifically, a deep-learning approach) with the aim of high-confidence detection of variant ARG sequences which may have never been observed before. We therefore regard DeepARG results as generally more speculative, and indeed have used these results in effect as supporting data to the principal (RGI) results (see below).

However, there is also the consideration that any single reference database is unlikely to be comprehensive. In general, the use of two (or more) tools/databases provides a greater perspective than one alone, and helps to identify any systemic problems which might occur with the ARG-prediction analysis.

Further information on ARG databases, categories and names of ARGs can be found in Appendix 3c

2.8.4 Two approaches to analysis of the read data: unassembled reads versus assemblies

2.8.4.1 Unassembled Reads

One approach to the analysis of metagenomics sequencing data for the detection of ARGs is to compare each sequencing read to a database of ARGs. This has the advantage that each read of sufficient quality is used to identify ARGs; a disadvantage is that the short read nature of this approach may be more likely to lead to false positive ARG assignments, since shorter fragments are less likely to discriminate between ARGs and non-ARGs. Where an appropriate match is found, this ARG is assigned to that sequence.

For this approach, RGI (ran in BWT mode, referred to as “RGI BWT” throughout) and “DeepARG-SS” were used. We note that at the time we performed these analyses (beginning May 2020), RGI BWT was formally still beta-release software.

Further information describing the methodologies and limitations of unassembled read analysis can be found in Appendix 3d.

2.8.4.2 Assembly of reads

An alternative and widely used approach to metagenomics sequence analysis is to assemble the reads into sets of longer contiguous sequences (“contigs”). Contigs have the advantage of being longer, more complete and more accurate than unassembled reads, which will ultimately provide more confidence when identifying ARGs. One notable drawback, however, is that fewer contigs may be assembled if there is insufficient sequencing depth. Here, we refer to the sets of assembled contigs for each sample as "assemblies".

ARGs in the assemblies are identified by comparing contigs to a database of ARGs, using the principal mode of RGI ("RGI MAIN") and the long-sequence specific version of DeepARG, "DeepARG-LS".

Further information describing the methodologies and limitations of assembled read analysis can be found in Appendix 3d

2.8.5 Metagenome Assemblies

The main purposes of performing metagenome assembly are:

- to assemble the short reads into at least gene length sequences, which in general makes recognition of AMR (or any) genes more reliable
- to provide information on co-location of AMR genes and other AMR-relevant sequence features (various mobile genetic elements, for example).

2.8.5.1 Expectations of metagenome read assembly

The outcomes of metagenome assembly are difficult to anticipate. The number of assembled sequences ("contigs") and their length distribution depend on a number of factors, as does the total proportion of the short read-sequenced nucleotides which correspond to any of the contig sequences. These considerations are expanded upon in the Discussion. Further information describing the distribution of contig length and the detection of ARG sequences in assembled metagenomes be found in Appendix 3e

2.8.6 Quantitation

2.8.6.1 Unassembled reads

It is not uncommon for ARG-incidence publications to report unnormalised read counts, i.e. expressed only as a proportion of total reads. Other studies employ a length-based normalisation, while more rigorous approaches to normalisation, following the same tradition as RNA-seq, have also been gaining ground in the literature.

However, as described in Section 2.9, the subsequent modelling work requires either reliable true-positive, false-positive error rates in order to make use of incidence frequencies (such as based on read counts); or else, must be treated in a binary fashion, i.e. presence (anything non-zero) versus absence. Therefore, we have performed rigorous read-by-read assessment of all samples, to attempt to eliminate false positive matches (read ↔ reference alignments). This is described in more detail in Sections 2.8.7.2 and 2.8.7.3.

2.8.6.2 Assemblies

To obtain frequencies of each contig of an assembly, more sophisticated methods are necessary. Complexities arise from the fact that some mapped positions may be ambiguous, not all mapped positions make perfect matches, the frequencies of back-mapped reads may vary considerably along the contig's length, and other factors. In short, methods which effectively model the process in order to produce the best estimate of each contig's frequency in the sample, are required.

We used KALLISTO (Bray et al., 2016) which both performs the mapping and estimates "true" quantities from the results (this has been used in other metagenomics studies, for example (Bell et al., 2018)). The metric reported by KALLISTO is "transcripts (of the gene in question) per million total transcripts" (TPM).

We emphasise that the downstream modelling described here requires reliable **presence/absence** data, rather than quantitative data. Therefore, these data were not used quantitatively for the modelling.

However, the back-mapping results serve as a good indicator of consistency and quality of the assemblies. As described later, we performed back-mapping to both the full-length assembled contigs, and to the predicted ORFs therein.

For a small number of long contig sequences, we attempted to identify the species of origin using the Progenome classifier software (Mende et al., 2020, Mende et al., 2013). This is designed for single genome sequences, by typing using as many of 40 universal prokaryote marker genes as can be identified.

2.8.7 Screening RGI BWT read-ARG matches

As noted previously, the downstream modelling described here requires reliable presence/absence data. The simplest approach would be that presence of an ARG is indicated by one or more sequence reads which have been identified by the ARG-prediction software as being sufficiently similar to (part of) the sequence of that ARG. A more stringent approach would be to require a minimum of both reads of a single pair to match, and for more than one observation (read pair) to occur in the sample. Moreover, our *a priori* assumption was that in any given sample, a non-zero number of matches may be false positives, and that some of those may be easily recognisable as such on inspection. For these reasons, we applied strict screening criteria to the results output by the software, as described in the following sections.

2.8.7.1 RGI BWT output

RGI BWT outputs a "per-ARG" table for each sample, where each row represents a distinct ARG (in the CARD database). (There is also an analogous table at the allele rather than ARG level).

Here, we briefly describe this output and other output produced by RGI BWT, to clarify how we have processed this data.

RGI BWT creates the following:

- Various intermediate files which arise as part of the RGI processing.
- Tables (one row per ARG) which summarises averaged metrics (such as mean quality of matches between short-read pairs and the reference sequence(s) for that ARG).

In each row of the ARG table for a sample, the number of matching reads is specified in various contexts (such as wholly-matching and partially-matching; Appendix 3 contains further information on this). This effectively provides relative quantitative information, within the sample. Various other columns state some ARG-specific attributes (cross-references to ARO accessions, etc) and metrics describing the matches between the reads/read pairs and the ARG reference sequence (such as average coverage of the reference sequence in terms of percentage length and average length; average mapping-quality score of completely-matching reads; etc).

These various per-read (or per-read pair) quality metrics are necessarily *averaged*, since there is no *per-read* data. Therefore, individual read ↔ ARG reference matches cannot be inspected, and nor can they be screened further for the purpose of additional quality control.

However, a component of the temporary output of RGI BWT is a standard SAM-format alignment file (The SAM/BAM Format Specification Working Group, 2021), created by the short read-mapping software BowTie2 (Langmead et al., 2009, Langmead and Salzberg, 2012) This file contains details for each read that is mapped to (matched with) a reference ARG sequence. RGI BWT then uses this data to create the various summary tables.

The RGI BWT process and its output is described in more detail in Appendix 3. That also includes further information on how we assessed all the read-to-reference matches, and discarded some of these based on the details of each match, contained in the SAM file.

2.8.7.2 Assessing causes of identifiable false positives

Attributes of the read ↔ reference mapping to be considered can be broadly categorised as follows:

- Lengths of matching read segments and any mismatching segments
- Uniqueness of the mapping
- Plausibility of the sequences themselves irrespective of whether the read and reference sequences are very similar
- Sequence identity of the matching read segments.

Lengths of segments: When reads are mapped by BowTie2, it is common for segments (at either or both ends) to be "clipped" and omitted from the mapping, because they fail to match the reference sequence sufficiently well. There are essentially two reasons for this: part of the read extends beyond the end of the reference sequence ("overhangs"), i.e. that segment represents genomic sequence which is not part of the gene in question; or the segment is too dissimilar to the reference. Unless the latter type of segment (referred to here as an "unmapped segment") is very short, this is an indication that overall, the read does not represent a good match with the reference, even if the matched (mapped) segment is a perfect match. Note that mapped segments are not necessarily identical to the aligned part of the reference, nor should they be expected to be in all cases. The mapped and unmapped segments collectively constitute the parts of the read which notionally should have been aligned to the reference (whereas the overhanging segments would not be expected to be).

Mapped, overhanging and unmapped segments are illustrated in Appendix 3. Irrespective of the presence of unmapped or overhanging segments, the mapped segment needs to be

of sufficient length to have discriminatory value, in terms of uniquely identifying an ARG. This consideration is not only of discriminating between reference sequences in the database (CARD), but also in general; for example, a very short segment might occur not only in different bacteria but also in various other kingdoms of life. Further, we considered the relative length of any unmapped read segment in comparison with the total mapped + unmapped length.

Uniqueness: Some reads may equally well match multiple reference sequences; in brief, whether or not a read was uniquely mapped is apparent in the MAPQ score assigned by BowTie2. RGI BWT appears to ensure that only a single mapping (or no mappings) is present for each read, indicating that for some reads, equally good matches may not appear. Our approach is to discard these ambiguous reads, even though this may lead to some false negatives. This is part of the overall rationale to retain only reads which can be clearly matched to an ARG.

Plausibility of read and matching reference sequences: The issue here concerns long homopolymeric segments and also related long segments of sequences which are of very low complexity (for example dominated by one nucleotide base but interspersed with occasional instances of a different base). In terms of absolute frequencies, such segments are not rare in the sequence data sets and are also a known issue (especially with poly-G) with two-colour sequencing platforms including NovaSeq (De-Kayne et al., 2020).

Even if there were only a single long homopolymer sequence (i.e. assumed to be erroneous) in the reference database, then in a large NGS data set there are likely to be many instances of the same homopolymer sequence, which will match the reference well. This will result in an inflated count of this reference, and indeed often a false positive in terms of presence versus absence. Low-complexity segments can also match homopolymers (or each other) very well even if not completely identical.

Homopolymers in mapped read segments are easy to detect; low-complexity sequences require a different approach such as a sequence entropy calculation (here we use an

entropy calculation on the overall proportion p of each base, i.e. $\sum -p_x \ln(p_x)$ for x = { A, C, G, T }).

Sequence identity of the matching read segments: The required level of sequence identity between the mapped read segment and the aligned reference is conditional on the nature of the resistance attributed to the ARG. In brief, unless the sequence segments are very short, the levels of sequence identity that will necessarily occur for most mapped segments will be sufficient to infer homology and often the likely function. That will not always amount to identifying a particular ARG, but high identities, even if considerably below 100%, will often indicate that the ARG or a variant thereof has been identified.

However, some ARGs, referred to as "variant-mutation ARGs" have sequences near-identical to those of non-ARGs, but exhibit a small number of SNPs (possibly one) which prevent some antibiotics from binding to them. Besides these target molecule-encoding genes, it is possible that other AMR types are conferred by such mutations (the range of antibiotic substrates of degrading enzymes could conceivably be broadened in this way). These "variants" are curated in CARD, and when assessing reads mapped to such references, 100% identity is required.

The ARO ontology (maintained alongside the CARD data) under the "determinant of antibiotic resistance" branch (ARO:3000000), includes a term ARO:0000031, "antibiotic resistant gene variant or mutant".

Of course, with short-read data, even a 100%-identical match does not usually confirm that the referenced AMR gene (AMR variant allele) is present; it merely indicates that that part of the gene that was sequenced is *consistent* with that allele. This can still lead to false positives if the sequenced segment of the gene is identical in the AMR and non-AMR alleles. Nonetheless our approach is to reject matches only where there is evidence that they are not a known AMR variant of the gene (i.e. identity < 100%).

2.8.7.3 Quality criteria used for screening

The *retention* quality criteria used for screening are as follows; refer to section 2.8.7.2 for definitions of the "mapped", "overhanging" and "unmapped" segments. Unless otherwise stated, these criteria apply to the mapped segments.

- Absence of any homopolymers longer than a maximum threshold (12 bp)
- Sequence entropy of a magnitude greater than a minimum threshold (1.1)
- MAPQ score of at least a minimum threshold (2)
- Total length of all unmapped segments does not exceed a threshold proportion (25%) of the total unmapped + mapped segments length
- Mapped segment length of at least a minimum threshold (45 bp)
- Total mapped segment length of both reads of the pair of at least a minimum threshold (75 bp)
- Conditional upon the AMR type (as annotated by the ARO term), a minimum percentage identity between the mapped segment and the aligned reference segment (if the ARG is annotated as ARO:0000031 or as any descendant ARO term, then the minimum identity is 100%; for all other ARGs, no minimum identity is required).

Excepting the penultimate criterion (the only one which considers reads as a pair), any single read which fails any of the above is discarded, irrespective of the performance of its paired read. Both reads are discarded if the pair fails the minimum combined-length requirement.

With the rationale of requiring independent sequence read observations of each ARG within each sample, we applied a final filter on the basis or read-pair counts: an ARG was counted as positive only if at least two read pairs passed the previous filters, where both reads of the pair mapped to the same ARG.

2.8.8 Antibiotics of particular concern

During the analysis stage and as an addition to the work originally proposed, FSA requested that genes encoding resistance to six antibiotics, groups of antibiotics, or genes conferring AMR phenotypes of particular concern were flagged. These were;

- Colistin
- Methicillin
- Vancomycin
- Carbapenem antibiotics
- Fluoroquinolone antibiotics
- ESBL (Extended Spectrum Beta Lactamase) ARGs

The antibiotic resistance literature is vast, and it was beyond the scope and resource of the current project to manually assess the antibiotics to which all the genes identified conferred or may confer resistance. Instead, it was decided to utilise the ARO ontology, which flags the antibiotics to which a particular ARG confers resistance. This is extremely useful, although there are a number of limitations to this approach, which largely depend on hierarchical level of the antibiotic/antibiotic class of interest. Fluoroquinolone and carbapenem antibiotics are directly flagged in the results from RGI (see Appendix 8). However, the others were not, and this necessitated an inspection of ARO to identify ARO terms that were annotated as conferring resistance to the antibiotics of interest. For methicillin and vancomycin, a text search of the ARG names from our analysis for mention of these antibiotics was also performed.

ESBLs were even more challenging, as there is not a single, agreed definition of ESBL (Paterson and Bonomo, 2005), and ESBL phenotype is not consistently annotated within ARO. For these genes it was agreed with FSA to highlight genes for which some alleles can confer an ESBL phenotype and label them as potential ESBL genes. These were CTX, SHV, TEM, OXA, VEB, GES, PER, IBC, BES and TLA.

Finally, for vancomycin and colistin, it was observed that a number of genes which are known to confer resistance were present, but not flagged by the ARO terms. In these

instances, van genes (for vancomycin resistance) and MCR genes (for colistin resistance) were identified by a further text search.

It is therefore possible (and indeed likely) given the limitations of the ARO that some genes conferring resistance to these antibiotics of interest are present but have not been identified. However the approach outlined above was the only feasible way to undertake this analysis given the starting point of the project, which was to estimate the presence and burden of AMR genes, rather than of resistance to antibiotics.

2.8.9 Priority Taxon Detection

Refer to 1.2 Aims and objectives. This part of the analysis does not relate to the AMR study, and was performed on request by the FSA for food-safety reasons. Potentially, the results could be used in future to relate to findings regarding ARGs in the samples concerned, but that was not an objective here.

The object was to identify priority taxa of concern in any samples still within the use-by date. At the time of purchase all samples were within the expiry date (if stated) and were processed for DNA extraction promptly. The expiry date of only a few samples (such as ice cream) had not yet passed by the time the sequence data was available for analysis. The analysis used the short-read NovaSeq metagenomics data, and the contig sequences assembled from them.

The list of priority taxa was supplied by the FSA, and was the same as that used for the earlier screening of the 1,001 16S metabarcoding data sets (2.5.2): *Campylobacter*, *Enterococcus faecalis*, *Salmonella*, *Enterococcus faecium*, *Shigella*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Clostridium botulinum*, *Staphylococcus aureus*, *Clostridium perfringens* and *Yersinia enterocolitica*.

Briefly, the procedure followed these steps:

- Analysis of the **unassembled short reads of all samples**, using a relatively fast, widely used tool designed for this purpose.

- For those samples which were still within expiry data and tested positive for any of the priority taxa in (1), the following analyses were conducted on all of the sample's **assembled contigs:**
- Contigs were compared to the sequences of a small number of "classical marker genes", i.e. the Multi Locus Sequence Typing (MLST) loci (where available) since these are essentially diagnostic of the organisms' DNA
- Since MLST genes are few in number and are thus often likely to not be sampled by metagenomics sequencing due to chance, the contigs were also compared with a more general sequence database; many more of the contigs are likely to yield databases matches, with the disadvantage that many database sequences may not discriminate between taxa, and that the provenance of taxonomic annotation of these database sequences is on average much less authoritative than those in an MLST database.

Further details of the methodology are in Appendix 4.

2.8.10 Assessment of the use of 16S Metabarcoding to screen samples prior to metagenomic sequencing

The 54 samples belonging to food types labelled as 'low bacteria' by 16S sequencing (Table 6) were analysed, to see if these same samples also had low bacterial concentrations when sequenced metagenomically. The level of bacteria was defined by inspecting the distribution of bacteria and host reads across each sample type. ‘Low bacteria’ was assigned to food categories that had the majority of the samples with 75% or greater host reads. In order to estimate the number of bacterial and host reads in each sample, Kraken2 (Wood et al., 2019), a taxonomic sequence classifier, was run on all 256 samples. Reads counts for any reads which were labelled as either bacteria or unclassified were extracted for subsequent analysis. As the supplied Kraken2 database is intended for identifying bacteria, archaea and viruses, 'unclassified' hits can generally be attributed to host sequence, and thus can be used as a proxy for host sequences.

The 10 samples which had failed 16S sequencing QC steps and were selected for metagenomic sequencing (section 2.6.1) were also analysed. Total read numbers from metagenomic sequencing for all 256 samples were ranked from high to low, and the ranks of the 10 'failed' samples were inspected.

2.8.11 PromethION Data Analysis

The order of analysis for the PromethION data is shown in Figure 3.

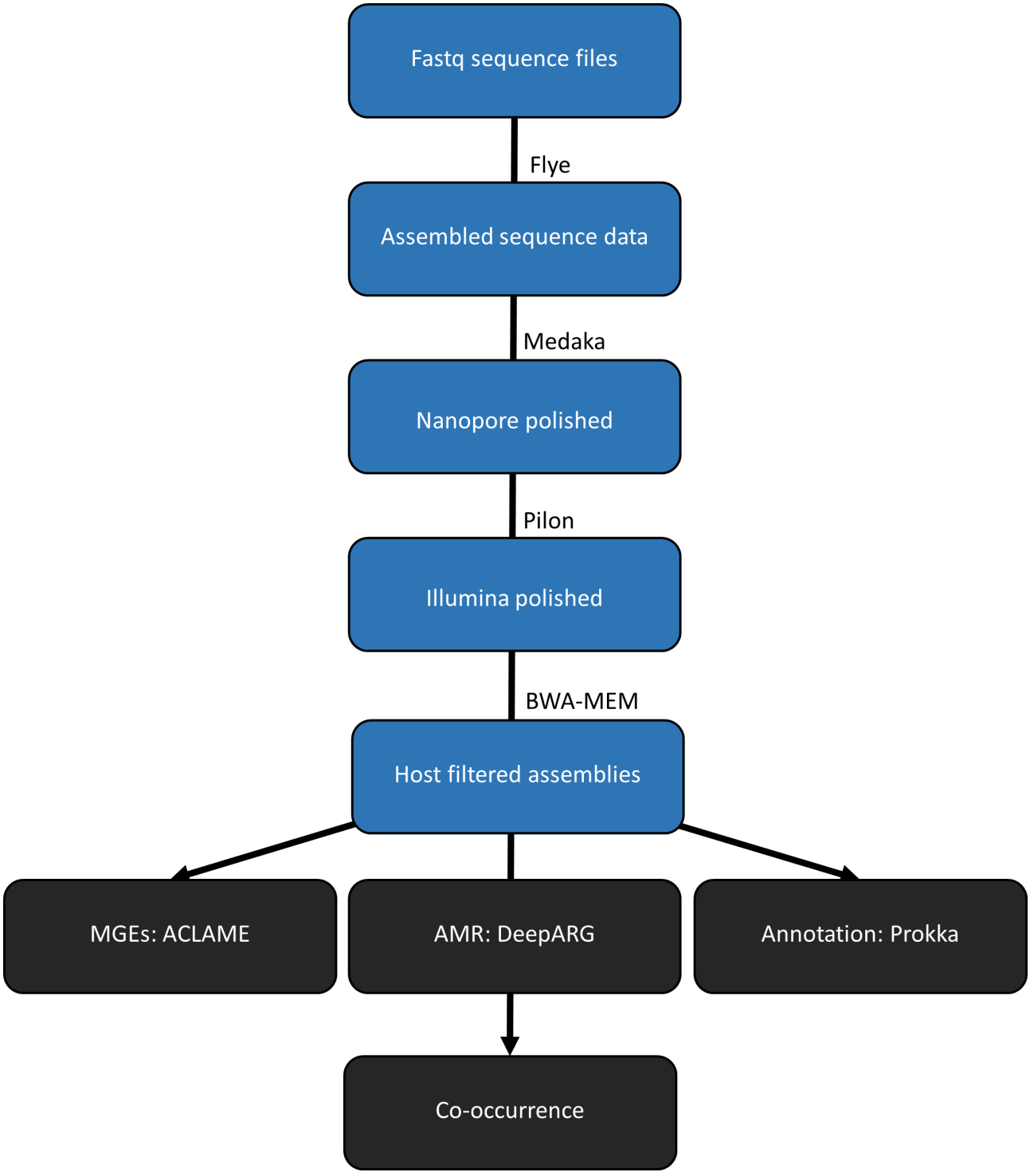

Fastq sequence files
Flye
Assembled sequence data
Medaka
Nanopore polished
Pilon
Illumina polished
BWA-MEM
Host filtered assemblies
MGEs: ACLAME
AMR: DeepARG
Annotation: Prokka
Co-occurrence

Figure 3 Flowchart outlining the main steps taken in the analysis of the long-read data. Blue boxes indicate the outputs of bioinformatics tools. Grey boxes indicate the outputs relating directly to ARGs and MGEs.

2.8.11.1 Assembly

As the average read quality for the run was good, but the total number of reads generated was low, it was decided that no quality threshold would be imposed prior to the assembly step, therefore allowing all of the reads to contribute towards assemblies. The removal of host sequences at this step was also deemed unnecessary, as this would not negatively impact the assembly time, and can be subsequently removed after this step.

The Flye assembler (Kolmogorov et al., 2020) was used to assemble the samples. The 'nano-raw' parameter was used to address the fact that input data had not been error corrected, and the 'meta' parameter was used to run the assembler in metagenome aware mode. Of the 24 samples sequenced, 18 produced sufficient data for assemblies to be generated.

Polishing is a means to increase the accuracy of assemblies, and can be accomplished both with short and long reads. Firstly, the long read polishing tool, Medaka, (Oxford Nanopore Technologies Ltd, 2018) was used to increase the accuracy of the assemblies, before subsequent polishing with the short read polisher, Pilon (Walker et al., 2014). BWA-MEM (Li, 2013) was used to remove host sequences from the assembled and polished data. Prokka (Seemann, 2014) was used to annotate the assemblies with gene references where possible. However, as this is low-coverage metagenomic nanopore data, prokka's ability to annotate accurately will be somewhat reduced due to indels (insertions/deletions) and homopolymer errors causing incomplete genes to be identified, or possibly entirely missed.

2.8.11.2 DeepARG and NanoARG

DeepARG (Arango-Argoty et al., 2018) is a machine learning tool that uses deep learning to characterise and annotate antibiotic resistance genes in metagenomes. Due to its deep learning nature, DeepARG was used on this initial set of long read data as an experimental approach, to evaluate how suited it is to handling the unique problems of Nanopore sequencing. NanoARG (Arango-Argoty et al., 2019) is a web service provided by the same group as DeepARG, which features an adapted version of the DeepARG pipeline. Samples were also submitted to this web service to assess the differences between the two analysis pipelines. To identify instances of co-occurrence, AMR genes which were identified by DeepARG as belonging to the same contig were extracted, and the start and end positions of the ARG were noted. The ACLAME database (Leplae et al., 2010) was used to check for any Mobile Genetic Elements (MGEs) present in the dataset. This was performed by first converting the database into a BLAST (Altschul et al., 1990) database, and then running a BLASTn (megablast) search to identify any matches. Due to indels and homopolymer errors of Nanopore sequencing, coupled with the low coverage of the data, it is difficult to convincingly separate errors from real MGE hits.

2.8.11.3 Hybrid-Spades vs Meta-Spades Assembler Comparison

As the amount of data generated from the PromethION was low, assemblies created using primarily the long-read data will result in less informative results. To evaluate how long-read data can instead be used to improve primarily short-read assembly tools, the best performing 14 long-read samples were assembled using Spades (Prjibelski et al., 2020) in both meta (for metagenomic assembly of illumina short read data) and hybrid (for assembly using both long nanopore and short illumina reads) modes. DeepARG was run on both assembly datasets, and co-occurrences were calculated.

2.9 Estimation of population burden

2.9.1 Dietary Consumption Data

Data from the UK National Diet and Nutrition Survey (NDNS) were extracted from the UK Data Archive (NatCen Social Research, 2019). The data included the same year 1-8 survey records as used in prioritising the sampling plan, collected between 2008/09 and

2015/16, plus the recently added year 9 records (2016/17). Sample weightings were recalculated to account for the differences in sample sizes between years. The total number of individuals included in the combined dietary dataset is 13350. The NDNS sampling strategy ensures these are representative of the UK population, including children and adults. The consumptions of the sampled RTE items were extracted for each individual, to provide information about the combinations of those items consumed per person within the population.

2.9.2 Assumptions Based on Sample Design

The sampling strategy is described in Section 2.1.2. It covers sample food types such that 90% of consumption of RTE foods was covered, and sample numbers were in proportion to consumption with a minimum of five samples per food type. The total number of samples is 256. While fewer types of dairy were sampled than types of produce, more than half of the samples taken were dairy samples. The number of samples of each food type taken from each UK region was in proportion to its population. Samples were taken from the eight largest supermarket retailers according to market share. Hence the collection of samples was designed to be and is assumed to be representative of the overall population consumptions of RTE products in the dairy, produce and meat categories.

2.9.3 Possible Measures Related to AMR Burden in the UK Diet

We can refer to burden in a single ready to eat food type as the incidence and prevalence of AMR genes in that food. In order to compare with other food types, the measures used should be consistent between foods. For a single ARG, in general terms we define incidence as the presence/absence of the ARG and prevalence as the frequency with which it appears. More precise definitions are required depending on whether the incidence and frequency relate to a single food type or to the overall population diet as a whole.

For incidence, we identify the ARGs found at least once within the samples of a single food type (incidence per food) or found at least once across samples of all consumed types (incidence in UK diet)

For prevalence, there are two possible quantities of interest. The first is the proportion of retail samples of individual ready to eat food types (for example what proportion of semi-skimmed milk samples in the UK contain a particular ARG or ARG type). Second is the prevalence in the UK diet overall (what proportion of individuals are exposed to a particular ARG or ARG type). Prevalence in the ready to eat portion of the UK diet is estimated from multiple foods and accounts for the consumption amounts of each food type as recorded in the UK dietary survey (NDNS). The calculation is defined in equation (1) below. The two measures of burden defined here can be further refined by considering subsets of ARGs according to classifications of interest. Following discussions with the FSA the following were identified as classes of interest and led to separate burden calculations:

- all ARGs
- ARG gene families
- ARGs linked to colistin resistance
- ARGs linked to carbapenem resistance
- ARGs linked to methicillin resistance
- ARGs linked to vancomycin resistance
- ARGs linked to fluoroquinolone resistance
- Antibiotic resistant gene variant or mutant
- Potential ESBL activity

2.9.4 Incidence Summaries at Sample/Food Level

First, we estimate the proportion of samples (of a given food type) containing a specific ARG. This is consistent with previous literature on measurement of AMR in foods. After calculating the incidence of individual ARGs in all samples (and within a food type) the following summaries can be derived by simple aggregation

- Number of ARGs in each single sample, and the between-sample range seen in this number.

- Number of unique ARGs or ARG families found across all samples of a given food type

2.9.5 Incidence: Total UK Diet

The 52 sampled food types are consumed within the UK and appear in the NDNS consumption diary data. Therefore, the incidence and the number of distinct ARGs in the diet can be estimated directly from the data. A lower bound estimate is simply the number of unique ARGs combined across all the collected samples. It is possible that others are present in the wider diet but not measured due to the limited sample sizes.

2.9.5.1 Prevalence Calculations, per Food

At the individual food level, we can compute the proportion of samples containing individual ARGs (direct count of samples, relative to the total number of samples obtained for any given food type). This is important for understanding which ready to eat food types in UK retail are potential sources of AMR, and the relative levels of different AMR genes.

2.9.5.2 Frequency (Relative Number of Samples) of a given ARG in a Food-Specific Dataset

These can be compared across food categories by plotting or tabulating the data (for example see Figure 16).

2.9.6 Prevalence Calculations, Population Level

When considering a particular ARG, the prevalence is defined as the proportion of UK individuals whose diet contain that ARG. This accounts for the typical combinations of RTE products that are consumed by single individuals and also the incidence of AMR found in the measured samples. It is relevant to assess how often there is potential for transmission of AMR genes in the human gut, and which ARGs are most prominent. Each individual consumption is linked to the AMR incidence of the combined samples for the relevant food type. This assumption is appropriate when considering the long term prevalence, because each individual will consume different products over time.

For some individuals, multiple consumptions for the same food type occur within the survey. They may be from the same source and have similar ARG profile, or may be from different items. If an individual consumes multiple items with the same ARG present, this is considered the same as if only one of those items contained the ARG. For the purpose of this modelling exercise, it is assumed that there is no aggregation effect (prevalence is proportion of individuals, not proportion of eating events). We are also considering the overall population, with many different dietary preferences. Individual subgroups with special dietary habits, for example vegetarians, could be analysed separately if the AMR burden for those subgroups were of particular interest.

Suppose we have N individuals $i = 1,2, \dots, N$ in the dietary survey with survey sampling weights w_i. Consider the food types $k = 1,2, \dots, K$ $(K = 52)$ included in the 256 analysed samples. Let j index a particular ARG ($j = 1,2, \dots, J$). We define the incidence indicator I_{ijk} to be 1 if ARG j is present in food k consumed by individual i and 0 otherwise. Another indicator C_{ik} is set to 1 if food k is consumed by individual i and 0 otherwise. A measure of the total prevalence of ARG j can then be defined as

$$B_j = (1/\sum_{i=1}^{N} w_i) \sum_{i=1}^{N} w_i \max_k (I_{ijk}\, C_{ik}) \qquad (1)$$

For each ARG, the empirical proportion of all NDNS diets containing the ARG was calculated using (1) to estimate B_j. Diary survey values for the consumptions of food items were used for C_{ik}. Because all individuals are assumed to consume from the same pool of samples over a long time period, $I_{ijk} = I_{jk}$ (same for all individuals) where

$$I_{jk} = \begin{cases} 1 & 1 \text{ or more samples of food type } k \text{ contains arg } j \\ 0 & \text{otherwise} \end{cases}$$

An indication of the sampling uncertainty in these estimates is provided by generating 100 bootstrap samples (for each food type, re-sampling with replacement from its original data). The 100 prevalence estimates were used to plot approximate 95% confidence intervals.

These uncertainties do not include measurement uncertainty (i.e. in the absence of an explicit estimate of the false positive and negative rates associated with the identification of ARGS in samples we assume that the detection of the presence of ARGS is reliable) or sampling uncertainty in the dietary consumption surveys. The latter is expected to be extremely small compared to the sampling uncertainty in ARG measurements, as there are 13350 individuals surveyed. The numbers of RTE foods sampled are much smaller and often show variability between measurements (for example as seen in Figure 12). The uncertainty intervals are included only to highlight the possible impact of sampling uncertainty. Due to the nature of the burden definition in (1), the true proportions are more likely to be at the upper end of the interval under the assumption of a low false positive rate, because increasing the number of samples for a particular food would only lead to higher proportions of diets containing the ARG.

2.9.6.1 Possible Unquantified Uncertainties

There are multiple potential sources of uncertainty associated with the sample workflow which may affect how representative the list of AMR genes presented in this report are of the AMR genes which are present in the largest proportion of UK diets. While these cannot be quantified from the data obtained in this study, some of these sources are discussed in section 4.1.1.

3. Findings

3.1 Sampling Strategy

3.1.1 Literature Review

Only a minority of the publications provided by our literature search provided useful quantitative data on the incidence of AMR in foods, as follows.

Our list of search terms was arrived at by a process of trial and refinement. Although our final query involves a lengthy series of precise terms, it was still difficult to differentiate between studies of the retail context and various other research purposes. For example, some published studies investigated efficacy of various post-harvest treatments on produce, which involved experimental inoculation of vegetables prior to assaying for bacteria/AMR. Further false positives, despite being necessarily positive for “ready to eat” or “food” terms, included several laboratory studies on antimicrobial activity of novel bacteriocins (often derived from *Lactobacillus* or *Lactococcus* strains from “milk”, for example). Other studies sampled as-is produce or meat products etc, but in the pre-retail context including both pre- and post-harvest, and within processing factories, sometimes at numerous stages of production.

Numerous other studies can be assumed to have produced quantitative data of the kind we required, but simply did not report them in sufficient detail. Commonly, data on the proportion of AMR isolates of a specified bacterial species made no reference to the number of samples from which they were obtained, either explicitly or as a proportion of the total samples of that food type. Also commonplace was the still less informative reporting (in multi food-type studies) where these AMR isolate proportions did not even reference the food types from which they were obtained.

Data on AMR presence in ready to eat foods was gathered from 48 sources describing studies in which ready to eat foods had been tested for the presence of AMR bacteria. Information about 30 foods (lettuce, cucumber, spinach, carrot, salami, tomato, celery, apples, peppers, grapes, dates, peaches, watermelon, pears, nectarines, roast duck, beef

salami, ham not smoked, iceberg lettuce, ice cream, smoked salmon, chicken slices unsmoked, milk (skim status unspecified), strawberries, melon, shrimp, orange juice freshly squeezed, butter, yoghurt, cream), 46 microbiological taxa and 110 *types* (gene or antibiotic) of resistance was provided.

Figure 4 shows a summary of the information provided by the 48 studies. Table 3 gives the references used for each food type.

In general, several foods were tested within each study for the presence of a number of bacterial taxa. In some studies the initial detection of taxa was undertaken by growing bacteria on media that contained antibiotics. In other studies taxa were detected and then tested for AMR. For the purposes of this review a sample was counted as positive for bacteria if it tested positive for any of the bacterial taxa tested, and positive for the presence of AMR if any of the AMR tests were positive. Because of the way that information was presented in many of the studies we reviewed, we could gain information on the number of samples in which particular types of AMR were detected, but not number of different types of AMR present in individual samples. Table 4 and Figure 5 show a summary of the proportion of samples in which AMR presence was found in the 25 studies for which we were able to estimate this quantity.

There is considerable variation in the proportion of samples of the same food type found to contain AMR across studies. Part, but not all of this variation is caused by estimating proportions from small numbers of samples. A more important source of variation may be related to studies having different scopes: Table 5 shows a summary of 14 studies that examine AMR in *Lettuce*. Studies report between 0 and 94% of samples contain AMR. The main driver for this is differences in bacterial taxa studied between studies, with higher AMR rates being associated with the less-targeted (Bacterium, species not specified) study and more common taxa (for example *Staphylococcus* spp.). There is also variation that is not associated with differences in bacterial taxa, or random variation driven by small sample sizes. For, example the two studies reporting the presence of AMR associated *Salmonella enterica* reported very different proportions (5%, 0.003% $p<0.0001$).

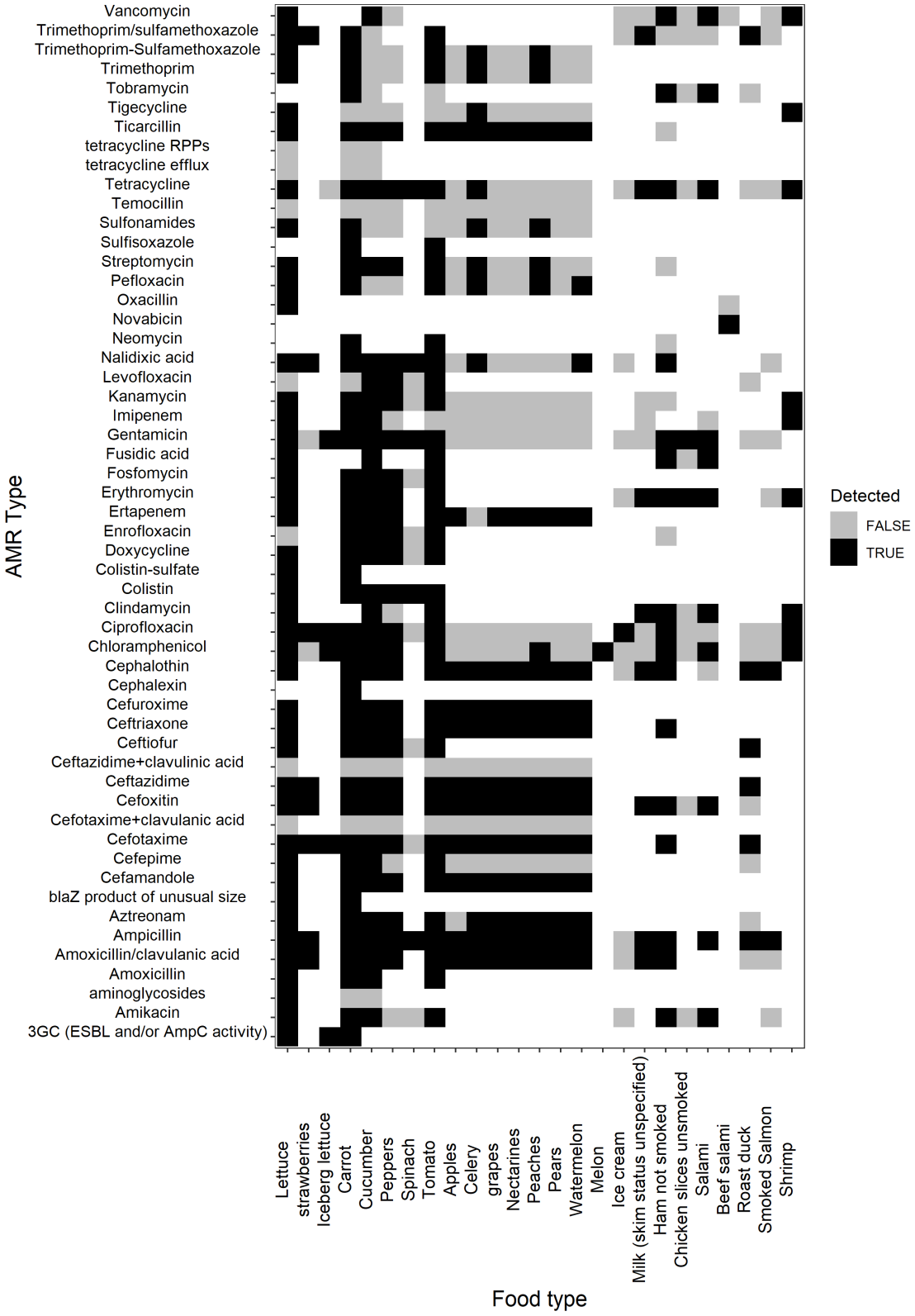
No evidence was found of large differences in the proportion of samples found to contain AMR in different food groups (dairy, meat, produce, seafood). However, given the foregoing discussion and the small number of studies, this is probably better characterised as absence of evidence than strong evidence of absence.

Table 3. References from which AMR data were extracted for each food type.

Food Type	References Reviewed
Apples	(Mesbah Zekar et al., 2017)
Beef Salami	(Organji et al., 2018)
Butter	(Cetinkaya et al., 2014)
Carrots	(Liu et al., 2019) (van Hoek et al., 2015) (Bamidele et al., 2017) (Gutierrez-Alcantara et al., 2016a) (Jones-Dias et al., 2016) (Karumathil et al., 2016) (Akoachere et al., 2018) (Mesbah Zekar et al., 2017) (Ali, 2019)
Celery	(Mesbah Zekar et al., 2017)
Chicken slices unsmoked	(Fijalkowski et al., 2016)
Cream	(Cetinkaya et al., 2014)
Cucumber	(Chen et al., 2015) (Bamidele et al., 2017) (Akoachere et al., 2018) (Liu et al., 2019) (Wu et al., 2018) (Mesbah Zekar et al., 2017) (Ye et al., 2018) (Ling et al., 2018)
Dates	(Mesbah Zekar et al., 2017)
Grapes	(Mesbah Zekar et al., 2017)
Green Pepper/Peppers	(Akoachere et al., 2018) (Liu et al., 2019) (Park et al., 2018) (Mesbah Zekar et al., 2017)
Ham not smoked	(Iulietto et al., 2016) (Domenech et al., 2015) (Fijalkowski et al., 2016) (Pesavento et al., 2014) (Cetinkaya et al., 2014)
Ice cream	(Domenech et al., 2015)

Food Type	References Reviewed
Iceberg lettuce	(van Hoek et al., 2015) (Bhutani et al., 2015) (Berthold-Pluta et al., 2017) (Ortega-Paredes et al., 2018)
Lettuce	(Niyomdecha et al., 2016) (Almeida et al., 2015) (Cesar et al., 2015) (Chen et al., 2015) (van Hoek et al., 2015) (Igbeneghu and Lamikanra, 2015) (Higgins et al., 2017) (Bamidele et al., 2017) (Bokai et al., 2017) (Jones-Dias et al., 2016) (Karumathil et al., 2016) (Byrne et al., 2016) (Adzitey, 2018) (Akoachere et al., 2018) (Liu et al., 2019) (Wu et al., 2018) (Yi et al., 2018) (Park et al., 2018) (Mesbah Zekar et al., 2017) (Berthold-Pluta et al., 2017) (Ortega-Paredes et al., 2018) (Wood et al., 2015) (Ling et al., 2018)
Melon	(Reddy et al., 2016)
Milk (skim status unspecified)	(Gao et al., 2018) (Wu et al., 2019)
Nectarines	(Mesbah Zekar et al., 2017)
Orange juice freshly squeezed	(Berthold-Pluta et al., 2017)
Peaches	(Mesbah Zekar et al., 2017)
Pears	(Mesbah Zekar et al., 2017)
Roast Duck	(Ye et al., 2018)
Salami	(Fijalkowski et al., 2016) (Federici et al., 2014) (Cetinkaya et al., 2014) (Rahimi et al., 2017)
Shrimp	(Igbinosa and Beshiru, 2019)
Smoked Salmon	(Domenech et al., 2015)

Food Type	References Reviewed
Spinach	(Liu et al., 2019)
Strawberries	(Kurtboke et al., 2016) (Jones-Dias et al., 2016) (Ortega-Paredes et al., 2018)
Tomato	(Gutierrez-Alcantara et al., 2016b) (Reddy et al., 2016) (Estepa et al., 2015) (Liu et al., 2019) (Wu et al., 2018) (Mesbah Zekar et al., 2017) (Ye et al., 2018) (Ortega-Paredes et al., 2018) (Ling et al., 2018)
Watermelon	(Mesbah Zekar et al., 2017)
Yoghurt	(Cetinkaya et al., 2014)


AMR Type
Vancomycin
Trimethoprim/sulfamethoxazole
Trimethoprim-Sulfamethoxazole
Trimethoprim
Tobramycin
Tigecycline
Ticarcillin
tetracycline RPPs
tetracycline efflux
Tetracycline
Temocillin
Sulfonamides
Sulfisoxazole
Streptomycin
Pefloxacin
Oxacillin
Novabicin
Neomycin
Nalidixic acid
Levofloxacin
Kanamycin
Imipenem
Gentamicin
Fusidic acid
Fosfomycin
Erythromycin
Ertapenem
Enrofloxacin
Doxycycline
Colistin-sulfate
Colistin
Clindamycin
Ciprofloxacin
Chloramphenicol
Cephalothin
Cephalexin
Cefuroxime
Ceftriaxone
Ceftiofur
Ceftazidime+clavulinic acid
Ceftazidime
Cefoxitin
Cefotaxime+clavulanic acid
Cefotaxime
Cefepime
Cefamandole
blaZ product of unusual size
Aztreonam
Ampicillin
Amoxicillin/clavulanic acid
Amoxicillin
aminoglycosides
Amikacin
3GC (ESBL and/or AmpC activity)
Detected
FALSE
TRUE
Lettuce
strawberries
Iceberg lettuce
Carrot
Cucumber
Peppers
Spinach
Tomato
Apples
Celery
grapes
Nectarines
Peaches
Pears
Watermelon
Melon
Ice cream
Milk (skim status unspecified)
Ham not smoked
Chicken slices unsmoked
Salami
Beef salami
Roast duck
Smoked Salmon
Shrimp
Food type

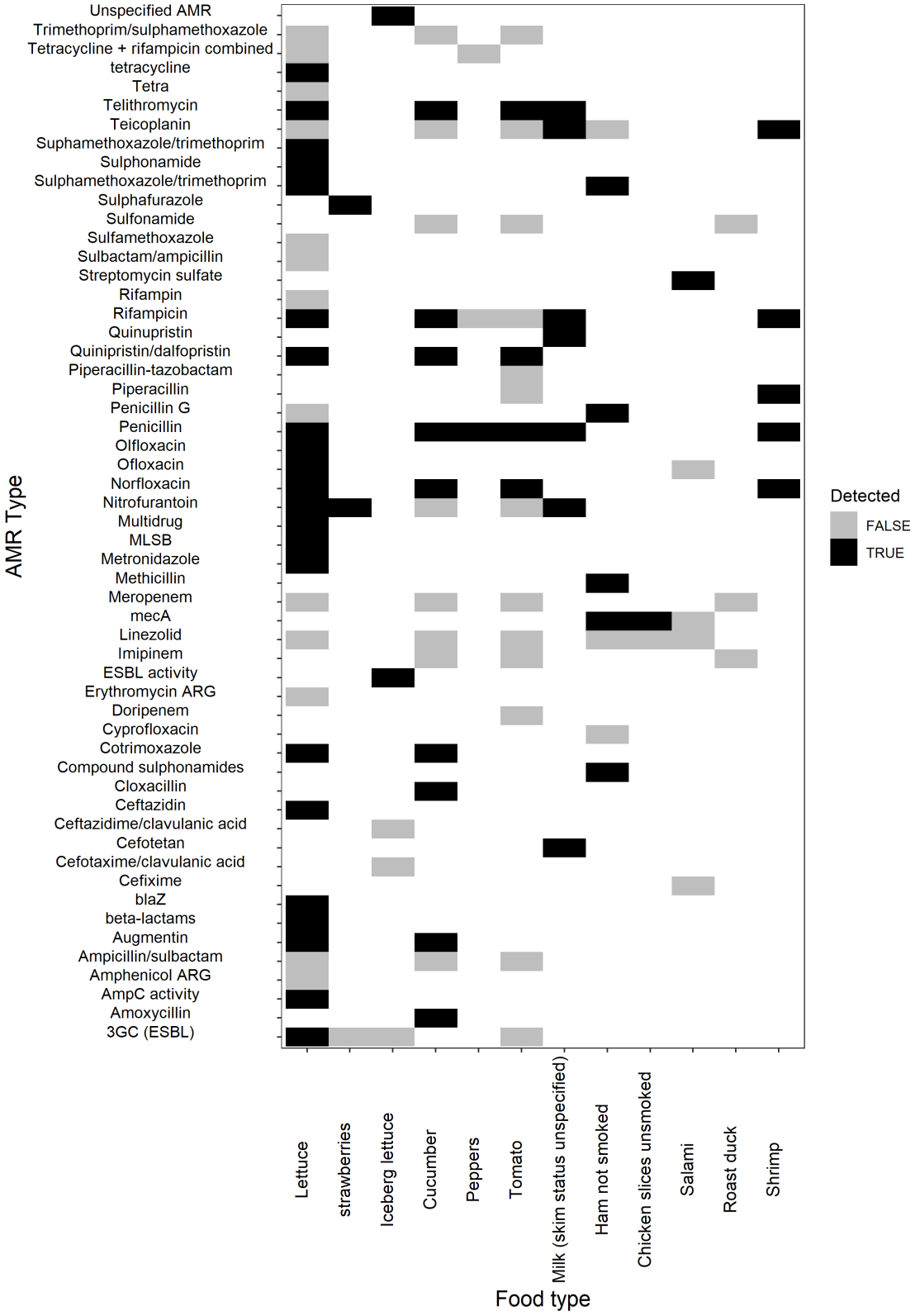

Unspecified AMR
Trimethoprim/sulphamethoxazole
Tetracycline + rifampicin combined
tetracycline
Tetra
Telithromycin
Teicoplanin
Suphamethoxazole/trimethoprim
Sulphonamide
Sulphamethoxazole/trimethoprim
Sulphafurazole
Sulfonamide
Sulfamethoxazole
Sulbactam/ampicillin
Streptomycin sulfate
Rifampin
Rifampicin
Quinupristin
Quinipristin/dalfopristin
Piperacillin-tazobactam
Piperacillin
Penicillin G
Penicillin
Olfloxacin
Ofloxacin
Norfloxacin
Nitrofurantoin
Multidrug
MLSB
Metronidazole
Methicillin
Meropenem
mecA
Linezolid
Imipinem
ESBL activity
Erythromycin ARG
Doripenem
Cyprofloxacin
Cotrimoxazole
Compound sulphonamides
Cloxacillin
Ceftazidin
Ceftazidime/clavulanic acid
Cefotetan
Cefotaxime/clavulanic acid
Cefixime
blaZ
beta-lactams
Augmentin
Ampicillin/sulbactam
Amphenicol ARG
AmpC activity
Amoxycillin
3GC (ESBL)
AMR Type
Lettuce
strawberries
Iceberg lettuce
Cucumber
Peppers
Tomato
Milk (skim status unspecified)
Ham not smoked
Chicken slices unsmoked
Salami
Roast duck
Shrimp
Food type
Detected
FALSE
TRUE

Figure 4. Summary of AMR information provided by 48 studies

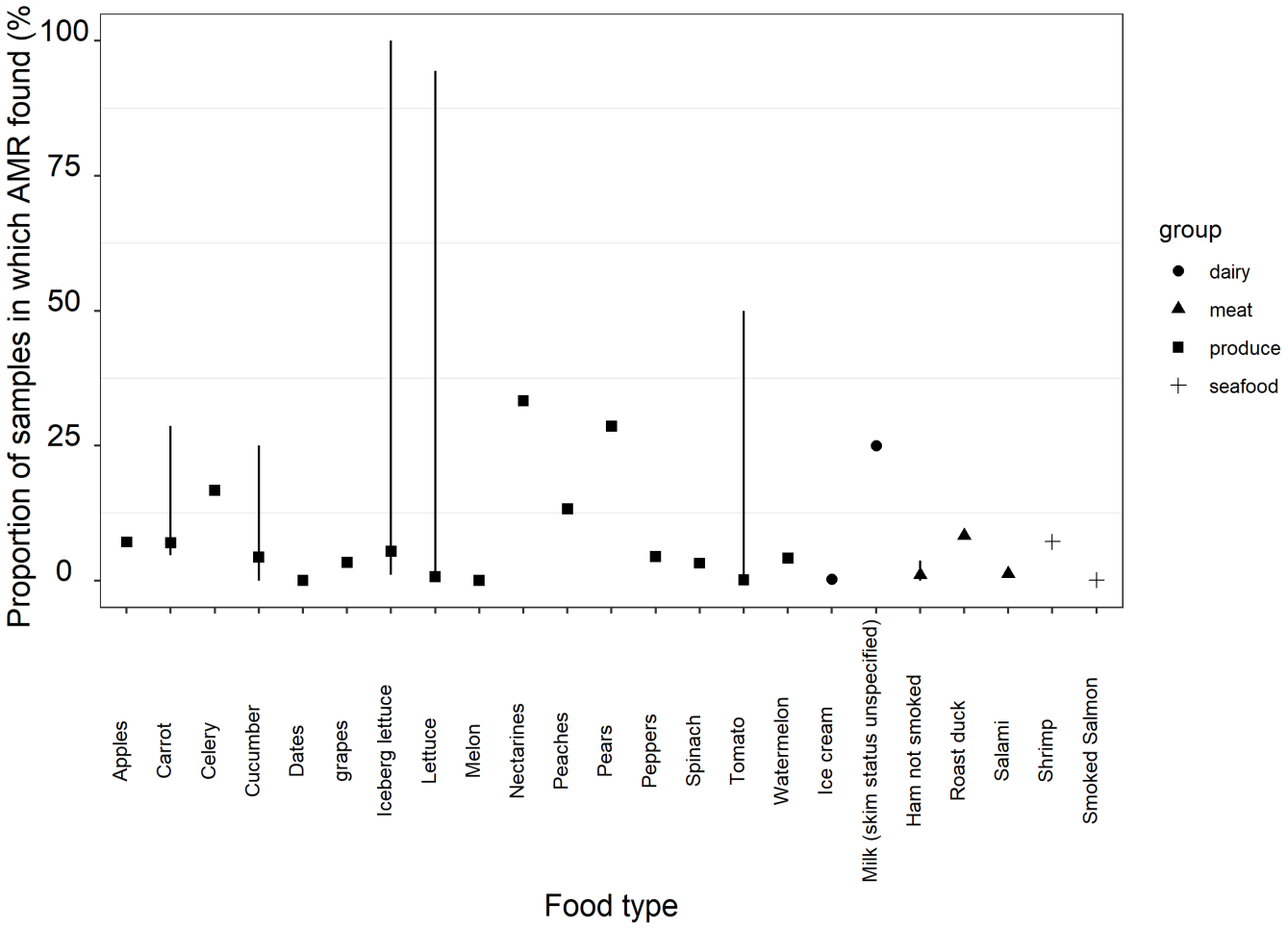


Figure 5. Proportion of food samples in which AMR presence was detected. Points show mean across studies, lines show maximum and minimum rate reported in individual studies

Table 4. Samples tested for AMR in 25 studies

Group	Food type	Studies	Number of samples tested	Number of samples: positive bacteria	Number of samples: positive AMR	Samples positive for AMR (%): mean	Samples positive for AMR (%): minimum	Samples positive for AMR (%): maximum	Samples positive for AMR among samples containing bacteria (%): mean	Samples positive for AMR among samples containing bacteria (%): minimum	Samples positive for AMR among samples containing bacteria (%): maximum
dairy	Ice cream	1	758	6	2	0.264	0.264	0.264	33.3	33.3	33.3
dairy	Milk	1	276	70	69	25	25	25	98.6	98.6	98.6
meat	Ham Not Smoked	2	674	20	7	1.04	0	3.74	35	0	58.3
meat	Roast duck	1	12	1	1	8.33	8.33	8.33	100	100	100

Group	Food type	Studies	Number of samples tested	Number of samples: positive bacteria	Number of samples: positive AMR	Samples positive for AMR (%): mean	Samples positive for AMR (%): minimum	Samples positive for AMR (%): maximum	Samples positive for AMR among samples containing bacteria (%): mean	Samples positive for AMR among samples containing bacteria (%): minimum	Samples positive for AMR among samples containing bacteria (%): maximum
meat	Salami	2	160	2	2	1.25	0.833	2.5	100	100	100
produce	Apples	1	14	1	1	7.14	7.14	7.14	100	100	100
produce	Carrot	5	413	29	29	7.02	4.74	28.6	100	100	100
produce	Celery	1	6	1	1	16.7	16.7	16.7	100	100	100
produce	Cucumber	4	231	46	10	4.33	0	25	21.7	0	100
produce	Dates	1	2	0	0	0	0	0	NA	NA	NA
produce	grapes	1	30	1	1	3.33	3.33	3.33	100	100	100

Group	Food type	Studies	Number of samples tested	Number of samples: positive bacteria	Number of samples: positive AMR	Samples positive for AMR (%): mean	Samples positive for AMR (%): minimum	Samples positive for AMR (%): maximum	Samples positive for AMR among samples containing bacteria (%): mean	Samples positive for AMR among samples containing bacteria (%): minimum	Samples positive for AMR among samples containing bacteria (%): maximum
produce	Iceberg lettuce	2	202	11	11	5.45	1.04	100	100	100	100
produce	Lettuce	14	32228	316	231	0.717	0	94.4	73.1	0	100
produce	Melon	1	19412	15	1	0.005	0.005	0.005	6.67	6.67	6.67
produce	Nectarines	1	3	1	1	33.3	33.3	33.3	100	100	100
produce	Peaches	1	15	2	2	13.3	13.3	13.3	100	100	100
produce	Pears	1	7	2	2	28.6	28.6	28.6	100	100	100

Group	Food type	Studies	Number of samples tested	Number of samples: positive bacteria	Number of samples: positive AMR	Samples positive for AMR (%): mean	Samples positive for AMR (%): minimum	Samples positive for AMR (%): maximum	Samples positive for AMR among samples containing bacteria (%): mean	Samples positive for AMR among samples containing bacteria (%): minimum	Samples positive for AMR among samples containing bacteria (%): maximum
produce	Peppers	2	67	4	3	4.48	3.57	5.13	75	66.7	100
produce	Spinach	1	31	1	1	3.23	3.23	3.23	100	100	100
produce	Tomato	7	32680	52	34	0.104	0	50	65.4	0	100
produce	Watermelon	1	24	1	1	4.17	4.17	4.17	100	100	100
seafood	Shrimp	1	720	59	52	7.22	7.22	7.22	88.1	88.1	88.1
seafood	Smoked Salmon	1	803	1	69	0.125	0.125	0.125	1.45	1.45	1.45

Table 5. AMR data obtained from 14 Studies testing lettuce for AMR. "Bacterium" refers to unspecified bacteria identified.

Bacterial taxa	Number of AMR types	Samples	Proportion AMR (%)
Bacterium	16	125	94.4
Staphylococcus spp.	11	36	83.3
Escherichia coli, *Salmonella*	8	120	20.8
toxigenic *Clostridium difficile*	5	297	13.8
Klebsiella pneumoniae Enterobacter cloacae	29	41	7.32
Citrobacter freundii, *Serratia* spp.	16	137	5.11
Salmonella enterica	7	40	5.00
Escherichia coli	14	65	3.08
Acinetobacter baumanii Stenotrophomonas maltophilia	5	100	2.00
Salmonella enterica	4	31099	0.003
Listeria monocytogenes	8	9	0.000
Cronobacter spp.	9	4	0.000
Cronobacter spp.	9	87	0.000
Escherichia coli	5	68	0.000

3.1.2 Sampling Strategy

Based on the results of the literature survey, the sampling strategy was to be based on the total population consumption of food types within four broad categories (dairy, fresh produce, cooked meats, seafood). These broad categories were proposed in the response to tender, as they encompassed the diversity of different RTE food types on retail sale in the UK. The 1000 samples to be taken were to be divided evenly between these broad food categories (250 samples of each type), and then samples were to be taken within

each food category based on consumption. However, when consumption of these different broad categories was compared, it was observed that consumption varied widely between them. Dairy accounted for approximately 55% of consumption, produce for 42%, cooked meat for 3% and seafood less than 0.5%. Based on this the FSA decided to refocus sequencing effort on high consumption food items, and pick food types from all RTE foods according to consumption. This improves the ability to estimate the average consumption of AMR foods, while reducing the ability to say as much about AMR content of certain food types.

The sampling strategy was then amended to sample food types such that 90% of consumption of RTE foods was covered, with a minimum of five samples per food type. This resulted in 52 different food types being sampled, comprising 33 produce types, 17 dairy types, and two cooked meat types. While fewer dairy types were sampled than produce types, more than half of the samples taken were dairy samples. The final list of samples to be taken was distributed across UK regions based on population and agreed with the FSA.

3.2 Sampling

A total of 1042 samples were collected. Of these 41 were rejected, either by Hallmark (n=9), or by Fera (n=32), leaving a total of 1001 samples to be processed. Samples were rejected for two main reasons: the quality of the product had deteriorated in transit (for example squashed fruit, leaking ice cream, samples arrived after their use-by date); incorrect sample type had been purchased (for example semi-skimmed milk rather than skimmed, fat spread of mixed dairy and plant origin).

3.3 16S Metabarcoding

3.3.1 Microbial Community Analysis

The microbial community analysis was undertaken for two purposes (see section 2.4.1); to determine relative proportions of host and bacterial DNA, and to identify any taxa of

concern to FSA. We specifically did not choose to bias the selection of samples for metagenomic sequencing based on the taxa present. Therefore, additional analysis and interpretation of the taxa identified was beyond the scope of this project. For this reason, and due to the large amounts of data generated by the metabarcoding analysis of 1001 samples, the findings are presented in Appendix 5. Appendix 5 contains the following documents:

- AMR_all_stats
- Pie_charts
- Genus_tables

Additionally, we provide the interactive bar plots, representing taxonomic profiles, produced by the analysis. These files are large and so we provide them in a high-compression format archive file (7-Zip, which requires the 7-Zip or compatible software):

- Qiime2_bar_plots.7z

3.3.1.1 AMR all stats

The AMR_all_stats file contains summary statistics for all samples sequenced. The workbook is made up of two worksheets – Summary Stats and Complete Stats.
The contents of the summary stats and complete stats worksheets are described in tables in the AMR_all_stats_key file in Appendix 5.

Post Quality Filtering and Post Denoising headers are the most important stages. The number of reads retained after quality filtering represents the total number of good quality useable DNA sequences and is an indication of the overall quality of the dataset.

The denoising step includes singleton removal, chimera removal, dereplication and minimum length filtering, which can substantially reduce the number of reads available for taxonomic assignment. This can be seen most clearly in milk-based products, where a large number of cow mitochondrial sequences are found and removed from the final dataset. We perform an additional chimera checking stage, thus the number of reads

retained after chimera removal is the number of reads that will be used to make taxonomic assignments.

3.3.1.2 Genus Tables

The Genus_tables directory contains 52 .csv files – 1 file per food category. Each file contains a list of sample IDs, taxa assigned to that sample and some metadata columns (Table 6). Normally, taxonomic resolution cannot be expected at the species level. However, in some cases genus level assignment is not possible.

Table 6. Description of the headers in the Genus tables files.

Header	**Description**
index	The sample ID.
<Taxonomic assignments>	1 or more columns which contains the results of the taxonomic assignments. Each column header contains the taxonomic lineage of the assignment (D_0__ to D_5__, 0 representing Kingdom and 5 representing genus). The content of the columns shows the number of reads assigned to that taxon.
Food_Group	The food group (either Produce_Samples or Milk_Samples).
Food_Category	The food category.
Raw_Reads	The raw reads associated with that sample. This can indicate if a sample has sequenced poorly.

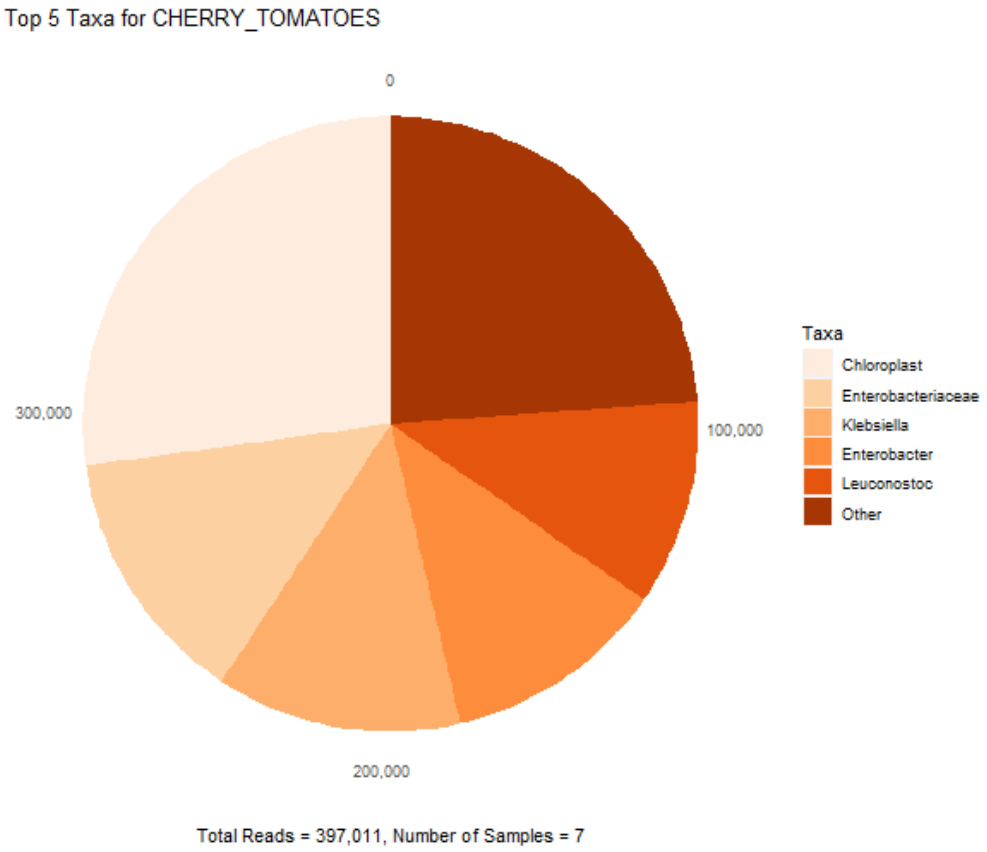
3.3.1.3 Qiime2 Bar Plots

The Qiime2_bar_plots directory contains 52 .qza files – 1 per food category. Each file can be opened by dragging the file into the designated area on the Qiime2 View website (https://view.qiime2.org/). Please use a compatible browser (Google Chrome) when

viewing these files. Once loaded, Taxonomic Level (Kingdom to Species) can be selected, and samples can be sorted based on Sample Metadata (Raw Reads is often useful) or Taxonomic Abundance. The bar plots provide an overview of what each food category looks like. (N.B. use of these files and Qiime2 View does not involve any data being uploaded to the qiime2.org website; all data resides solely within the browser on the user's computer.)

3.3.1.4 Pie Charts

The Pie_charts directory contains 52 .png files – 1 per food category. Each file can be opened to reveal a pie chart and a summary table. An example is shown below, in Figure 6. For each food category, the top 5 taxa (by total reads) are displayed, which provides an estimate of which taxa are found. This is indicative and should not necessarily represent a precise measure of proportional incidence. Generally, the pie charts are useful for an 'at a glance' view of each food category, but can also help to highlight foods that are dominated by one frequently occurring taxa (e.g chloroplast). These results are a useful indication of the food types which are most challenging in terms of obtaining non-host DNA.



Taxa	Total_Reads
Chloroplast	107816
Enterobacteriaceae	53923
Klebsiella	51163
Enterobacter	46622
Leuconostoc	42809
Other	94678

Figure 6. Pie chart showing the top five taxa identified across all Cherry Tomato samples, in addition to an 'other' category which sums the reads for every other taxa identified, and their relative proportions.

3.3.2 Priority Taxon Detection

This concerns the detection of 16S metabarcode reads which may indicate the presence of DNA from foodborne pathogens of concern, and is not part of the AMR research (refer to Section 1.2, Aims and objectives).

None of the taxa present in the FSA's priority list were able to be confidently identified in this analysis. Details of the determination of distinguishable taxa, the frequency of matched sequences and further inspection of selected taxa can be found in Appendix 4b.

3.4 Metagenomic Sequencing

3.4.1 Sample Selection for Metagenomic Sequencing

Based on the 16S data presented in Appendix 5, food types were assigned to those which had high levels of host DNA present, low levels of host DNA present, and those which displayed a range or intermediate levels of host DNA:bacterial DNA (Figure 7, Table 7). Samples were then selected for sequencing as described in section 2.6.1.

Figure 7. Proportion of the 16S amplicon reads in each sample, across all food types, which are of bacterial origin.

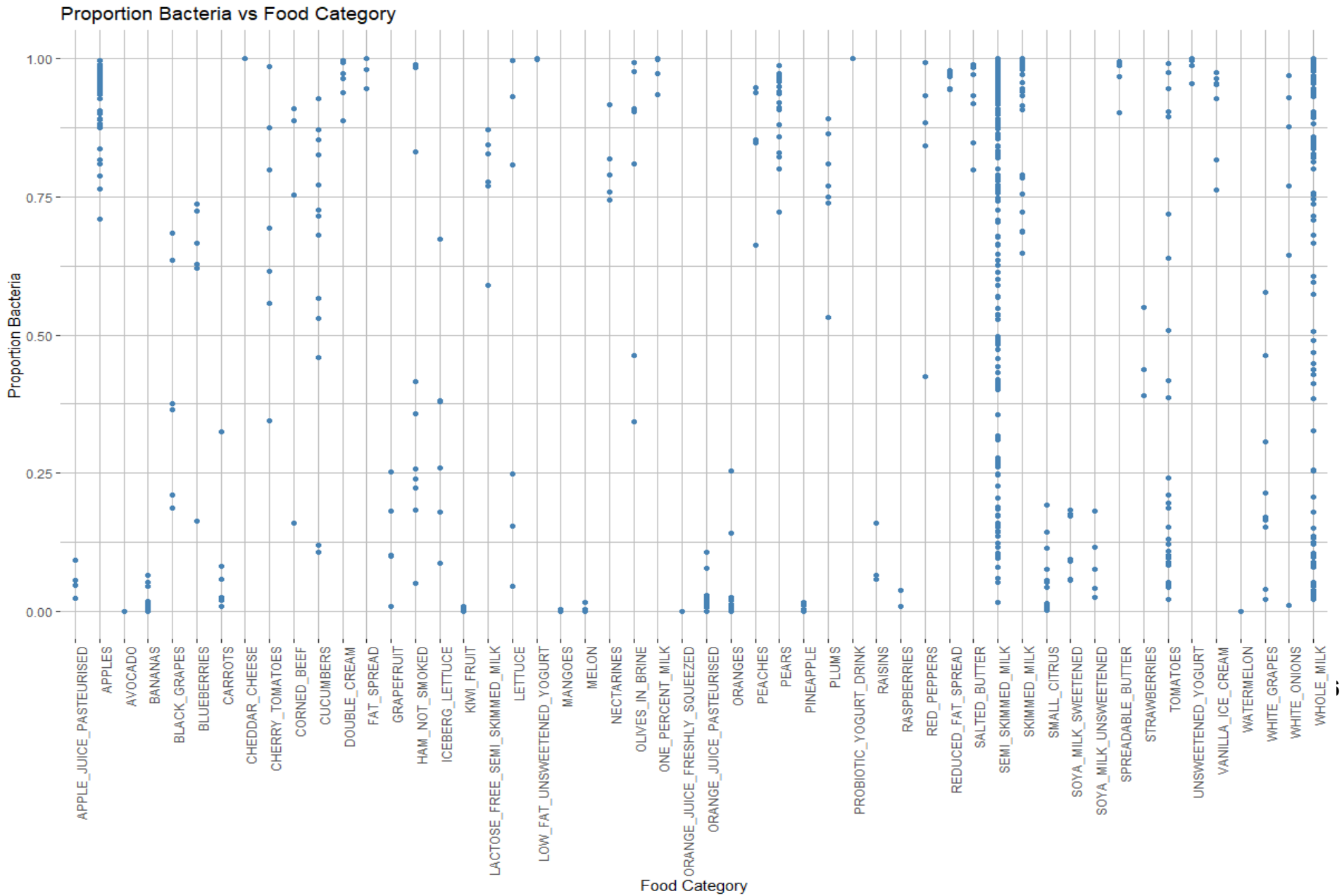

Proportion Bacteria vs Food Category
Proportion Bacteria
1.00
0.75
0.50
0.25
0.00
APPLE_JUICE_PASTEURISED
APPLES
AVOCADO
BANANAS
BLACK_GRAPES
BLUEBERRIES
CARROTS
CHEDDAR_CHEESE
CHERRY_TOMATOES
CORNED_BEEF
CUCUMBERS
DOUBLE_CREAM
FAT_SPREAD
GRAPEFRUIT
HAM_NOT_SMOKED
ICEBERG_LETTUCE
KIWI_FRUIT
LACTOSE_FREE_SEMI_SKIMMED_MILK
LETTUCE
LOW_FAT_UNSWEETENED_YOGURT
MANGOES
MELON
NECTARINES
OLIVES_IN_BRINE
ONE_PERCENT_MILK
ORANGE_JUICE_FRESHLY_SQUEEZED
ORANGE_JUICE_PASTEURISED
ORANGES
PEACHES
PEARS
PINEAPPLE
PLUMS
PROBIOTIC_YOGURT_DRINK
RAISINS
RASPBERRIES
RED_PEPPERS
REDUCED_FAT_SPREAD
SALTED_BUTTER
SEMI_SKIMMED_MILK
SKIMMED_MILK
SMALL_CITRUS
SOYA_MILK_SWEETENED
SOYA_MILK_UNSWEETENED
SPREADABLE_BUTTER
STRAWBERRIES
TOMATOES
UNSWEETENED_YOGURT
VANILLA_ICE_CREAM
WATERMELON
WHITE_GRAPES
WHITE_ONIONS
WHOLE_MILK
Food Category

Table 7. Categories of bacterial DNA levels to which the different food types were assigned.

Low Bacteria	High Bacteria	Range or intermediate
Apple juice pasteurised	Apples	Black grapes
Avocado	Cheddar cheese	Blueberries
Bananas	Corned beef	Cherry tomatoes
Carrots	Double cream	Cucumbers
Grapefruit	Fat spread	Ham not smoked
Kiwi fruit	Lactose free semi skimmed milk	Iceberg lettuce
Mangoes	Low fat unsweetened yoghurt	Lettuce
Melon	Nectarines	Semi skimmed milk
Orange juice freshly squeezed	Olives in brine	Strawberries
Orange juice pasteurised	One percent milk	Tomatoes
Oranges	Peaches	Whole milk
Pineapple	Pears	
Raisins	Plums	
Raspberries	Probiotic yoghurt drink	
Small citrus	Red peppers	
Soya milk sweetened	Reduced fat spread	
Soya milk unsweetened	Salted butter	

Low Bacteria	High Bacteria	Range or intermediate
Watermelon	Skimmed milk	
White grapes	Spreadable butter	
	Unsweetened yoghurt	
	Vanilla ice cream	
	White onions	

3.4.2 Metagenomic Sequencing

Sequencing summary statistics for NovaSeq and PromethION sequencing are shown in Appendix 2. Sequences are deposited in the European Nucleotide Archive under accessions ERP128088 and ERP128787.

3.5 Bioinformatic Analysis of Metagenomes

3.5.1 Overview of metagenomic read data

A total of 8.47 billion raw read pairs were generated in total from the 256 samples, thus a mean of 33.08 million per sample, with a median of 32.57 million (Table "metagenomics_sequencing_by_sample.xlsx" in Appendix 2). Three extreme outliers were the fat spreads, with only 30,336 to 40,747 read pairs. All other samples had more than 2 million raw read pairs, with 247 in excess of 10 million pairs. In more detail, 6 samples had 2-10 million pairs, 13 samples had 10-20 million pairs, 81 samples 20-30 million, 92 samples 30-40 million, 46 samples 40-50 million, 10 samples 50-60 million, 4 samples 60-70 million and one sample of 71,816,590 raw pairs.

3.5.1.1 Basic quality control (QC)

Few samples were problematic in terms of high proportions of read pairs being affected by poor quality (Table "metagenomics_sequencing_by_sample.xlsx" in Appendix 2). The four worst-performing samples had a pass-rate of 62% - 78% of read pairs (iceberg lettuce,

corned beef, apple juice pasteurised and bananas), and were left with between 12 million to 30 million pairs post-QC. The next 18 worst samples in this regard had pass-rates of 84% - 90% (highest 89.99%); thus the pass-rate was ≥ 90% for 234 of the 256 samples. In more detail, 142 samples have a rate 90% - 95%, and 92 samples have a rate 95% - 98% (highest rate 97.89%). In total, 7.87 billion read pairs remained, i.e. 92.93% of the raw pairs.

The number of post-QC reads in each sample can also be seen in Figure 8 (*x*-axis).

3.5.1.2 Host-read filtering

The "host" is the food organism, whose DNA sequences may be present in the metagenomic data.

Figure 8 shows the post host-filtering number of reads versus the original (i.e. post-QC) number of reads for each sample.

For those samples where relatively few reads were removed, this could be either due to the original sample being low in host organism reads, or due to difficulties in detection of host reads (due to reference genome sequence quality, for example).

In general, fewer host reads were detected and removed from the produce category than from dairy or meat.

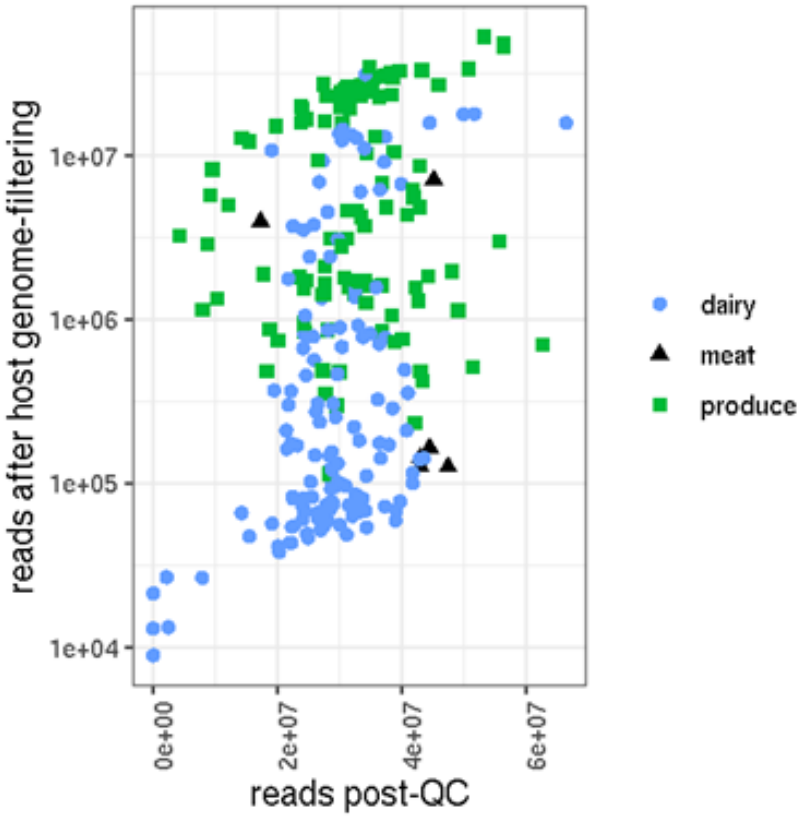


Figure 8. Indication of host genome-originating reads. For each sample the number of reads after basic quality control (x-axis) is plotted against the number of reads remaining after reads identified as being of host origin were removed (y-axis).

The numbers of read pairs remaining in each sample after host-filtering is provided in Table “metagenomics_sequencing_by_sample.xlsx” in Appendix 2, both as absolute numbers and as a percentage of the number of post-QC read pairs (not raw read pairs); a brief summary follows.

There are many samples which have a very low "pass" rate, i.e. a very high rate of identified host read pairs. 90 samples had < 1% of post-QC read pairs remaining after host-filtering, but these number 13,000-420,000 read pairs, with 37 of these samples > 100,000 pairs). The absolute numbers of remaining read pairs are the pertinent metric in

terms of the usefulness of the metagenomics data; these are summarised for all samples in Figure 9.

Overall (all 256 samples), post host-filtering only one sample has < 10,000 read pairs, a total of 57 samples < 100,000, and 109 samples < 500,000.

125 samples have > 1 million post host-filter read pairs, of which 12 samples > 30 million.

The distribution is not unimodal and notably reflects different trends among the food types. Of the 30 samples with > 20 million reads, all but one (probiotic yoghurt drink) are of 'produce' type. Conversely, of the 100 samples with the lowest post host-filtering read counts, only four are produce samples (watermelon, 63rd lowest; and three mango, 88th , 93rd and 98th lowest counts), four are the meat samples (68th, 69th, 74th, 79th lowest) and the remainder are dairy. The two lowest read-count samples are the reduced fat spreads, and the fourth lowest is the other 'fat spread'; with 8,938; 13,040 and 21,311 read pairs respectively. However, these represent respectively 32%, 46% and 55% of the post-QC read counts (the raw read counts were only 30,000 to 41,000 pairs). In contrast, the two spreadable butter samples have the 39th and 36th highest post host-filter read counts, with around 16 million and 18 million read pairs.

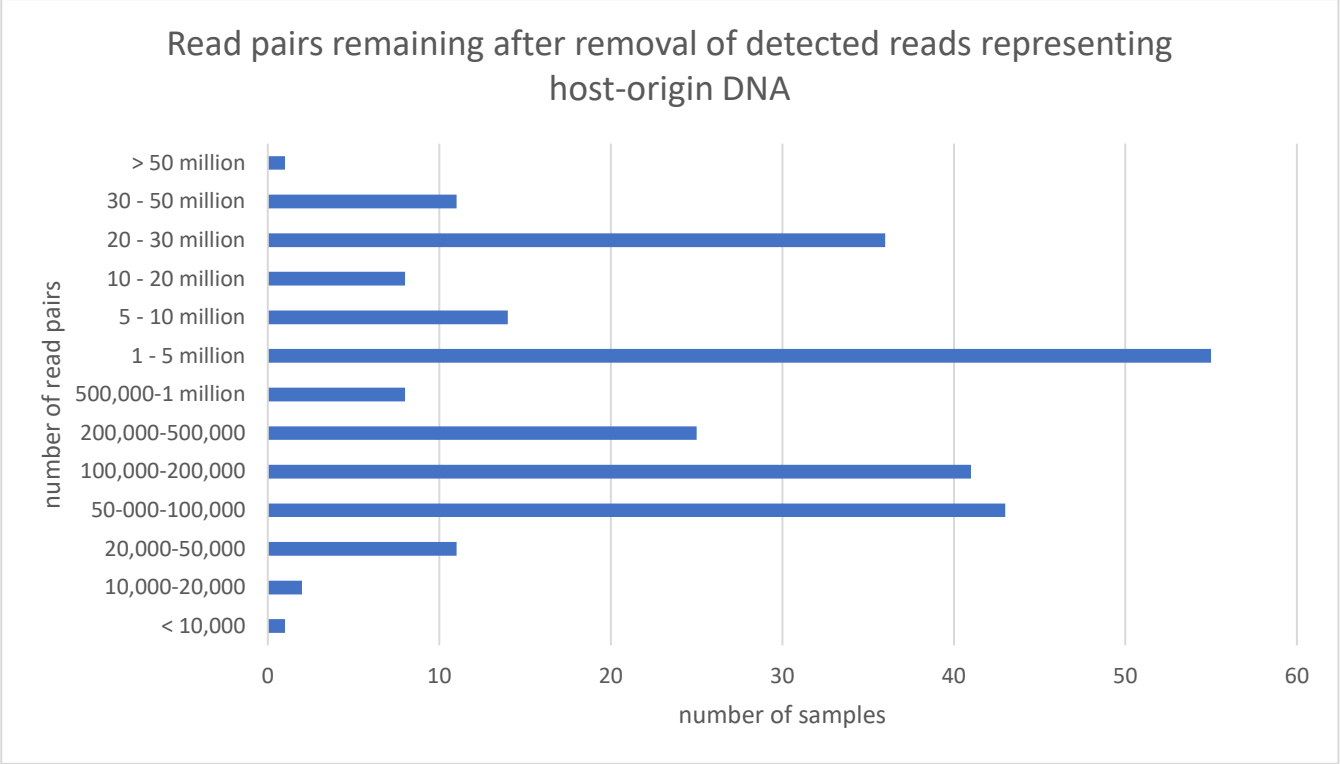


Figure 9. Frequencies of read-pair counts in each sample following the host-DNA filtering stage.

These data highlight that:

- there is a very large range in the number of suitable read pairs remaining for analysis, following firstly basic quality-control, and subsequently removal of reads identified as of host-organism origin
- that the range appears not to be a random effect but depends on food type; this indicates that our efforts to obtain microbial DNA from the produce types has been quite successful, whereas this was not possibly for many dairy samples such as milk.

3.5.2 Metagenome assemblies

Notwithstanding the previously noted limitations of N50 and L50, we have included these metrics in Appendix 6.

Sample #2672718 (red peppers) contains the longest single contig, which is > 4.2 Mbp (more than twice the length of any other contig in any sample), and also 5 other contigs of > 1 Mbp (only 13 samples have any contigs of > 1 Mbp). However, the N50 is 3,041 bp, which is only the 56th best N50 of all 256 samples; its N50 is 6,142, which is the 60th lowest (best) value. This suggests a relatively long "tail" of low-abundance genomes in a metagenome dominated by one or a few taxa. The total post host-filter read count is unexceptional (23 million pairs).

Conversely, #343701 is the sample with the highest N50 (125,295) and has an L50 of only 8 contigs (there are only 184 contigs in the assembly). Its longest contig is 434,779 bp, one tenth of the longest contig in any sample (above). This is actually the joint-lowest (best) L50. This sample #343701 has around 31 million read pairs after host-filtering. This is neither exceptionally high nor low, but this sample is the sole 'probiotic yoghurt drink' and might be assumed to have a very skewed metagenome, dominated by one or a very small number of bacteria originating from a production culture. Notably, the other sample (#2672554) with L50 = 8 in fact has the worst N50 of any sample (390), but key to this is that there are only 19 contigs in the entire assembly, ranging from 203 bp to only 727 bp in length. This is indeed the sample (reduced fat spread) with the lowest read count of all (only 8,938 post host-filter).

In summary, the two samples with the equal best L50 value consist of the sample with the best N50 and the sample with the worst N50. This illustrates the very different natures of these metagenomes, and how they cannot necessarily be anticipated from any single metric such as read counts, contig counts, mean contig lengths, N50, L50, etc. A further observation is that the highest N50 in any sample is not especially high, representing perhaps 2% to 10% of the length of a bacterial genome. There are therefore no metagenomes approaching "completeness" even if some may (possibly) be dominated by one or a few genomes which are individually near-complete.

3.5.2.1 Contig lengths in the context of ARG sequence detection

A consideration of the contig lengths in relation to typical gene lengths is important, since if most of the contigs are shorter than ARGs, necessarily few ARGs can be found. Even contigs of approximately the same length as ARGs would often not enclose the entire ARG length (if an ARG were present) and would be more likely to represent only a segment of it. Ideally, our data would include a large number of contigs whose length is of an order of magnitude greater than ARG lengths. To mitigate this, we had run the detection software RGI MAIN in a mode which aims to predict partial genes for short contigs instead of requiring full-length matches (section 2.8.6.3).

The nucleotide sequences in the principal section of CARD range from 162 to 4,359 bp (median 861 bp; mean 946.6 bp). In the 'wildcard' section of CARD, the nucleotide sequences are between 60 bp and 6,237 bp (median 1,194; mean 1,431.9).

The highest median contig length of any sample is only 1,023 bp, which is in a sample (#343520) with a relatively short maximum contig length (71,534 bp) and 2,651 contigs in total. The next longest median is 860.5 bp, and in all only 8 samples have median lengths > 700 bp. A further 81 have a median between 500 bp - 700 bp, 129 between 400 bp - 500 bp, and the remaining 38 between 348 bp - 400 bp. (For comparison, many read pairs have a combined length of 302 bp, i.e. two non-adjacent segments of 151 bp).

Therefore, regarding the use of the assemblies as input data, it appears that the length distribution is not especially encouraging for the data set overall. Regarding finding matches with full-length gene sequences or even partial matches to segments which are long enough to identify the ARGs of origin, some samples are much more fragmented than others and so will be inherently likely to yield fewer positives.

Looking at other aspects of the length distributions, Figure 10 summarises the lengths of the single longest contig in each sample; this is useful as it indicates the best case-scenario within a sample regarding the ability to detect full-length gene(s). 49 samples lack any contigs > 10,000 bp (10 kbp). 87 samples have a longest contig > 100 kbp. Three

samples have at least one contig exceeding 1 Mbp (1,130,022 bp; 1,975,816 bp; 4,213,328 bp).

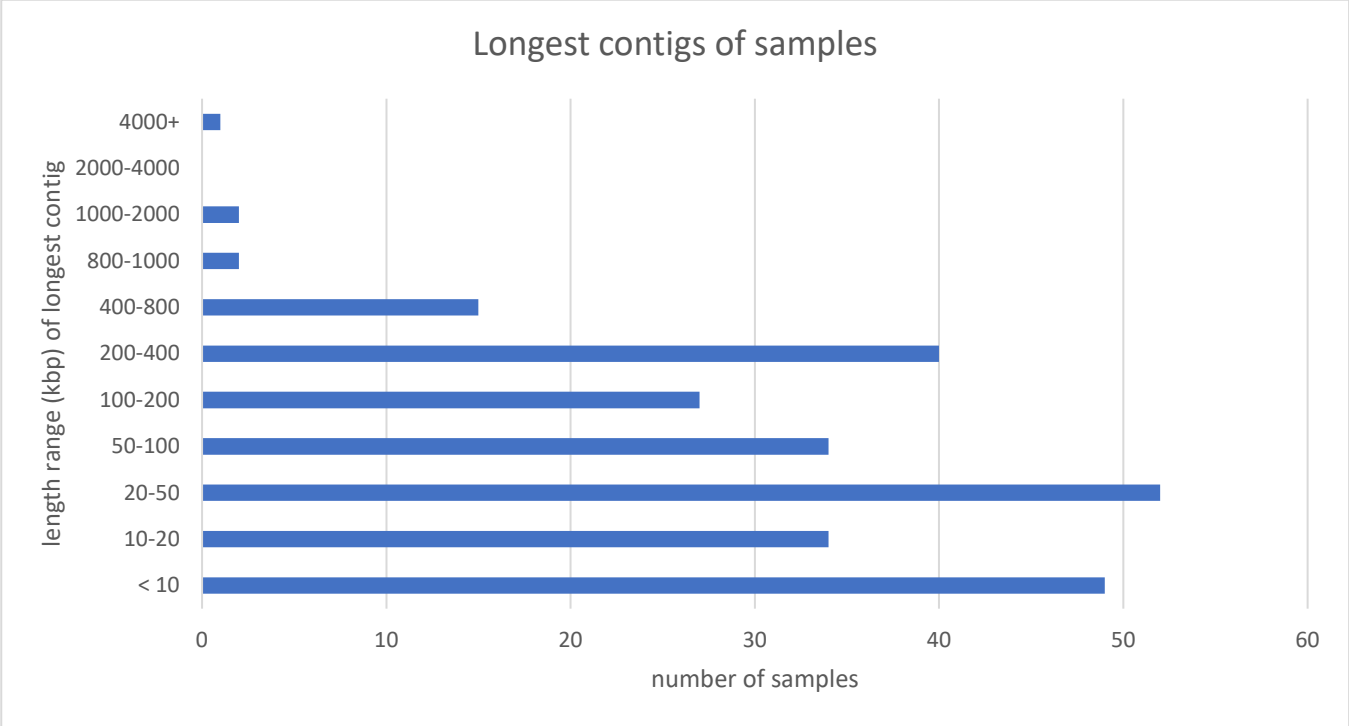


Figure 10. Frequencies of length-range of the single longest contig of each sample.

Therefore, most samples could in principle harbour at least one full-length ARG sequence, if these genes happen to occur in the longer contigs, but clearly the maximum possible number of full-length sequences is intrinsically very limited in many samples. Again, the expectation is that detection of ARGs may be inherently much less likely in a considerable number of samples, compared to other samples.

For the purpose of detecting co-located ARG sequences (or ARGs with other AMR-related moieties), the above considerations are accentuated.

3.5.2.2 Assessing assemblies: back-mapping reads

As part of its ARG-detection/prediction procedure, the RGI MAIN software predicts open reading frames (ORFs) in the input sequences (in this context, the assembled contigs).

Back-mapping is usually performed using the *assembled contigs* as the references. In the context of our analyses, it was also useful to back-map the reads using only the *predicted ORF sequences* as references.

The proportion of reads which can be back-mapped to the sequences which arose from that collection of reads, indicates the quality of the assembly. Appendix 6 describes some detailed analysis of the back-mapping results, concerning both the mapping to contigs and mapping to ORFs, with the aim of evaluating the quality of the assemblies, as well as the amount of host (food-organism) DNA.

3.5.2.3 Conclusions on assembly quality and host contamination

Appendix 6 provides detailed analysis of the quality of the assemblies, since it is necessary to answer a fundamental question: are the assemblies of the 256 samples' metagenomes collectively suitable for the production analysis of ARG sequences?

The data suggest that there are many samples which fall in each of these categories:

- Poor-quality assemblies
- High-quality assemblies with a low gene-density (probably due to host-DNA contamination)
- High-quality assemblies with a high gene-density

Our view is therefore that for this data set overall, *prediction of ARGs from assemblies is not the ideal approach*, even though some samples will be ideal for that. Our principal production data (ARG-identifications) are *therefore the predictions from unassembled short-read data*.

Although we have thus not used RGI MAIN to generate production ARG-predictions, the results it produced regarding gene (ORF) predictions have proved useful as part of the assessment of the assemblies, as described in the previous sections.

3.5.3 Assigning taxonomy by marker genes using Progenome Classifier

Given that a number of samples' assembled metagenomes include some long contig sequences, we investigated whether the long contigs could be reliably identified taxonomically, using a dedicated typing method to identify marker genes. At least one of the assembled contigs is large enough to be a complete bacterial genome. The Progenome classifier / SpecI (Mende et al., 2020, Mende et al., 2013) uses a database of 40 universal marker genes to attempt to classify prokaryote genomes. The software reports the number of 'detected' genes, but relies on successfully 'mapping' marker genes, which is usually a smaller number.

3.5.3.1 Samples with at least one assembled contig exceeding 500 kbp

A taxonomic identification of a genome sequence should often be possible even if it represents only a fragment of the complete genome, if sufficient marker genes are present. The majority of known bacterial genomes exceed 1 Mbp in size, with many much larger than that (Mira et al., 2001), but some extreme examples are smaller than 200 kbp (Nakabachi et al., 2006).

The 13 samples with at least one assembled contig exceeding 500 kbp, and therefore constituting a large portion of a bacterial genome, are listed in Table 8, along with details of the 54 such contigs which occur in them. Only three of these samples could be taxonomically assigned (whether analysing the long contigs collectively or individually) by Progenome Classifier, respectively with confidence levels of 64%, 91% and 91% (in the sample where the genes occurred on more than one contig, each contig has a lower confidence when analysed separately).

The red peppers sample #2672718 has six contigs exceeding 1 Mbp, including a 4 Mbp contig (i.e. approximately bacterial genome-sized). However, there was no prokaryote assignment. We have not confirmed, but we suspect that at least some of these long contigs are of fungal origin, which may also apply to several of the other samples. Long contigs of plant origin may also be present.

The only sample with any contigs identified to a single named species is a yoghurt; this was from a relatively modest-sized contig, which is not unexpected for what is assumed to be a far less microbiome-diverse sample than the agricultural produce.

Table 8. The assembled contigs of length > 500,000 bp. There are 54 in all, in 13 samples. The contig IDs where any marker genes could be reliably assigned by Progenome Classifier are in bold, with corresponding species verdicts in the final column.

Sample ID	**Sample type**	**Contig lengths**	**Species designation (PROGENOME)**
6607	apples	747709 636734	*Agreia* sp. (either sp. Leaf244 or sp. Leaf283)
6658	apples	568319	
2664457	pears	516924	
2664666	strawberries	617549	
2664679	apples	796483 765582 588918 533808	
2664758	plums	826066 750948 650521 611041 520165	

Sample ID	Sample type	Contig lengths	Species designation (PROGENOME)
2664783	apples	708764	
2664800	cucumbers	841083 660100 641658 564539	*Agrobacterium* or *Rhizobium* sp. (either genomosp. 3, sp. LC34, sp. SUL3 or *Rhizobium* sp. Root651)
2672544	apples	578507 513806	
2672611	low fat unsweetened yoghurt	670317	*Lactobacillus acidophilus*
2672718	red peppers	4213328 1935613 1655039 1226349 1182851 1035342 913111 789740 787135 762392 653185	

Sample ID	Sample type	Contig lengths	Species designation (PROGENOME)
2672786	apples	1975816 1082071 1003947 728338 665947 665053 657523 629483 580652 580212 539651	
2685919	pears	1130022 1048195 1037017 951853 772916 762013 662510 619694 573615	

Sample ID	Sample type	Contig lengths	Species designation (PROGENOME)
		518886	

3.5.3.2 Additional samples analysed by Progenome Classifier

We also analysed (**collectively**) all four of the > 100 kbp contigs of **sample #2672455** (longest is 231,090 bp). We selected this due to the corresponding Nanopore long-read sequences being identifiable with Progenome (see Section 3.5.9.6). However, no assignment was possible for these four sequences, indicating that few or no marker genes could be identified within the assembled NovaSeq data.

Secondly, we analysed the long > 100 kbp contigs (9 in all; longest 434,779 bp) of the probiotic yoghurt drink **sample #343701**, since as noted previously this had the best N50 and joint-best L50 values. 14 marker genes were mapped, and a species assignment of *Lactobacillus casei/paracasei* [*Lactobacillus* sp. HMSC25A02/*Lactobacillus casei/Lactobacillus paracasei*] resulted, with 98% confidence. This was the highest number of marker genes assigned in any of these 'long contig-only' tests, and twice the next best number.

Finally, we made a small number of attempts at processing an entire set of contigs (irrespective of length) of a sample with Progenome Classifier. The software is designed to classify an isolate complete or near-complete genome rather than metagenomic data, and a lack of taxonomic assignment, or a "chimera" can thus be expected to occur in some cases. However, one sample, **#2664700** (olives in brine; 30,362 contigs; longest 349,338 bp), was assigned as *Stenotrophomonas maltophilia*, with all 40 marker genes found multiple times.

3.5.4 ARG predictions from RGI

We processed all 256 samples' quality-controlled, host-filtered unassembled short read sequence data with RGI BWT. We also processed (with RGI MAIN) all the samples' assembled metagenomes which had been derived from those.

For both the RGI BWT (short-read) and RGI MAIN (contigs) results, we also performed in-house filtering of the results.

As stated in Section 3.5.2, due to the nature of a significant proportion of the samples' assembled metagenomic data, we could not use the predictions from RGI MAIN as the production results.

3.5.4.1 ARG predictions from RGI MAIN

We could not use the RGI MAIN results (from assembled contigs) as the production data, due to issues with too many of the samples' assemblies (see 3.5.2).

We have maintained the references to RGI MAIN here and in other sections, because this indeed provided the invaluable data for assessing the qualities of the assemblies in the first instance (3.5.2.2). As the ARG predictions were not used in burden assessment (section 2.9) or interrogated further after the decision to use short-read RGI-BWT ARG data, and have therefore not been subject to the same degree of assessment as the RGI-BWT data, the RGI MAIN results are not presented further.

3.5.4.2 Production RGI results of short-read analysis (RGI BWT)

The following refers to the RGI BWT results after we had applied our in-house filtering (See section 2.8.6). The results are presented in Appendix 8.

In terms of distinct ARG names , 179 of the 256 samples had at least one ARG detected. 28 samples had only one ARG name present, and a further 159 samples had fewer than 10 different ARG names.

48 samples had between 10 and 49 ARG names; 34 samples had 50-99; 12 samples had 100-149; and 3 samples at least150 (highest 193).

In terms of positive read pairs (where either or both of R1 and R2 are positive for an ARG), naturally the 77samples in which no ARGs were identified have a zero count. A further 121 samples have fewer than 10 positive read pairs passing the filters in total, and a further 41 samples 10-99 read pairs. 44 samples have 100 - 999 read pairs, 47 samples 1,000 - 9,999 read pairs, 6 samples 10,000-19,999 and 3 samples > 20,000 (highest 25,049).

In all, 478 different ARG names passed all the filters. However, there were 477 unique ARO terms for the gene names; ARO:3000556 was present both as ‘tet(44)’ and ‘tet44’. Note that many ARG names can correspond to multiple variant gene sequences in the reference database, but usually these have identical names (in fact there are 4 variant sequences of tet44, including the tet(44) sequence, in CARD/WildCARD). More details of numbers of ARGs detected in different sample types are in Section 3.6.

3.5.4.3 Evaluating the screening of variant/mutant-type ARGs

We had applied screening (2.8.8) for good matches between the short reads and the reference ARG sequences, for all ARG types, to produce the data described in the previous section. As part of that production process, we had further applied a requirement for a very strict match (100% identity) to those matches to ARGs of the "antibiotic resistant gene variant or mutant" type (2.8.8). As this is an important aspect of dealing with putative sequence-based matches for ARG-prediction, we summarise the effect which this particular screen had on the results.

After the general screening applied to all ARGs, 782 different ARG names appear collectively in the 256 samples. Only 6 of these are categorised under the "antibiotic resistant gene variant or mutant" (term ARO:0000031 in the hierarchical classification; 285 of the genes in the reference database are annotated with this). Two of these in fact appear to be synonyms for the same gene. However, these appeared in a very large number of samples, but the great majority of samples became negative for these ARGs with the additional 100%-identity filter applied (Table 9). For simplicity, the numbers of samples containing at least one read-pair, where both reads match the ARG, are shown.

Table 9. The effect of applying the 100%-identity requirement to matches to ARGs under the "antibiotic resistant gene variant or mutant" category. All six genes of this type that appeared in the study are shown. *Two very similar ARG names are present in the reference database for ARO:3004480.

ARG name	**ARO succession**	**Positive samples after standard filter without VM-screen**	**Positive samples after standard filter with VM-screen**	**Positive samples after standard filter with VM-screen and minimum 2-prs filters**
Bifidobacterium adolescentis rpoB conferring resistance to rifampicin (*sic**)	3004480	23	0	0
Bifidobacterium adolescentis rpoB mutants conferring resistance to rifampicin (*sic**)	3004480	147	1	0
rpoB2	3000501	122	3	1
Bifidobacterium ileS conferring resistance to mupirocin	3003730	87	0	0
Streptomyces rishiriensis parY mutant conferring resistance to aminocoumarin	3003318	79	0	0

ARG name	ARO succession	Positive samples after standard filter without VM-screen	Positive samples after standard filter with VM-screen	Positive samples after standard filter with VM-screen and minimum 2-prs filters
Pseudomonas aeruginosa soxR	3004107	72	33	17
one or more of the above		189	35	18

Some of these genes such as the RNA polymerase B subunit (*rpoB*) and isoleucyl-tRNA synthetase (*ileS*) genes are essentially universal in bacteria, and so it is unsurprising that very similar sequences to these were found in many samples. Indeed, *rpoB* is one of the longest bacterial genes and so would be especially likely to be detected.

As previously emphasised, our approach would be expected to still result in some false positives for such genes: if a short-read originated from a non-AMR variant of one of these genes, but did not encompass the critical sites which differ from the AMR variants, then this would pass our screen. However, for five of the above genes, even if all of the remaining matches were indeed false positives, this would add the spurious positive to only a very few samples. In the case of *P. aeruginosa soxR*, the worst-case scenario would be 7% of the total samples gaining a spurious additional positive ARG. The caveat is that some or even all of these positives could be true positives in any case.

The fact that so few "variant or mutant" type genes are involved is reassuring since, generally, genes whose AMR characteristics do not depend on these precise differences are expected to be more reliably identifiable, without insisting on an extremely high sequence identity (which would, indeed, certainly falsely discard some true ARG matches). Nonetheless, it is valuable to determine the overall characteristics of our data in terms of sequence identities.

Overall, following the general screen and the variant/mutant-type 100% identity screen, both the mean and median identities of the short-read matches to the references sequences were 90.1% (total of 461,405 forward and 394,563 reverse reads). 96.4% of the reads match with a sequence identity of at least 75% (generally, this would be sufficient to infer a probably similar gene function). 51.6% of read-matches are of 90% identity or better; 31.5% of read-matches are of at least 97% identity, and 20.0% are of 100% sequence identity.

3.5.5 ARG predictions from DeepARG

DeepARG is a tool published in 2018, which uses a novel deep-learning methodology to predict ARGs. Our default position was to treat RGI / CARD predictions as the standard and DeepARG predictions as more speculative. We ran it with a probability cutoff of $P \geq 0.8$, and subsequently applied a filter of $P \geq 0.9$ to these results. As we consider DeepARG to be more experimental than RGI, and the results only used as a sense check of the RGI results (and not to inform burden analysis) we present a summary of the results in Appendix 11 for reference. As these results do not inform burden analysis, they are not considered further.

3.5.6 Co-occurrence

Quality filtered ARGs, identified by RGI, from the 256 assembled samples were inspected for evidence of co-location. Of these, 40 samples were identified as positive (where a positive is defined as 2 ARGs being present on the same contig) (Appendix 7). Overall, this resulted in 276 co-occurring ARGs. Interestingly, many ARGs flagged as co-occurring appear to belong to the same AMR gene family, which could suggest that the gene

detection software may be terminating gene detection early, possibly due to errors produced from low coverage assemblies. Thus, a number of these instances of co-occurrence may be false positives. There are still a number of co-occurring ARGs from different gene families identified on the same contig, but it would be prudent to inspect results of interest in more detail before drawing conclusions.

3.5.7 Priority Taxon Identification

Details regarding initial screening of metagenomic samples, identification with MLST sequences and similarity searches with nucleotide databases can be found in Appendix 4 and 4b.

3.5.7.1 Conclusions from metagenomics analysis

We conclude that there is strong evidence at the sequence level, both unassembled reads and assembled contigs, for the presence of *K. pneumoniae* DNA in sample #2672480 (vanilla ice cream), and no convincing sequence-based evidence for any of the other taxa of concern.

For sample #6412 (also vanilla ice cream), we conclude that there is strong evidence at the sequence level for the presence of DNA originating from *Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae* and *Yersinia enterocolitica,* and no other taxa of concern. The first four of these are supported by evidence from both unassembled reads and assembled contigs; *Y. enterocolitica* is supported by evidence from contigs alone.

We have analysed the contig sequences of these two samples carefully and have found no evidence to suggest that any of the MetaPhlAn3 positives (resulting from comparison of short reads to a relatively few marker sequences) are false positives; and have found evidence of only one false negative from MetaPhlAn (*Y. enterocolitica* in #6412).

With the exception of *K. pneumoniae* in #6412, we found evidence for at least one MLST locus sequence for each of the MetaPhlAn3-positive taxa in these samples.

3.5.8 Assessment of the use of 16S Metabarcoding to Screen Samples prior to Metagenomic sequencing

The 54 samples belonging to food types labelled as 'low bacteria' by 16S sequencing (Table 7) were analysed with Kraken2, to see if these same samples also had low bacterial concentrations when sequenced metagenomically. The average percentage of bacterial reads in these 54 samples was 14.4%, ranging from 77.3% to 1.3%, with a median value of 5.6%. Only 5 of these 54 samples had bacterial percentages of greater than 50%. For food types labelled as ‘not low bacteria’, the average percentage of bacterial reads was 44.2%, ranging from 99.9% to1.45%, with a median value of 38.9%. 85 of these samples had bacterial percentages of greater than or equal to 50%.

The 10 samples which had failed 16S sequencing QC steps and were selected for metagenomic sequencing (section 2.6.1) were also analysed. Total read numbers from metagenomic sequencing for all 256 samples were ranked from high to low, and the ranks of the 10 'failed' samples were inspected. The median rank was 104, the highest rank was 35, and the lowest rank was 255.

From the above, we can conclude that A) a sample with low bacterial percentages by 16S sequencing will most often produce a low number of metagenomic bacterial reads, and B) samples which fail 16S sequencing QC steps do not necessarily mean that the sample will fail when sequenced metagenomically.

3.5.9 PromethION Data Analysis

Note: The following results (3.5.9) were used to assess long read sequencing and its suitability for sequencing AMR. These results are NOT used for subsequent burden analysis outside of this section, and should be viewed as independent exploratory analysis.

3.5.9.1 Assembly

After assembly, polishing with both long and short reads, as well as the removal of host sequences, 14 of the initial 24 samples produced assemblies. The remaining 10 samples

did not produce enough data to form assemblies, so were not taken forward for further analysis. Assembly quality metrics can be seen in Table 10.

Table 10. Assembly statistics for PromethION data

Barcode_SampleID	Contigs	Bases	Max	Min	Average	Median
barcode01_2664549	65	2075150	75551	6757	31925.4	29120
barcode02_2664550	12	417086	58654	18909	34757.2	29740
barcode03_2664719	225	3561435	85478	117	15828.6	11490
barcode07_6574	31	1380426	113338	8565	44529.9	39810
barcode09_2664798	125	5870589	800374	276	46964.7	7610
barcode12_2664718	47	729356	49419	678	15518.2	10140
barcode14_2672664	52	1624518	103721	188	31240.7	26540
barcode15_2672455	37	3426904	2929534	309	92619	8220
barcode16_2672718	108	4396925	147512	221	40712.3	33100
barcode17_2664702	6	34344	13001	1279	5724	3820
barcode21_2664788	19	735264	69365	18778	38698.1	29910
barcode22_6620	11	198647	60673	1361	18058.8	14520
barcode23_2672522	224	4123662	155565	75	18409.2	8100
barcode24_2672512	51	2981855	1800200	228	58467.7	19520

3.5.9.2 DeepARG

DeepARG is a tool published in 2018, which uses a novel deep-learning methodology to predict ARGs. DeepARG results are presented unfiltered and can be seen in the DeepARG_summarised.tsv file (Appendix 9). Overall, 320 ARGs were identified across all samples, but when applying a filter such as an alignment length of 75 bases to an ARG in the database, the number of ARGs is reduced to the following 15 - BACA, COB(I)ALAMIN_ADENOLSYLTRANSFERASE, ROSB, VGAC, MULTIDRUG_ABC_TRANSPORTER, OQXB, ANT(3")-IIC, MEXT, OMPR, MARR, ADEJ,

RPOB2, EFRA, MAJOR_FACILITATOR_SUPERFAMILY_TRANSPORTER and PENA. Reasons for this filter include the high error rate of nanopore data, which can usually be reduced by sufficient coverage (which unfortunately this data does not provide). Furthermore, errors in nanopore data can make ORF (open reading frame) detection difficult for commonly used tools such as prodigal (Hyatt et al., 2010).

3.5.9.3 NanoARG

The NanoARG web service was tested, and an example of the information provided can be seen in the barcode15_2672455_NanoARG word document in Appendix 9. Access to information for the remaining samples will be shared with FSA project officer, who can disseminate appropriately. The NanoARG web service provides bar plots for ARG (Anti-microbial Resistance Gene), MRG (Metal Resistance Gene) and MGE (Mobile Genetic Element) hits, as well as providing some idea of which taxa are present in the sample. The biggest drawback to NanoARG is that analysis settings cannot be adjusted for sensitivity, and that the service is online (and thus will be slower than running the analysis on a high-performance computer). The advantages include an adjusted approach to identifying ARGs (by removing the dependence on ORF detection tools) which is well suited to error-prone nanopore data, and the easy to use nature of an online tool. Ultimately, for the flexibility of analysis required, NanoARG results were deemed to be too inaccessible and thus unsuitable in its current state. However, outputs are still reported here as something to explore when the tool is available offline.

3.5.9.4 Co-occurrence

Across all samples, there were a total of 218 instances where at least 2 ARGs were identified on the same contig. However, the absence of a strict definition of how closely located these genes must be to each other in order to be considered co-occurring results in this data being presented unfiltered. The median number of ARGs found on contigs which were positive was 2, with a mean, maximum and minimum of 5, 55 and 2 respectively. Due to the variable length of the assemblies, longer contigs are more likely to contain more ARGs, thus it is important for future research to address the issue of

distances between ARGs in order for confident numbers of co-occurrence to be reported. Details of which contigs, the genes and identified ARGs can be seen in the attached files (DeepARG-cooccurrence_distances.tsv, DeepARG-cooccurrence.txt, Appendix 9).

3.5.9.5 Mobile Genetic Elements

Of the 14 samples inspected for MGEs, three reported no hits (2664718, 2664702, 2664788). Across the remaining 11, samples there were 1760 MGEs identified when no filtering was applied. When filtering on percentage identity to an MGE in the ACLAME database of 85%, and a minimum alignment length of 200, 608 MGEs were identified. Unfiltered data is provided in the 'aclame_summary.tsv' file (Appendix 9).

3.5.9.6 Metagenome-assembled Genomes

With metagenomic sequencing, it is possible to assemble MAGs (Metagenome-Assembled Genomes). Sample barcode15_2672455, with its maximum contig size of 2.9Mb, is one such example where a MAG has been assembled. Extracting this 2.9Mb contig and running it through the ProGenome classifier software (Mende et al., 2020, Mende et al., 2013) suggests that this is a complete genome of *Acinetobacter* sp. TTH0-4/*Acinetobacter albensis*. Interestingly, a MAG of this size is not present in either of the Spades assemblies for this sample, highlighting the power of long-read sequencing for capturing genome-sized contigs.

3.5.9.7 Hybrid vs Meta: Assembly

The 14 samples that produced long-read only assemblies were also assembled with Spades in meta mode (for metagenomic assembly of illumina short read data) and hybrid mode (for assembly using both long nanopore and short illumina reads), in order to assess the benefit of including longer PromethION reads in the assembly. Assembly quality metrics can be seen in Table 11, with more information available in the attached hybrid_vs_meta excel file (Appendix 9). Of note is the slightly increased average contig length achieved with the hybrid method, as well as a general decrease in the number of contigs in the assembly. This suggests that the long reads are helping to bridge gaps

present in the short-read data, which results in longer contigs. One of the main benefits of long read technologies is the ability to create longer unbroken sequences, resulting in longer contigs. Thus, as might be expected, the maximum contig length is on average greater in the hybrid assemblies – despite the low amount of data generated by the PromethION. If more data had been generated, this trend may have been observed more significantly.

Table 11. Descriptive assembly statistics from the 14 samples that were analysed using Spades in hybrid and meta mode.

Barcode_SampleID	Spades Method	Contigs	Bases	Max	Min	Average
barcode01_2664549	Hybrid	2106213	1072419557	755356	55	509.2
barcode02_2664550	Hybrid	1909648	792607874	916702	55	415.1
barcode03_2664719	Hybrid	3822275	1019463650	233460	55	266.7
barcode07_6574	Hybrid	265682	125321766	386306	56	471.7
barcode09_2664798	Hybrid	1027472	349670351	208553	55	340.3
barcode12_2664718	Hybrid	3204262	900853524	335541	55	281.1
barcode14_2672664	Hybrid	52188	43574793	357638	56	835
barcode15_2672455	Hybrid	43588	38109097	198979	56	874.3
barcode16_2672718	Hybrid	330292	188217240	2704692	55	569.9
barcode17_2664702	Hybrid	3482151	1253388657	243679	55	359.9
barcode21_2664788	Hybrid	1524407	586972551	507384	56	385
barcode22_6620	Hybrid	782242	237887275	351614	55	304.1
barcode23_2672522	Hybrid	12171	11908796	553239	56	978.5
barcode24_2672512	Hybrid	7027	5856950	543462	56	833.5
barcode01_2664549	Meta	2111258	1072539964	407195	55	508
barcode02_2664550	Meta	1912672	792828647	409368	55	414.5
barcode03_2664719	Meta	3824450	1019298289	214163	55	266.5
barcode07_6574	Meta	266413	125288122	293761	56	470.3

Barcode_SampleID	Spades Method	Contigs	Bases	Max	Min	Average
barcode09_2664798	Meta	1030380	349279826	168723	55	339
barcode12_2664718	Meta	3206281	900958430	408136	55	281
barcode14_2672664	Meta	53373	43501696	330067	56	815.1
barcode15_2672455	Meta	44172	38050101	192851	56	861.4
barcode16_2672718	Meta	330879	188026695	2248934	55	568.3
barcode17_2664702	Meta	3484131	1253429762	229457	55	359.8
barcode21_2664788	Meta	1531437	586635892	441271	56	383.1
barcode22_6620	Meta	782382	237849646	318351	55	304
barcode23_2672522	Meta	12935	11702155	173071	56	904.7
barcode24_2672512	Meta	7142	5823289	334730	56	815.4

3.5.9.8 Hybrid vs Meta: DeepARG

DeepARG was run on both the hybrid and meta assemblies for each sample. Without applying any filtering, the hybrid dataset identified 13859 ARGs, with the meta dataset identifying 13789 ARGs. When filtering the ARGs on an identity of 75% (a more liberal cut-off to allow for Nanopore sequencing errors), 1034 and 1027 ARGs were identified for hybrid and meta datasets. It would appear that the number of ARGs identified by each assembly method are broadly similar, with slightly more identified in the hybrid dataset.

3.5.9.9 Hybrid vs Meta: Co-occurrence

3824 ARGs were identified to be co-occurring in the hybrid dataset, whilst 3372 were found in the meta dataset. Here, co-occurring is defined as ‘present on the same contig’. The data is provided unfiltered so that more stringent measures can be applied in the light of a definition of co-occurrence. Interestingly, there are 452 more ARGs identified as co-occurring in the hybrid dataset than the meta dataset, which highlights the benefit of the long-read technology increasing the average contig length.

3.6 Estimation of UK population burden

A total of 477 unique ARGs were identified in the RTE samples. For each of the specific priority groups of ARG, smaller numbers were detected, as shown in Figure 11 and Table 12.

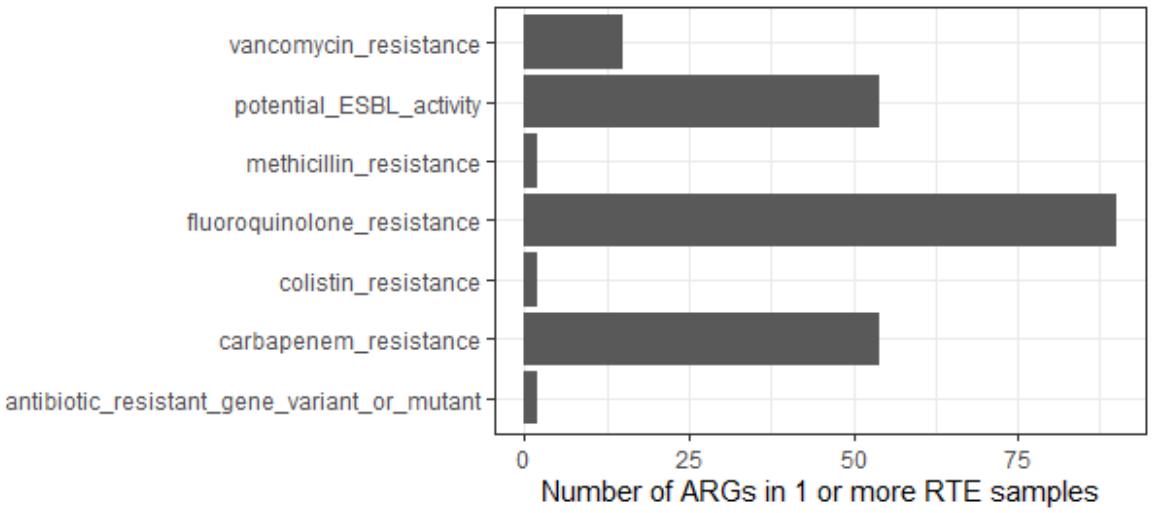


Figure 11. Number of ARGs (belonging to a particular category) found in at least one of the sampled RTE foods.

Table 12. Number of ARGs found in the sampled foods, within the defined sub-categories of ARG

ARG classification	Number of ARGs of this type identified within the samples
Fluoroquinolone resistance	90
Carbapenem resistance	54
Potential ESBL activity	54
Vancomycin resistance	15

ARG classification	Number of ARGs of this type identified within the samples
Methicillin resistance	2
Antibiotic resistant gene variant or mutant	2
Colistin resistance	2

Initially, we consider the prevalence in the diet for the full list of ARGs. In later sections, individual classes of ARGs (for example, methicillin resistant, colistin resistant or variant/mutant cases) are considered, leading to more targeted assessments.

3.6.1 ARGs detected in duplicate samples

11 duplicate samples (same product type, same batch code, bought at the one location during a single visit) were tested for ARGs Table 13. The aim of the duplicate analyses was to gain a first impression of the how consistently representative samples were of the "same product" (product, batch, location and time of purchase). While we do not expect representative duplicate samples to contain the same ARGs, representative unbiased samples can be expected to contain a similar number of distinct ARGs. The number of ARGs found in each duplicate sample was compared using a Poisson test. Significant values for the Poisson test (blueberries, iceberg lettuce), suggest that factors other than those used to describe the sample are affecting the number of ARGs detected. These may be associated with variation in the product or variation in testing. This may be a topic for further study

Table 13. Food categories for which duplicate samples were tested, showing the number of ARGs identified in each sample, the number shared in both samples, and the total number of different ARGs found across the pair of

samples. Poisson test (adjusted for multiple comparisons) significance values are shown.

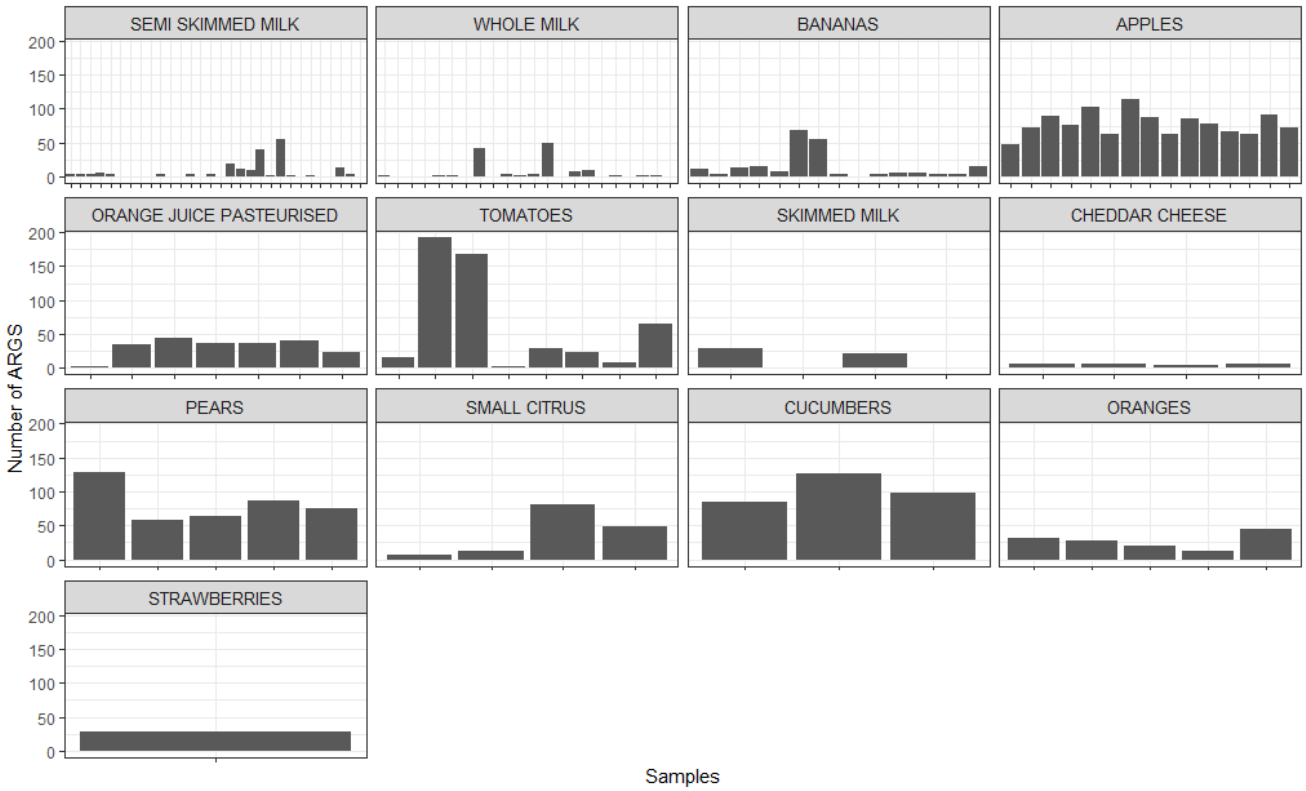
Food Category	ARGs in each sample	Number in both samples	Number in either or both samples	Poisson test
Apples	85, 79	56	108	1
Blueberries	57, 25	18	64	0.00535
Cherry tomatoes	154, 109	99	164	0.059
Double cream	1, 1	1	1	1
Iceberg lettuce	9, 55	8	56	3.89E-08
Lactose free semi skimmed milk	25, 21	18	28	1
Oranges	27, 21	14	34	1
Spreadable butter	10, 11	8	13	1
Tomatoes	193, 168	149	212	1
Unsweetened yogurt	7, 5	4	8	1
White onions	107, 139	94	152	0.383

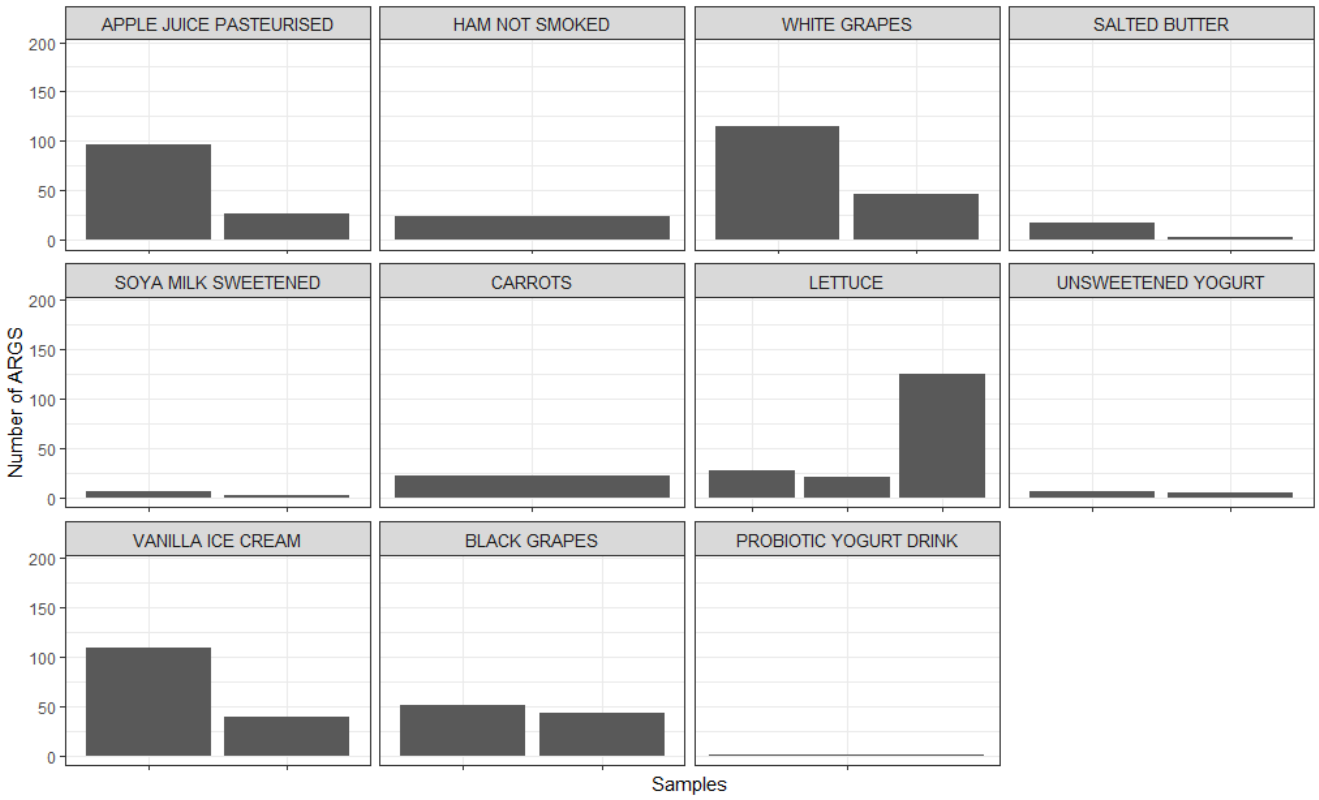
3.6.2 Incidence summaries at sample/food level

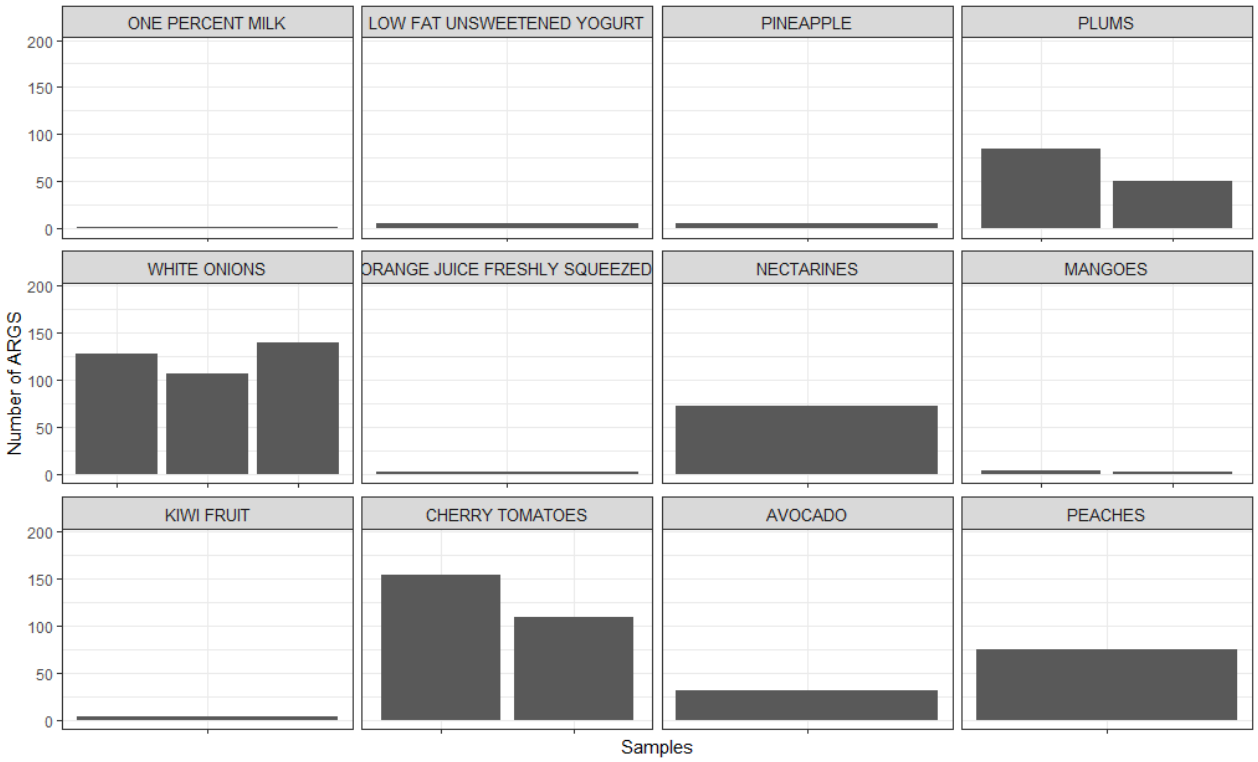
In Figure 12 we see that many produce items have larger number of unique ARGs than dairy products. Some have large variations between samples (for example bananas, tomatoes, milk). For example, there are two tomato samples (SampleID=2664549, 2664550) with 193 and 168 distinct ARGs whereas other tomato samples have fewer than 50 distinct ARGs. Pasteurised orange juice, pears, oranges appear to have similar numbers between samples, but have fewer samples. The 77 samples that did not contain any of the ARGs, and not shown in Figure 1, were for semi-skimmed milk (39), whole milk

(20), bananas (1), skimmed milk (3), ham (3), melon (1), reduced fat spread (2), mangoes (1), kiwi fruit (3), fat spread (1), avocado (2) and watermelon (1). For melon, watermelon, fat spread and reduced fat spread these were the only samples collected, so no ARGs at all were found in these 4 types. However, the sample sizes were very small in this case (Table 14).

This can also be shown as number of unique ARGs per food type, i.e. across combined samples within a food (Figure 12).


SEMI SKIMMED MILK
WHOLE MILK
BANANAS
APPLES
ORANGE JUICE PASTEURISED
TOMATOES
SKIMMED MILK
CHEDDAR CHEESE
PEARS
SMALL CITRUS
CUCUMBERS
ORANGES
STRAWBERRIES
Number of ARGS
0
50
100
150
200
Samples


APPLE JUICE PASTEURISED
HAM NOT SMOKED
WHITE GRAPES
SALTED BUTTER
SOYA MILK SWEETENED
CARROTS
LETTUCE
UNSWEETENED YOGURT
VANILLA ICE CREAM
BLACK GRAPES
PROBIOTIC YOGURT DRINK
Number of ARGS
0
50
100
150
200
Samples


ONE PERCENT MILK
LOW FAT UNSWEETENED YOGURT
PINEAPPLE
PLUMS
WHITE ONIONS
ORANGE JUICE FRESHLY SQUEEZED
NECTARINES
MANGOES
KIWI FRUIT
CHERRY TOMATOES
AVOCADO
PEACHES
Number of ARGS
Samples
0
50
100
150
200

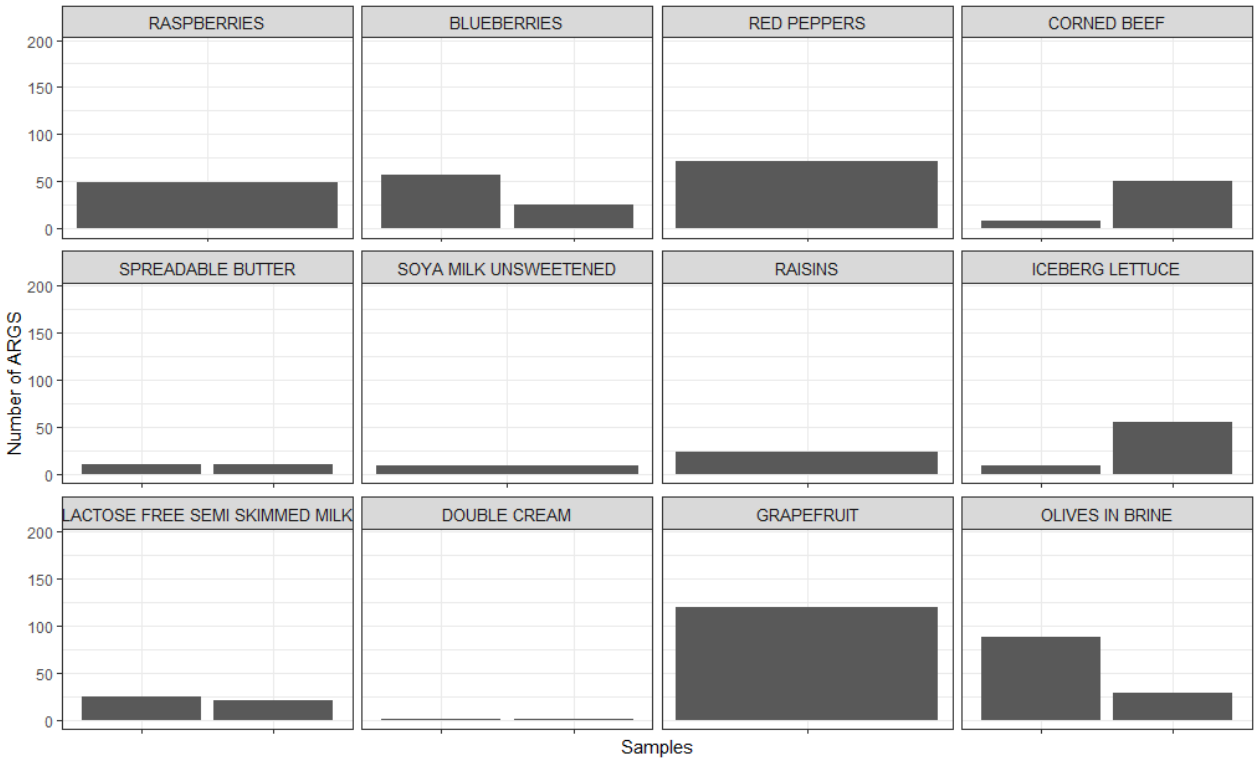


Figure 12. For each sample, the number of unique ARGs found. The x-axis represents unique sample numbers, which have been omitted here to save space. Samples are displayed together per food type, showing the between-sample variation in the number of ARGs. Values are shown for 179 samples (of the original 256 samples, 77 were not found to contain any of the ARGs, see Table 14).

Table 14. Summaries based on the number of ARGs per sample, by food type. The number of samples collected and the number of samples containing at least 1 ARG are also shown.

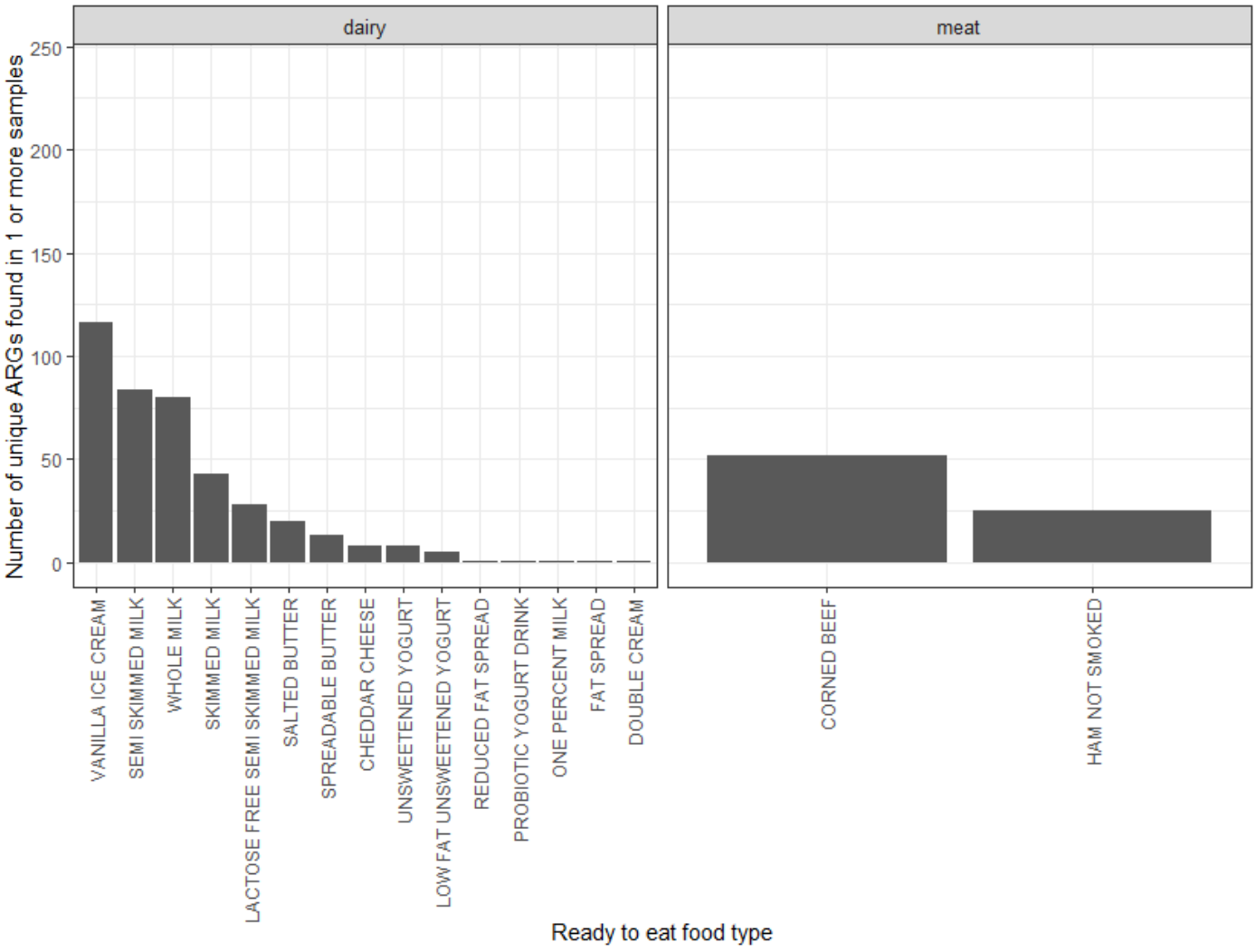
Food category	Mean number of ARGs per sample	Median number of ARGs per sample	Minimum number of ARGs per sample	Maximum number of ARGs per sample: maximum	Total number of samples	Samples with 1 or more ARG
Semi skimmed milk	2.9	0	0	56	69	30
Whole milk	3.3	1	0	49	42	22
Bananas	13.5	5.5	0	68	16	15
Apples	78.1	76	47	114	15	15
Orange juice pasteurised	31.3	36	3	45	7	7
Tomatoes	63.0	26	3	193	8	8
Skimmed milk	7.6	1	0	30	7	4
Cheddar cheese	5.8	6	5	6	4	4
Pears	82.4	76	58	129	5	5
Small citrus	36.8	30	7	80	4	4
Cucumbers	102.7	98	84	126	3	3
Oranges	27.2	27	12	44	5	5
Strawberries	29.0	29	29	29	1	1
Apple juice pasteurised	61.5	61.5	27	96	2	2

Food category	Mean number of ARGs per sample	Median number of ARGs per sample	Minimum number of ARGs per sample	Maximum number of ARGs per sample: maximum	Total number of samples	Samples with 1 or more ARG
Ham not smoked	6.0	0	0	24	4	1
White grapes	80.5	80.5	46	115	2	2
Melon	0.0	0	0	0	1	0
Salted butter	10.5	10.5	3	18	2	2
Soya milk sweetened	4.5	4.5	3	6	2	2
Carrots	22.0	22	22	22	1	1
Lettuce	57.7	27	21	125	3	3
Unsweetened yoghurt	6.0	6	5	7	2	2
Vanilla ice cream	74.0	74	39	109	2	2
Black grapes	47.0	47	43	51	2	2
Reduced fat spread	0.0	0	0	0	2	0
Probiotic yoghurt drink	1.0	1	1	1	1	1

Food category	Mean number of ARGs per sample	Median number of ARGs per sample	Minimum number of ARGs per sample	Maximum number of ARGs per sample: maximum	Total number of samples	Samples with 1 or more ARG
One percent milk	1.0	1	1	1	1	1
Low fat unsweetened yoghurt	5.0	5	5	5	1	1
Pineapple	6.0	6	6	6	1	1
Plums	68.0	68	51	85	2	2
White onions	124.7	128	107	139	3	3
Orange juice freshly squeezed	2.0	2	2	2	1	1
Nectarines	72.0	72	72	72	1	1
Mangoes	2.3	3	0	4	3	2
Kiwi fruit	0.8	0	0	3	4	1
Cherry tomatoes	131.5	131.5	109	154	2	2
Fat spread	0.0	0	0	0	1	0
Avocado	10.3	0	0	31	3	1
Peaches	74.0	74	74	74	1	1

Food category	Mean number of ARGs per sample	Median number of ARGs per sample	Minimum number of ARGs per sample	Maximum number of ARGs per sample: maximum	Total number of samples	Samples with 1 or more ARG
Raspberries	49.0	49	49	49	1	1
Blueberries	41.0	41	25	57	2	2
Red peppers	71.0	71	71	71	1	1
Corned beef	29.0	29	8	50	2	2
Spreadable butter	10.5	10.5	10	11	2	2
Soya milk unsweetened	9.0	9	9	9	1	1
Raisins	23.0	23	23	23	1	1
Iceberg lettuce	32.0	32	9	55	2	2
Lactose free semi skimmed milk	23.0	23	21	25	2	2
Watermelon	0.0	0	0	0	1	0
Double cream	1.0	1	1	1	2	2
Grapefruit	119.0	119	119	119	1	1

Food category	Mean number of ARGs per sample	Median number of ARGs per sample	Minimum number of ARGs per sample	Maximum number of ARGs per sample: maximum	Total number of samples	Samples with 1 or more ARG
Olives in brine	58.5	58.5	29	88	2	2



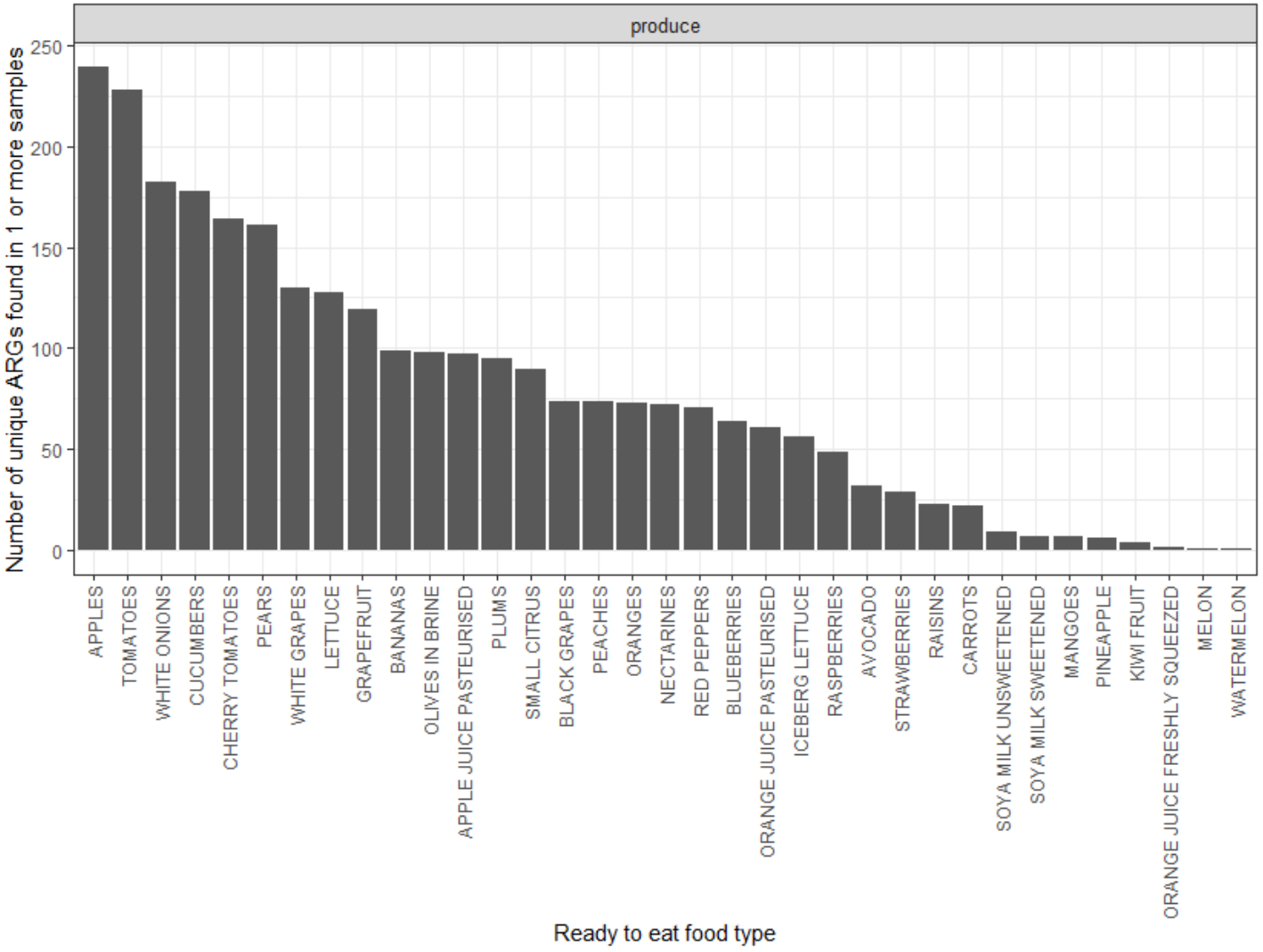
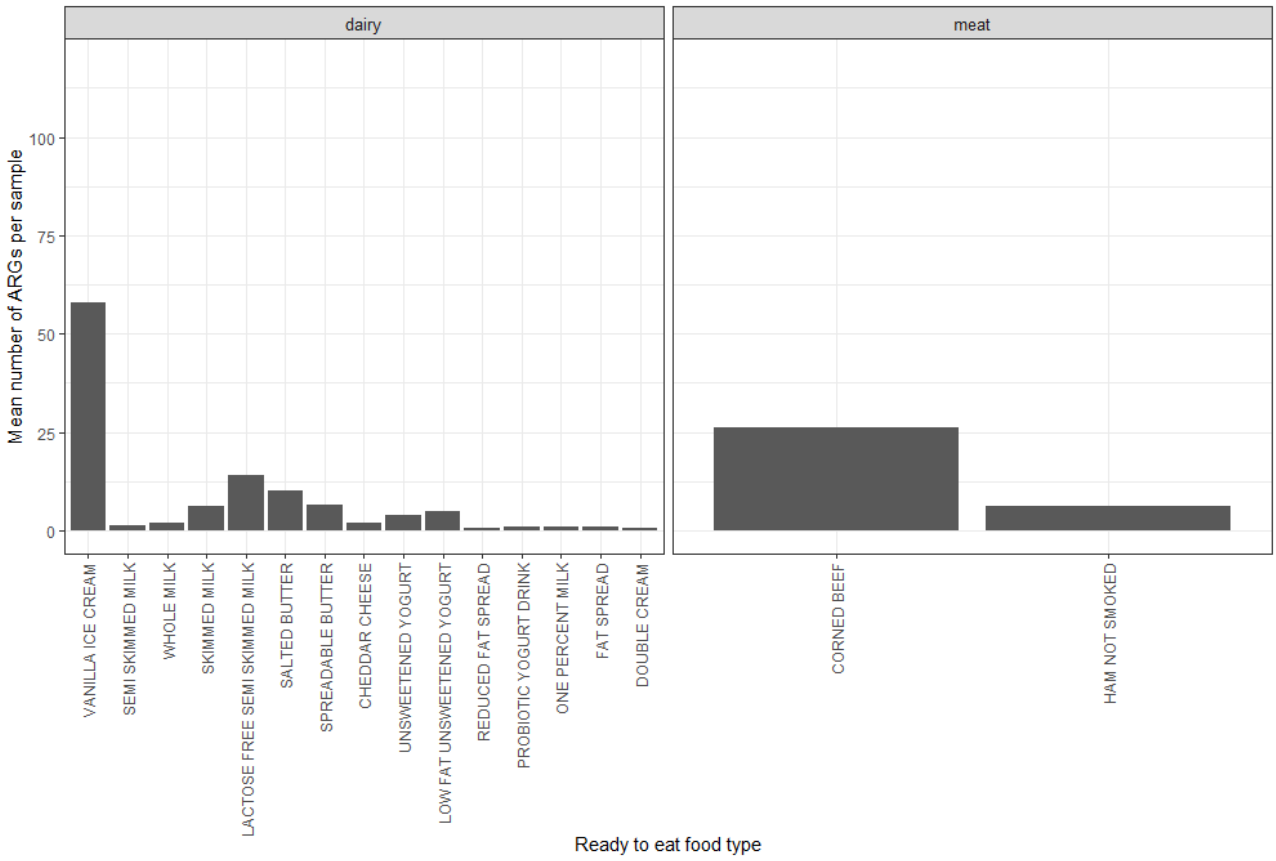


Figure 13. Total number of ARGs found per food type. These are displayed by food type (dairy, meat, produce) without accounting for sample size or population consumption.


dairy
meat
Mean number of ARGs per sample
100
75
50
25
0
VANILLA ICE CREAM
SEMI SKIMMED MILK
WHOLE MILK
SKIMMED MILK
LACTOSE FREE SEMI SKIMMED MILK
SALTED BUTTER
SPREADABLE BUTTER
CHEDDAR CHEESE
UNSWEETENED YOGURT
LOW FAT UNSWEETENED YOGURT
REDUCED FAT SPREAD
PROBIOTIC YOGURT DRINK
ONE PERCENT MILK
FAT SPREAD
DOUBLE CREAM
CORNED BEEF
HAM NOT SMOKED
Ready to eat food type

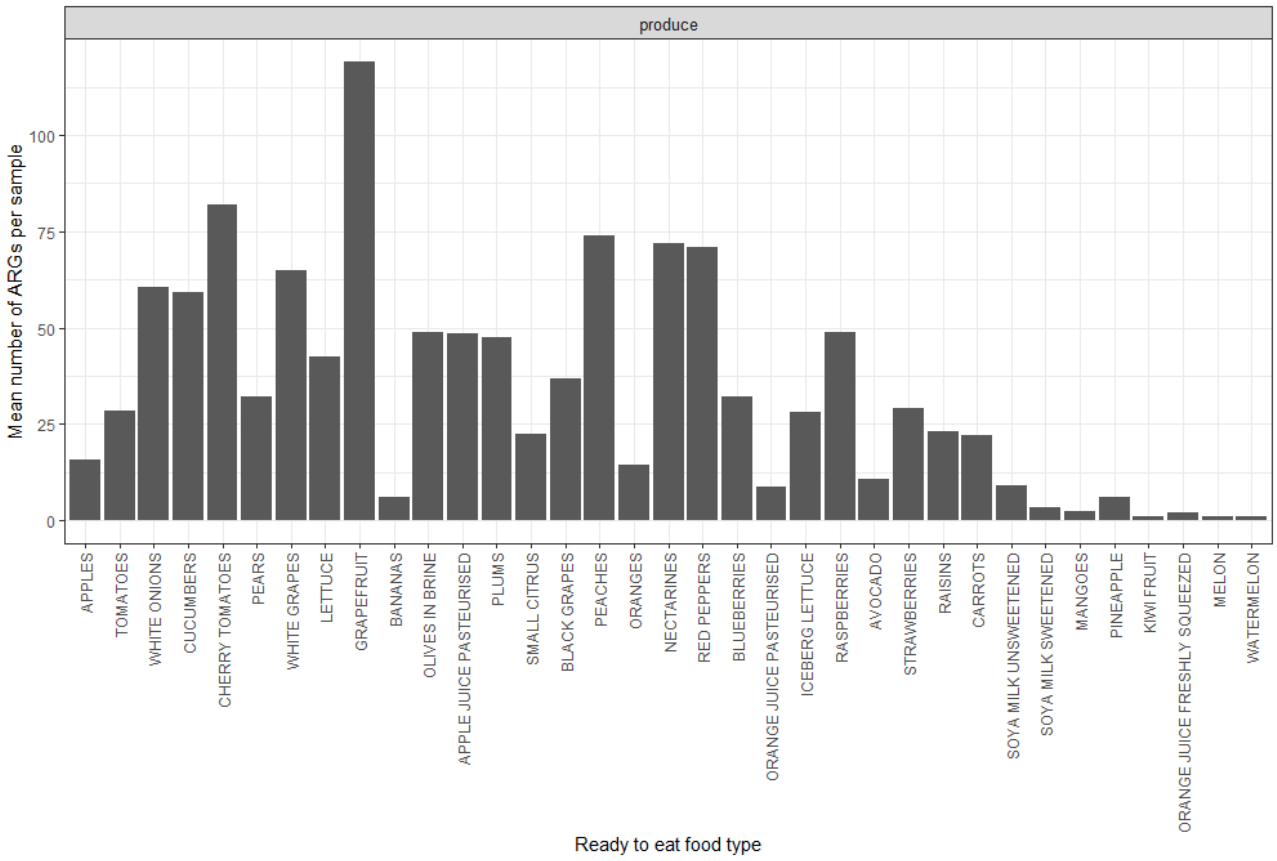


Figure 14. Average number of ARGs detected per sample, by food type. Note that the calculation does not include multiple instances of the same ARG. So, for example if 2 samples both contain the same ARG then the average is 0.5, not 1. The food types are arranged as in Figure 19, but highlight those food types for which the number of unique ARGs is high relative to the number of samples collected.

In Figure 15, the bar widths are proportional to the total UK consumption of each item. Labels of food corresponding to 10% of the overall consumptions of these foods have been left out for readability. In Figure 16 they are instead split into an 80% and 20% slice of cumulative consumptions, so that the less consumed items are also visible.

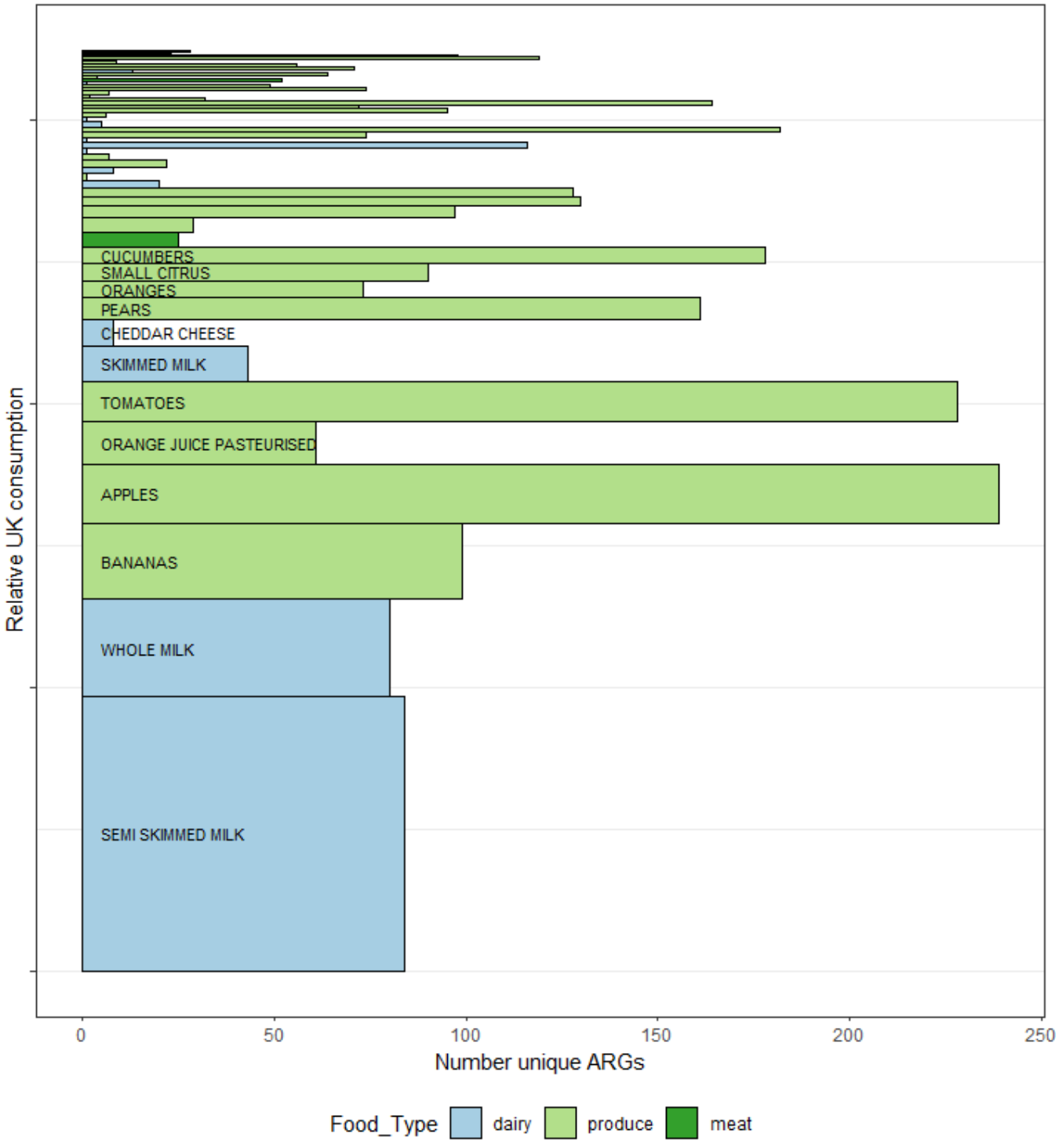
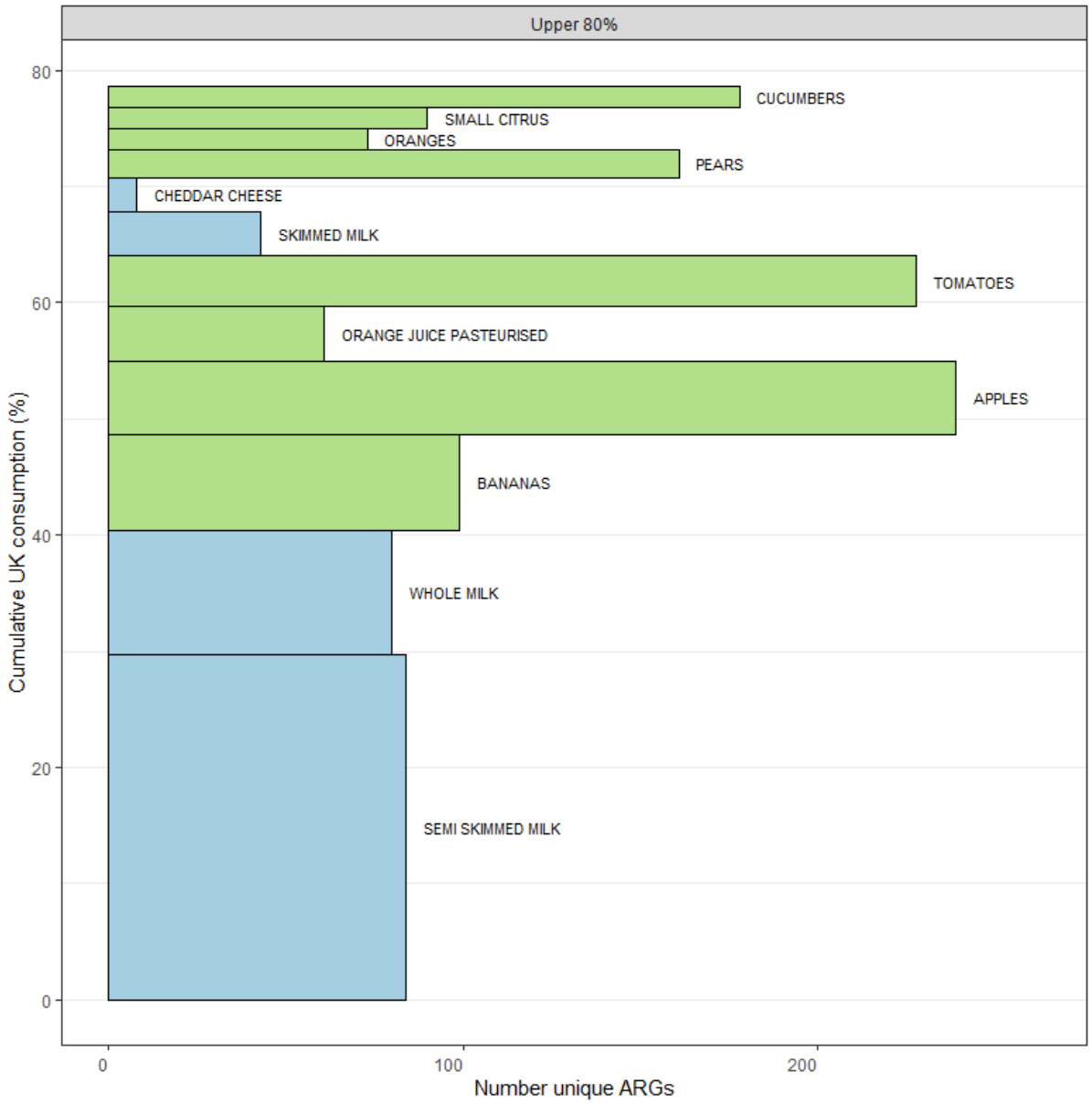


Figure 15. Number of ARGs found per food type. These are ordered by overall consumption amounts. The x-axis values are related to those shown in Figure 13, although

when considering combined samples per food type the number of ARGs tends to be larger than that seen in individual samples. This illustration is an alternative visualisation of the same information that highlights the relative contribution of the different ready to eat items to the total UK consumption



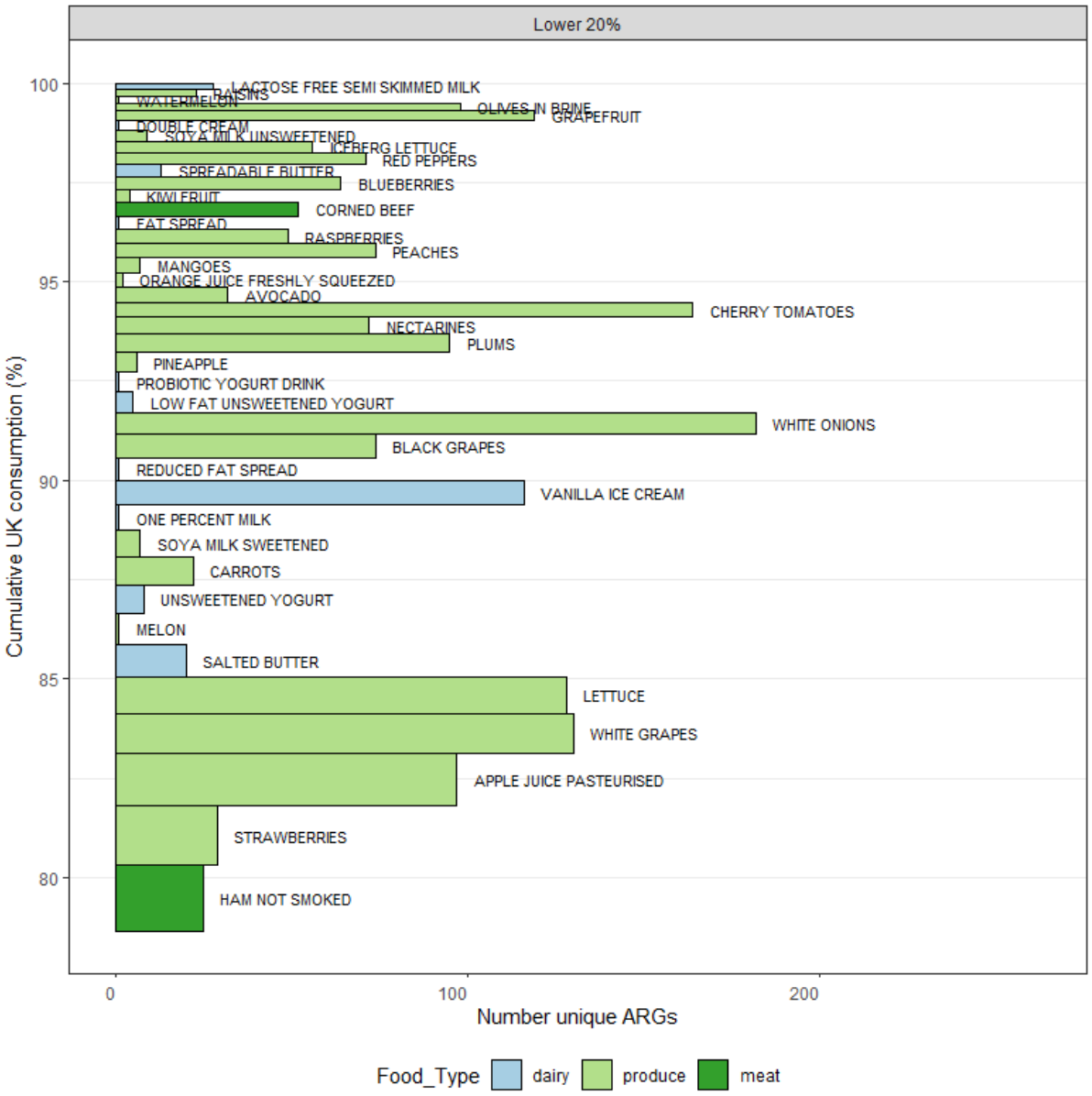
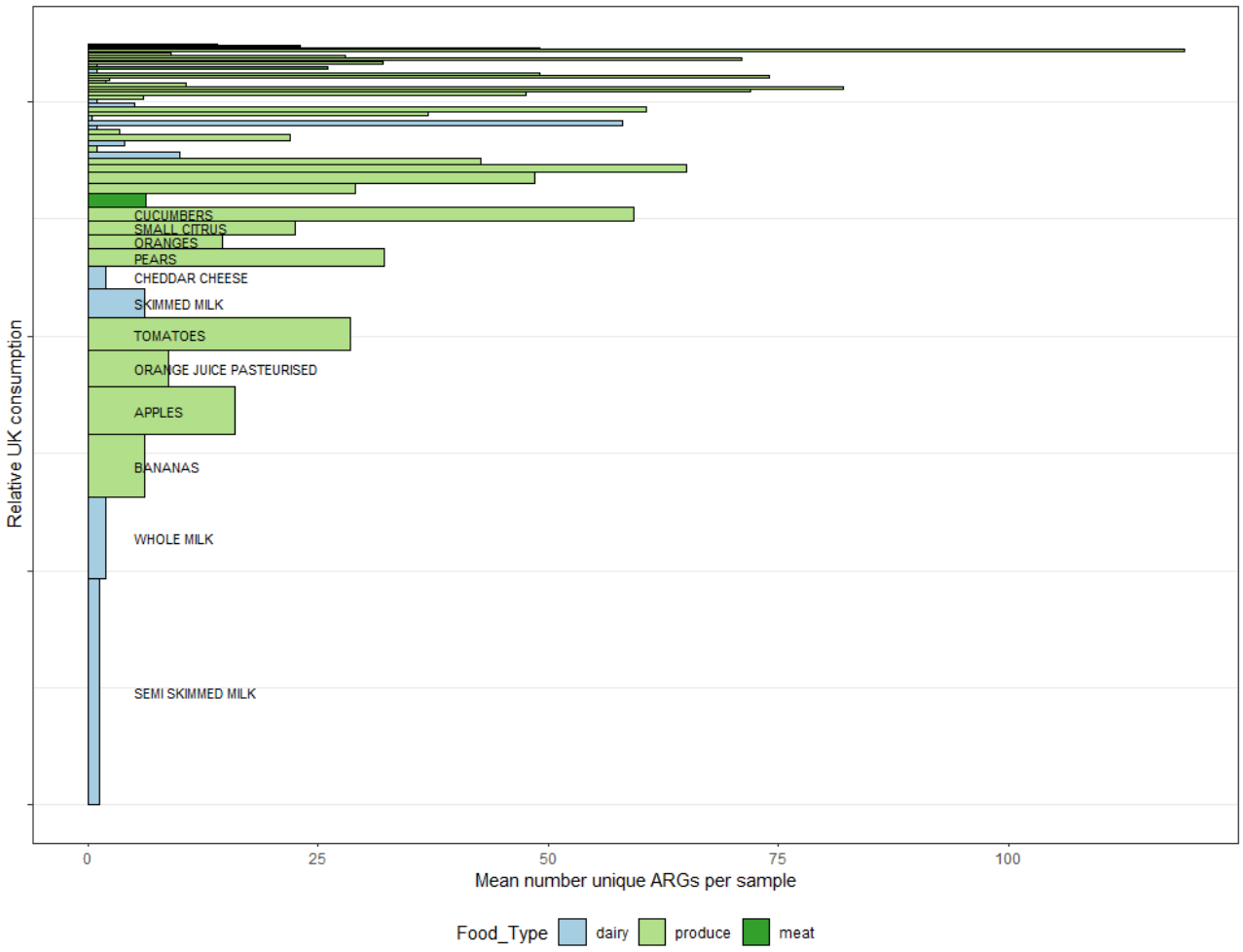
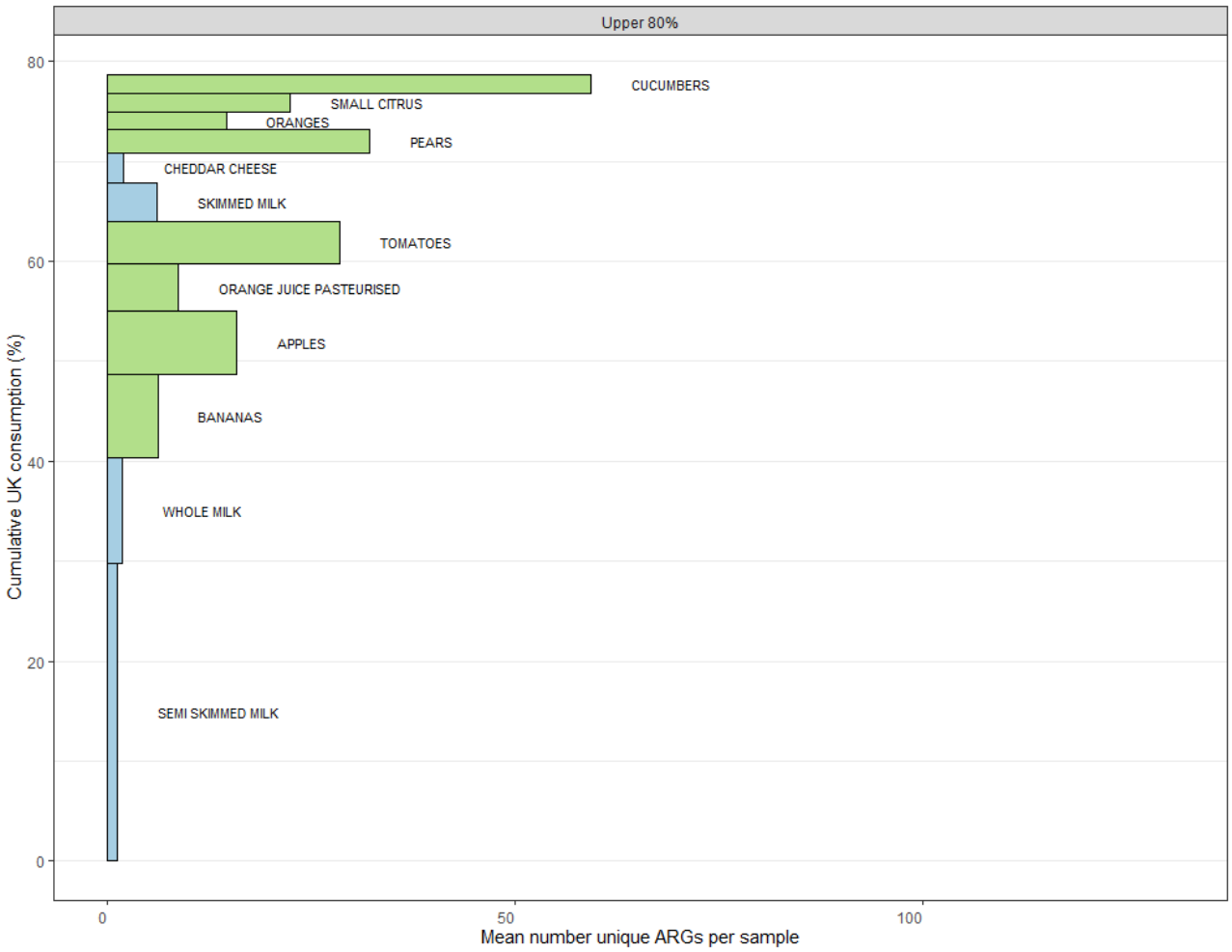


Figure 16. Number of ARGs found per food type. These are ordered by overall consumption amounts. Top 80% consumption (top) and bottom 20% consumption (bottom) relative to total consumption for the selected items.


CUCUMBERS
SMALL CITRUS
ORANGES
PEARS
CHEDDAR CHEESE
SKIMMED MILK
TOMATOES
ORANGE JUICE PASTEURISED
APPLES
BANANAS
WHOLE MILK
SEMI SKIMMED MILK
Relative UK consumption
0
25
50
75
100
Mean number unique ARGs per sample
Food_Type
dairy
produce
meat


Upper 80%
CUCUMBERS
SMALL CITRUS
ORANGES
PEARS
CHEDDAR CHEESE
SKIMMED MILK
TOMATOES
ORANGE JUICE PASTEURISED
APPLES
BANANAS
WHOLE MILK
SEMI SKIMMED MILK
Cumulative UK consumption (%)
0
20
40
60
80
0
50
100
Mean number unique ARGs per sample

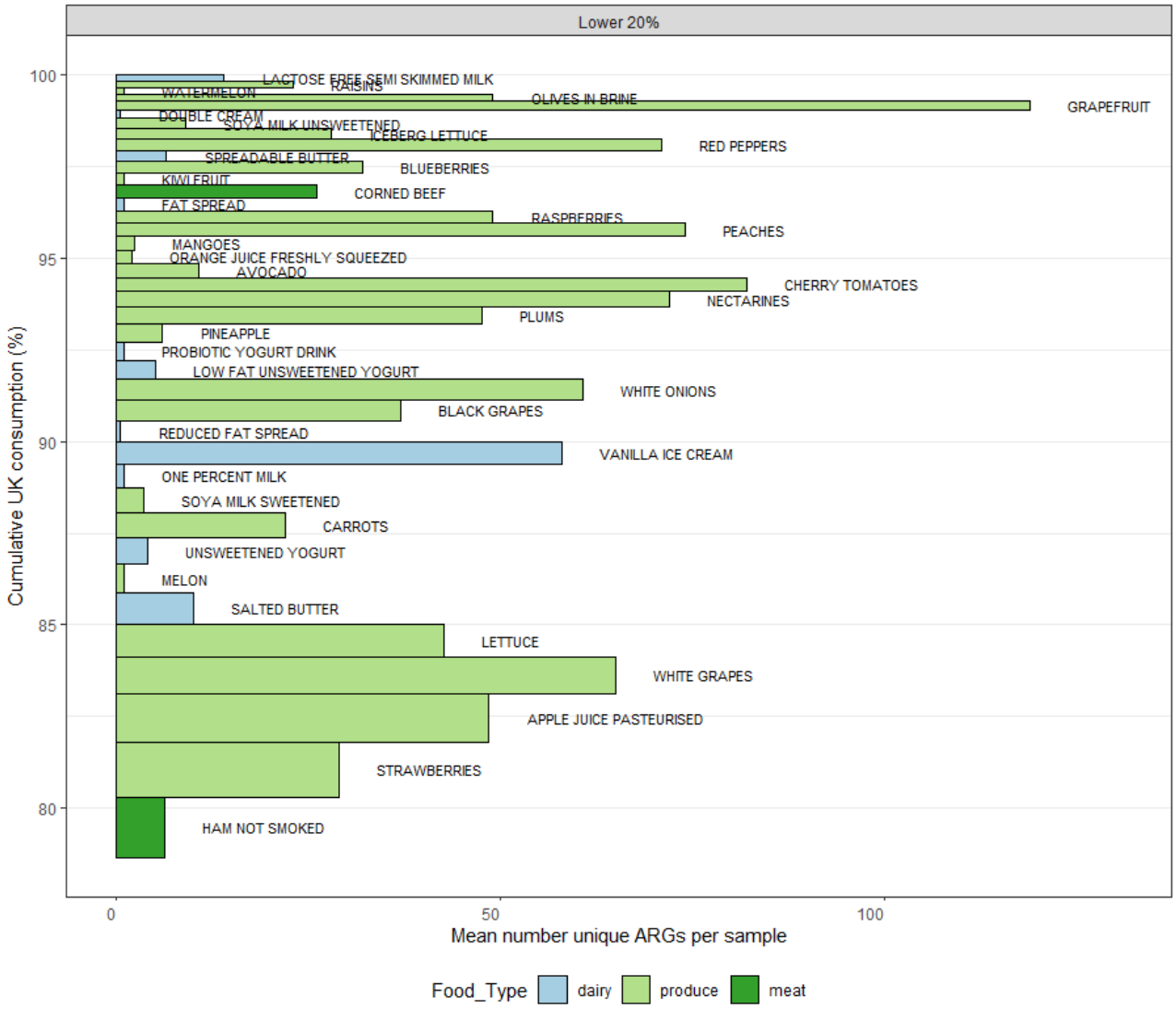
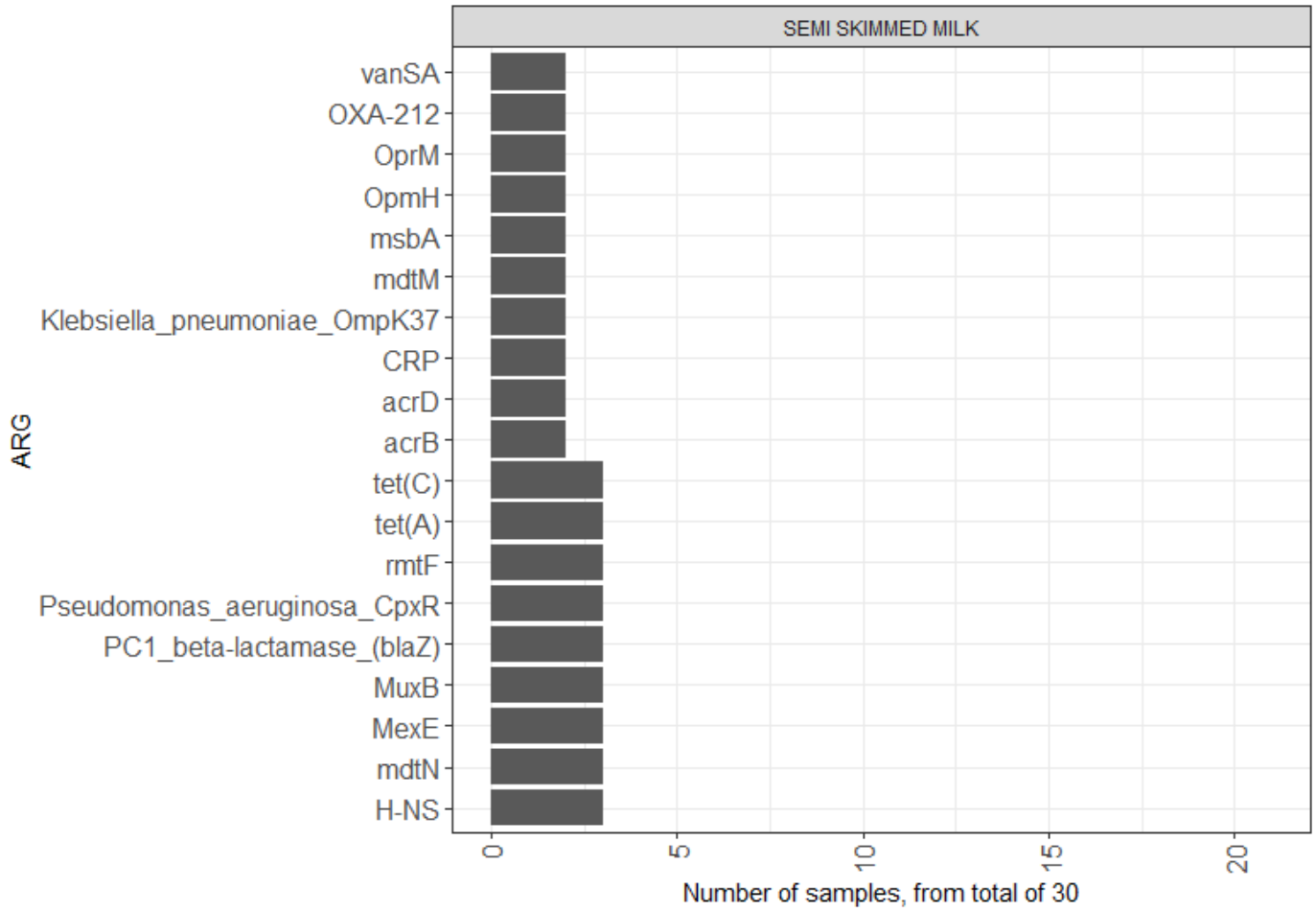


Figure 17. Mean number of ARGs found (per sample) within each food type. These are ordered by overall consumption amounts. Top 80% consumption (top) and bottom 20% consumption (bottom) relative to total consumption for the selected items.

A similar process was applied to summarise the number of **ARG families** (rather than ARGs) per food type, and these results are presented in Appendix 12.

3.6.3 Incidence: Total UK Diet

Examples of the frequency (relative number of samples) of a given ARG in a food specific dataset are shown for semi-skimmed milk (Figure 18), whole milk (Figure 19) and bananas (Figure 20). These food types were selected as the 3 most highly consumed ready to eat products included in the analysis. Analogous results for ARG families are provided in Appendix 12.



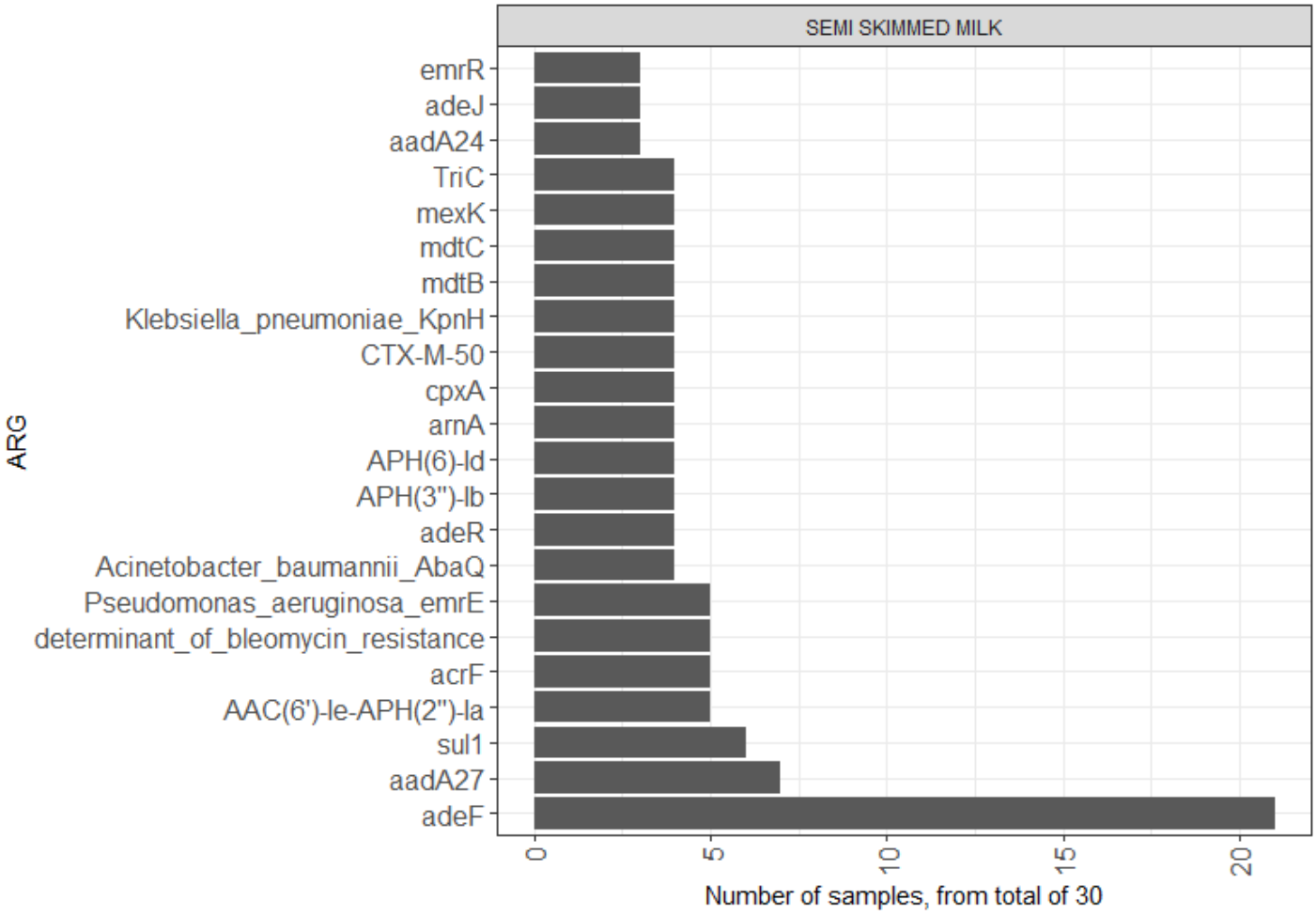
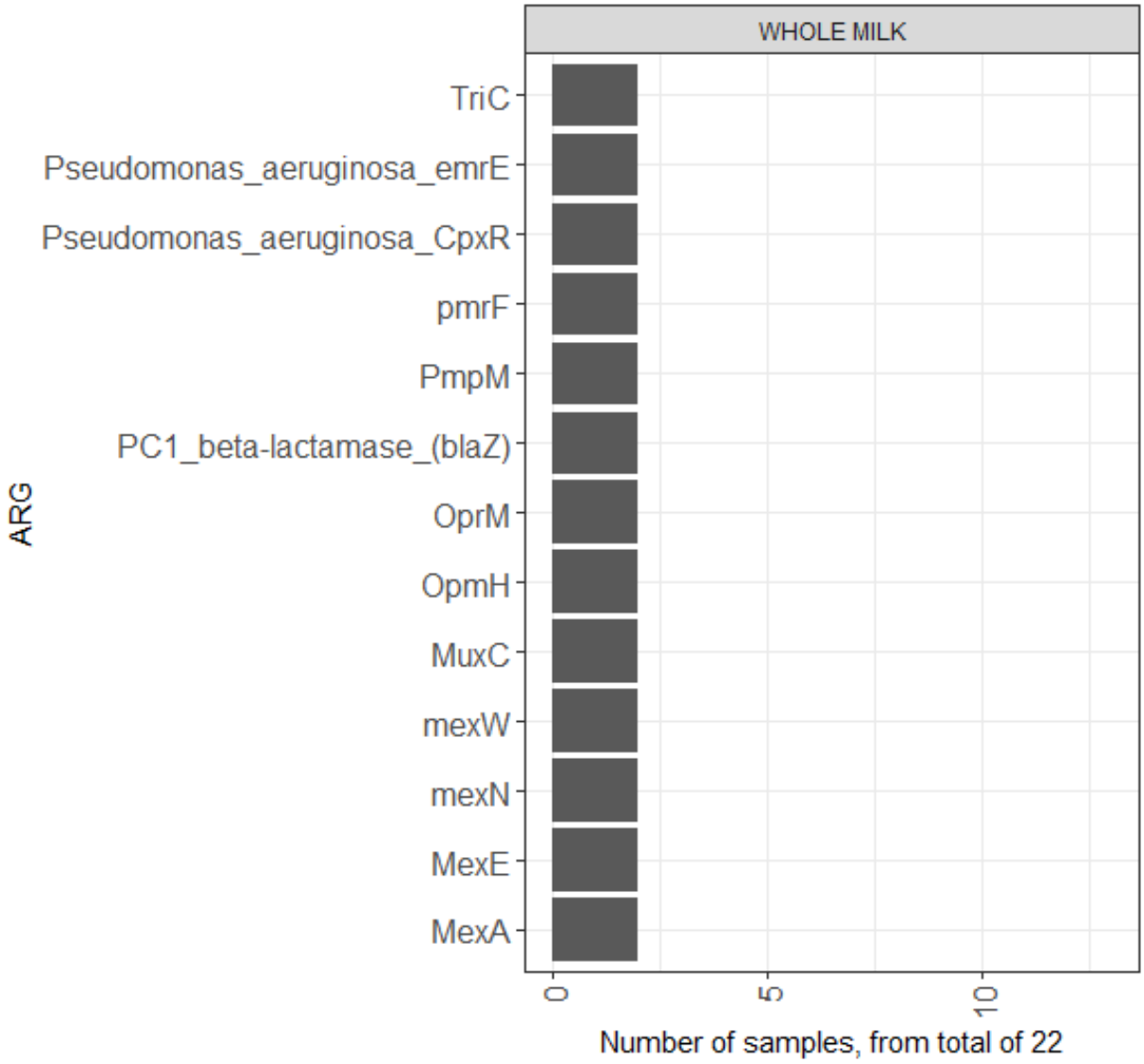


Figure 18. Number of semi-skimmed milk samples found to contain the named individual ARGs. (30 samples across a range of brands were found to contain one or more ARGs from a total of 69 measured original samples). For further information on each gene name, refer to the Antibiotic Resistance Ontology website.

For a further 41 ARGs, not shown in the graph, each was observed in only one of the 30 positive semi-skimmed milk samples: AAC(3)-Ia, AAC(3)-IIb, AAC(3)-IIe, aadS, adeK, amrA, bacA, baeS, catI, emrB, Enterobacter_cloacae_acrA, eptA, Escherichia_coli_ampC1_beta-lactamase, Escherichia_coli_mdfA, evgS, floR, kdpE, Klebsiella_pneumoniae_KpnF, Klebsiella_pneumoniae_KpnG, mdtO, MexB, mexI, mexN,

mexP, mexW, MuxC, OpmB, OprN, OXA-280, PmpM, pmrF, QnrB8, SHV-11, smeB, smeD, smeE, smeR, SRT-2, sul2, tolC, TriB, ugd.


WHOLE MILK
ARG
TriC
Pseudomonas_aeruginosa_emrE
Pseudomonas_aeruginosa_CpxR
pmrF
PmpM
PC1_beta-lactamase_(blaZ)
OprM
OpmH
MuxC
mexW
mexN
MexE
MexA
0
5
10
Number of samples, from total of 22

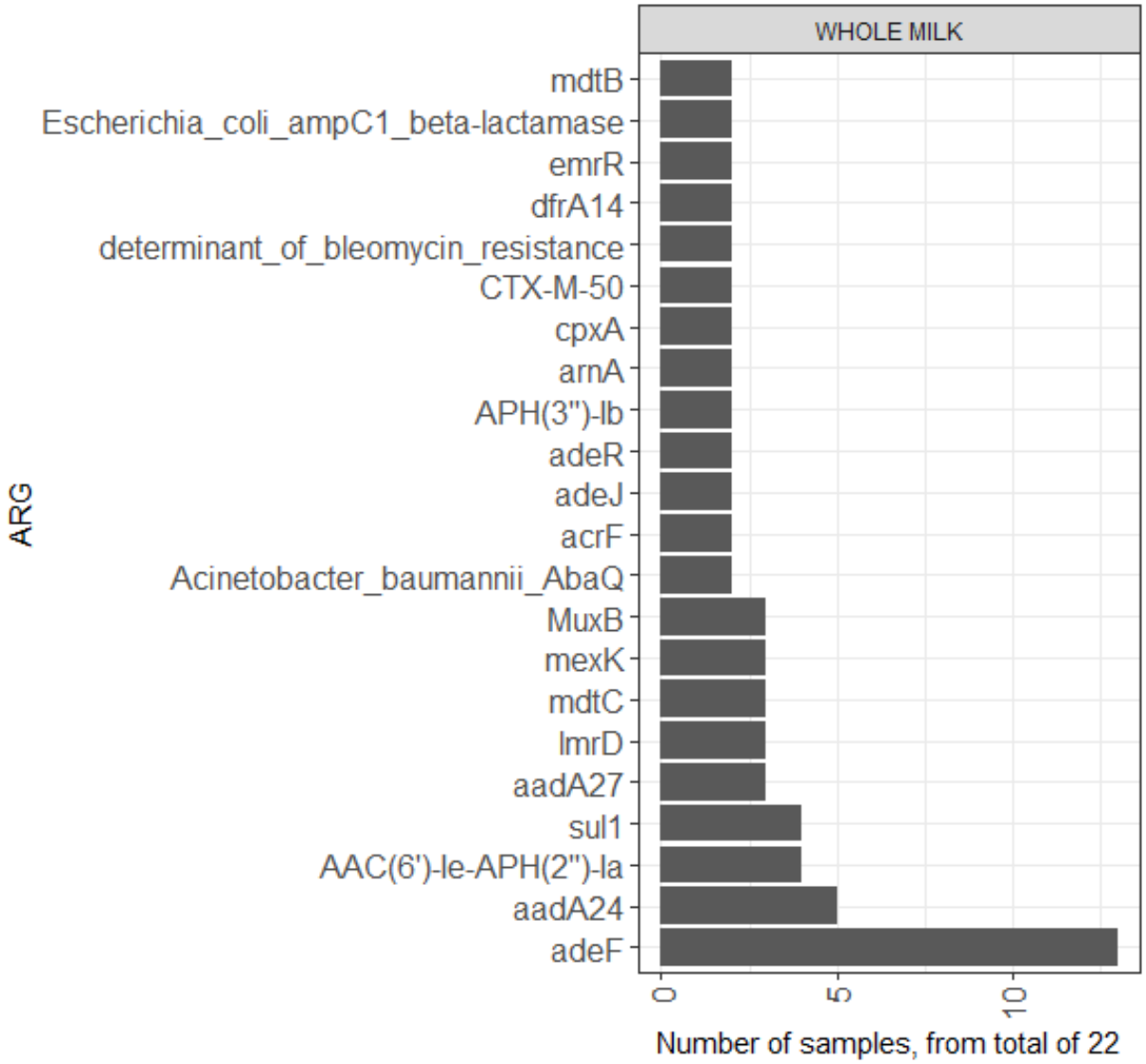
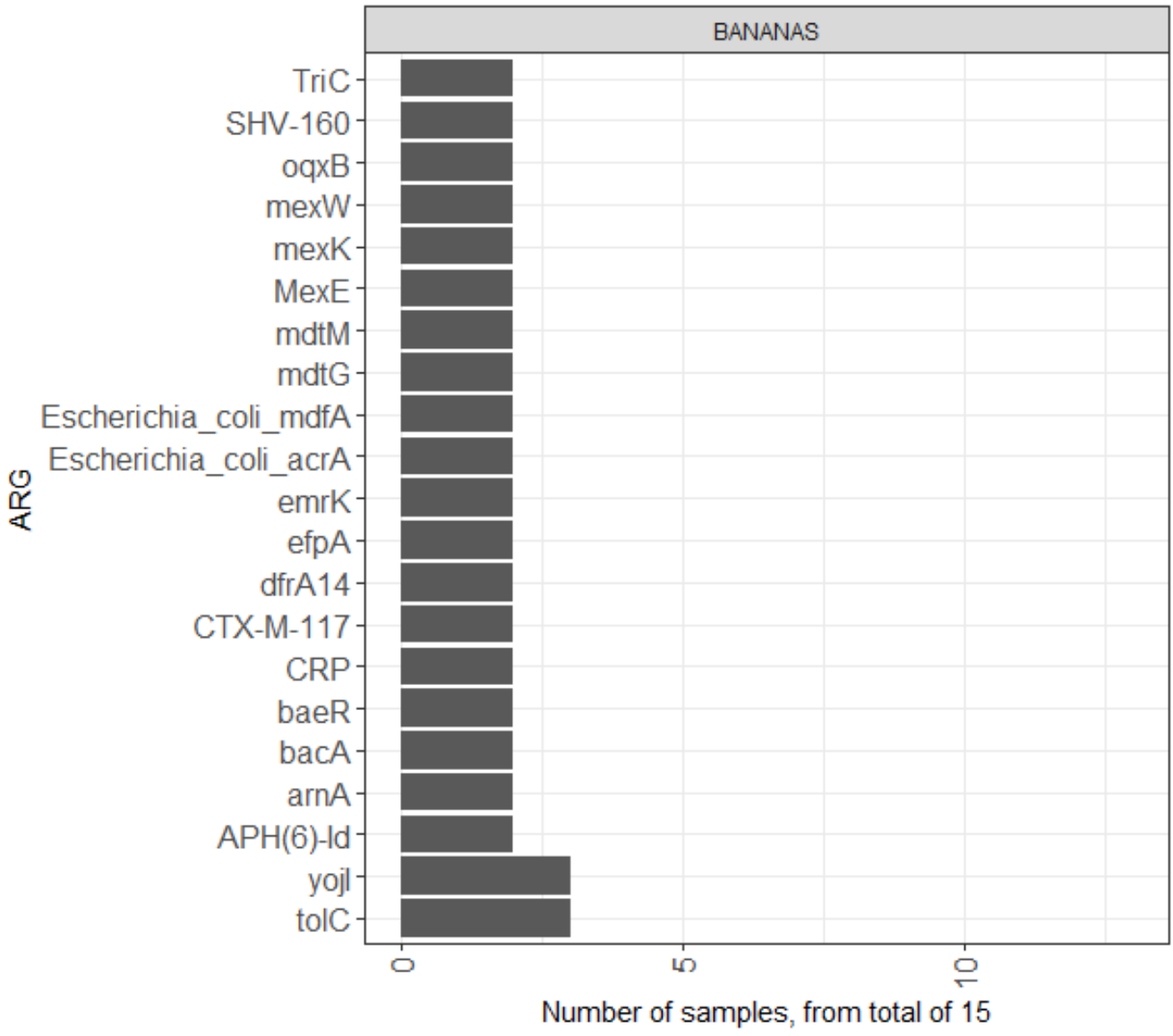


Figure 19. Number of whole milk samples (from a total of 22) found to contain individual ARGs (22 samples were found to contain one or more ARGs from a total of 42 measured original whole milk samples)

Another 44 were found in only one whole milk sample: AAC(3)-IIb, AAC(6')-Ib7, aadA17, aadA6, aadA7, abeM, Acinetobacter_baumannii_AbaF, acrB, adeG, adeI, adeK, ANT(3'')-IIa, ANT(3'')-IIc, APH(6)-Id, bcr-1, CRP, dfrA17, emrB, emrY, eptA, ErmB, Klebsiella_pneumoniae_KpnG, Klebsiella_pneumoniae_KpnH, mdtG, mdtM, mdtO, MexC,

mexI, mexL, mphE, msbA, msrE, OpmB, OprJ, OprN, OXA-274, OXA-275, OXA-65, rmtF, SHV-11, TEM-157, tet(A), TriA, TriB.



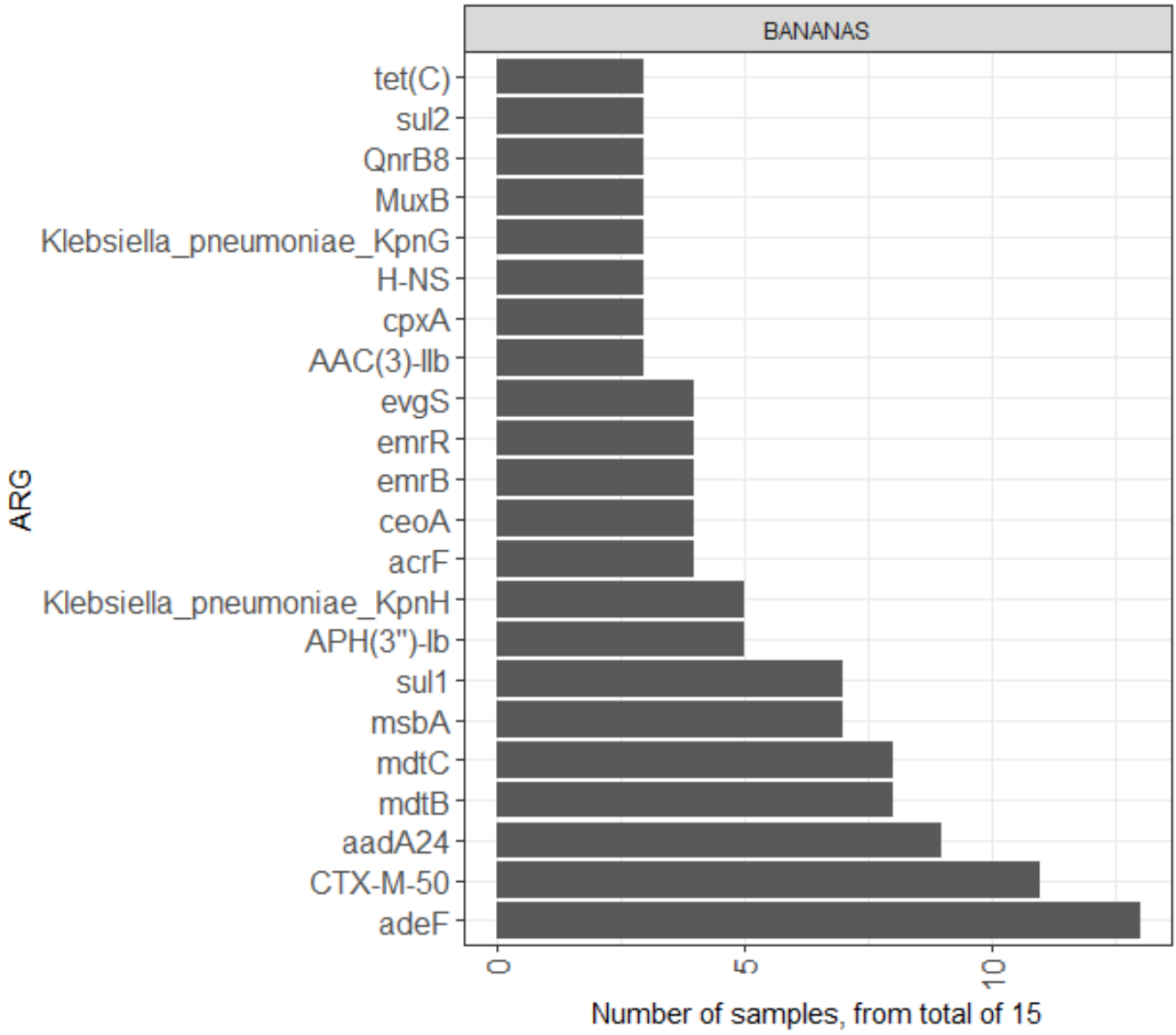


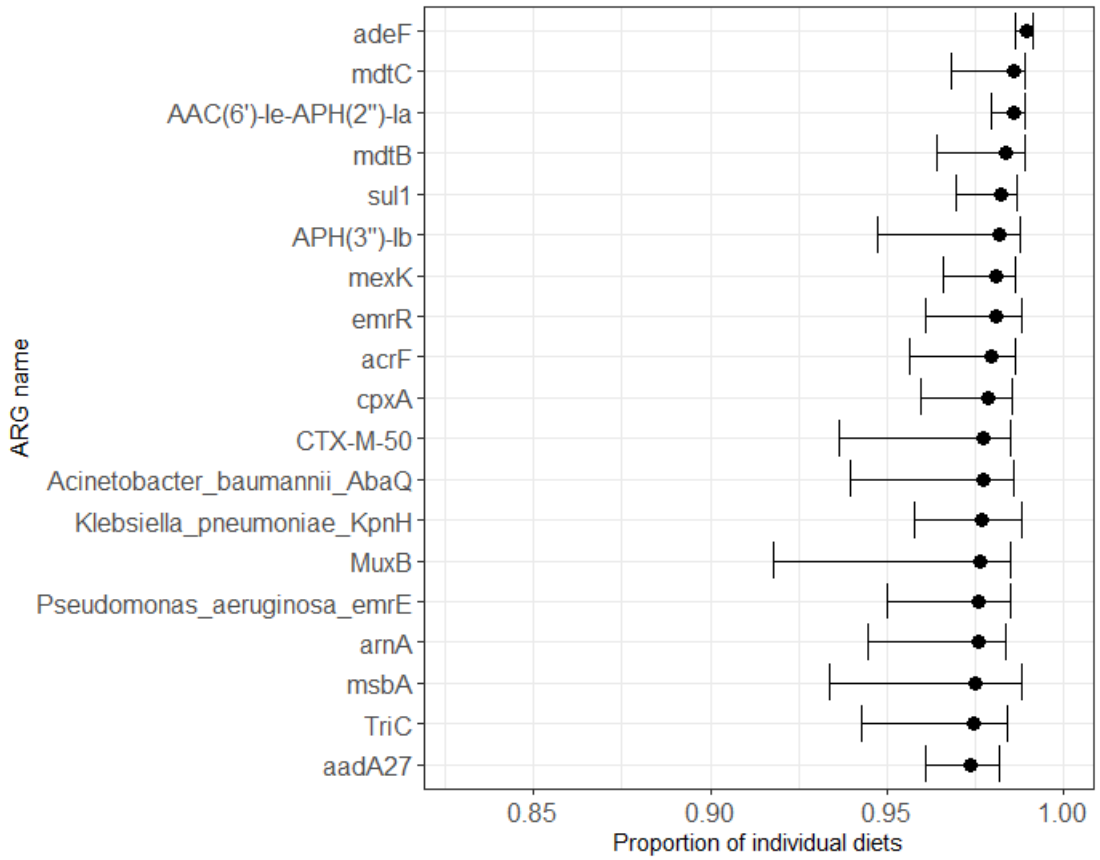
Figure 20. Number of banana samples (from a total of 15) found to contain individual ARGs (15 samples were found to contain one or more ARGs from a total of 16 measured original banana samples).

A further 55 ARGs were found in only one banana sample: AAC(6')-Ib7, aadA27, Acinetobacter_baumannii_AbaQ, acrB, adeJ, adeK, adeR, Agrobacterium_fabrum_chloramphenicol_acetyltransferase, APH(3')-Ia, baeS, catB3, catI, CTX-M-69, dfrA17, floR, fosA, FosA6, JOHN-1, kdpE, Klebsiella_pneumoniae_OmpK37,

lmrD, mdtN, mdtO, mdtP, MexB, MexD, mexN, mtrA, MuxC, novA, OKP-A-5, OpmB, OpmH, OprM, OprN, OXA-296, PER-6, pmrF, Pseudomonas_aeruginosa_CpxR, Pseudomonas_aeruginosa_emrE, Rm3, rmtF, SHV-11, smeB, smeD, smeE, SRT-2, TEM-102, TEM-132, TEM-47, tet(A), tet(V), tet(Z), THIN-B, ugd.

3.6.4 Prevalence Calculations, Population Level

The 52 RTE foods sampled in this study are representative of food groups corresponding to over 90% of the total RTE intakes of the UK population. These food types are estimated to cover 96.9% of those foods classified as RTE and informative (Section 2.1). Prevalence estimates for the proportion of individual diets in which individual ARGs appear are shown in Figure 21. Results for ARG families are included in Appendix 12.



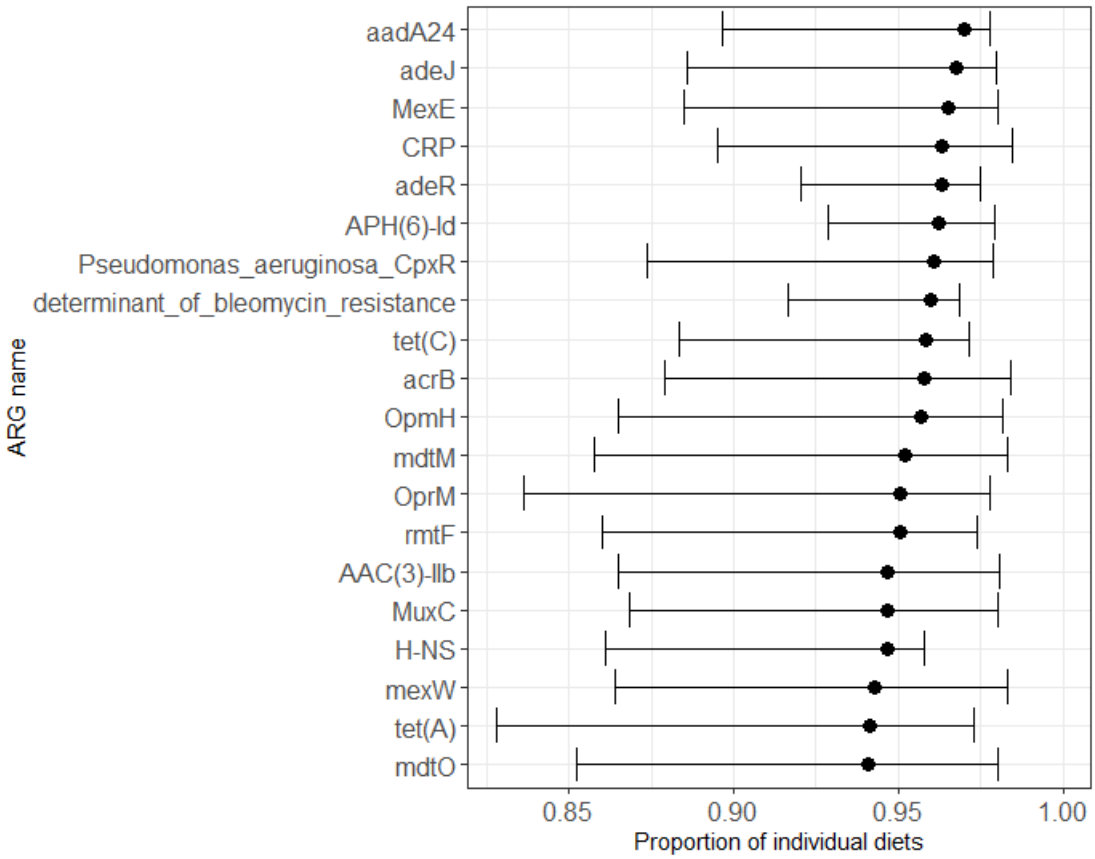


Figure 21. Estimated prevalence of ARGs in ready to eat foods, listing those that are estimated to occur in 95% or more of UK diets. The overall incidences per food were used here. We expect the ARGs found in milk and other high consumption items to be the main contributors with high prevalence, due to the frequency of consumption (see for example the list of most frequently observed ARGs for semi-skimmed milk). 95% confidence intervals are shown based on 100 bootstrap samples. For better readability, the plots have been divided into roughly equally numbers of points, with panels ordered from highest proportion of diets at the top to lowest proportion of diets at the bottom.

3.6.5 Colistin Resistance ARGs

A single colistin-resistant ARG (ICR-Mo) was found in 3 out of 15 samples of apple, 1 of 1 sample of nectarine and 1 out of 2 samples of cherry tomatoes. The gene family of ICR-Mo is ‘intrinsic colistin resistant phosphoethanolamine transferase’, and it appears to be a

chromosomally encoded determinant of colistin resistance (Wei et al., 2018). A second colistin resistant ARG (MCR-5.2) was found in the second sample of cherry tomatoes. The gene family of MCR-5.2 is MCR phosphoethanolamine transferase. The proportion of UK diets containing ICR-Mo is estimated as 46%, which is mainly driven by apple consumptions. The proportion of diets with MCR-5.2 is estimated as 7%.

3.6.6 Methicillin Resistance ARGs

A single methicillin resistant ARG was found in each of the 2 positive samples of pear (abcA in both samples). Similarly, a single case of methicillin ARG was found in the 1 sample of cucumber (mecA) and in the 1 sample of raisins (abcA). This led to the estimated burden in the UK diet of 37% of UK diets for mecA (gene family = methicillin resistant PBP2) and 17% UK diets for abcA (gene family = ATP-binding cassette (ABC) antibiotic efflux pump).

3.6.7 Antibiotic resistant gene variant or mutant

These are ARGs for which resistance is conferred by the presence of a mutation (for example in the target site for an antibiotic), rather than by the presence of the gene itself. To identify these as present we required 100% sequence identity to the reference gene sequences. Numbers of samples found to contain one or more ARGs in this category are shown in Table 15. In fact, for all 18 of these samples a single relevant ARG was measured. In 1 apple sample this was rpoB2 (gene family = rifamycin-resistant beta-subunit of RNA polymerase (rpoB)). In all other samples it was Pseudomonas_aeruginosa_soxR (gene family = ATP-binding cassette (ABC) antibiotic efflux pump;major facilitator superfamily (MFS) antibiotic efflux pump;resistance-nodulation-cell division (RND) antibiotic efflux pump). The estimates of UK dietary burden are 80% of diets containing Pseudomonas_aeruginosa_soxR and 41% of UK diets containing rpoB2.

Table 15. Foods and numbers of samples for which one or more ARGs, classed as variant or mutant, were measured

Food	Positive samples	Total samples measured	ARGs recorded
Apples	8	15	rpoB2 (1), Pseudomonas_aeruginosa_soxR (7)
Tomatoes	1	8	Pseudomonas_aeruginosa_soxR
Skimmed milk	1	7	Pseudomonas_aeruginosa_soxR
Pears	1	5	Pseudomonas_aeruginosa_soxR
Cucumbers	1	3	Pseudomonas_aeruginosa_soxR
Apple juice pasteurised	1	2	Pseudomonas_aeruginosa_soxR
White grapes	1	2	Pseudomonas_aeruginosa_soxR
Vanilla ice cream	1	2	Pseudomonas_aeruginosa_soxR
White onions	1	3	Pseudomonas_aeruginosa_soxR
Iceberg lettuce	1	2	Pseudomonas_aeruginosa_soxR
Olives in brine	1	2	Pseudomonas_aeruginosa_soxR

3.6.8 Carbapenem Resistance ARGs

Of the 52 RTE food types, 37 were found to contain one or more ARGs classified as carbapenem resistant, including all 15 of the apple samples (Appendix 10, Appendix 12) The total number of these ARGs found is often higher in the foods where fewer samples were taken, for example onions, cherry tomatoes. One of the most important dairy

consumption items (cheddar cheese) does not appear in this list, as there were no ARGs found in this category from the 4 samples analysed, and fewer ARGs were typically found in dairy products compared with produce samples. However, because the relative quantity of bacterial DNA extracted from samples compared with sample product DNA is much lower for dairy products than for produce products, numbers of ARGS found in dairy and produce samples are not comparable. The estimated proportions of individual diets containing ARGs and ARG families classed as carbapenem resistant are shown in Appendix 12.

3.6.9 Vancomycin resistant ARGs

Samples from 26 of the 52 RTE food types were found to contain one or more ARGs classified as vancomycin resistant (Figure 11 and Appendix 10). vanRO is the most frequently observed of these overall, and was found in many of the RTE produce samples. vanSA occurs in a high proportion of cheese, butter, yogurt but less frequently in milk samples. The distribution of van genes across food types is shown in Appendix 12. The relative frequencies of these and other ARGs, combined with the overall consumptions of all RTE foods, is reflected in the estimated dietary burden (proportion of individual diets) shown in Appendix 12.

3.6.10 Potential ESBL Activity

Of the 52 RTE food types 35 were found to contain one or more ARGs classified as having potential ESBL activity, including all 15 of the apple samples and 11 of the 16 banana samples (Appendix 10, Appendix 12). The total number of these ARGs found is often higher in the foods where fewer samples were taken, for example onions, cherry tomatoes. One of the most important dairy consumption items (cheddar cheese) does not appear in this list, as there were no ARGs found in this category from the 4 samples analysed. However, it is important to remember that ARG observations in dairy and produce are not directly comparable (see 3.6.7). The estimated proportions of individual diets containing potential ESBL ARGs or ARG families are also shown in Appendix 12.

3.6.11 Fluoroquinolone resistant ARGs

Of the 52 RTE food types 46 were found to contain one or more ARGs classified as fluoroquinolone resistant, including a high proportion (or all) of the measured samples for most food types (Appendix 10). The total number of these ARGs found is often higher in the foods where fewer samples were taken, for example onions, cherry tomatoes. Results are displayed in Appendix 12. The food types with largest variety of fluoroquinolone resistant ARGs, relative to the number of samples measured, are apples (55) tomatoes (62), white onions (58), cucumbers (52), cherry tomatoes (47), pears (44), white grapes (36), grapefruit (44), and apple juice pasteurised (43).

Overall the numbers of ARGs of this type are greater than for the other classes. The overall pattern of ARGs per food type are very similar to that seen for the overall ARG list (for example Figure 15 and Figure 16) although with fewer ARGs overall. The estimated proportions of individual diets containing fluoroquinolone resistance ARGs or ARG families are also shown in Appendix 12.

4. Discussion

This project was commissioned and designed to estimate the diversity of Antimicrobial Resistance Genes in selected ready-to-eat foods. What this project is not able to do is provide estimates about the risks to consumers of Antimicrobial Resistance itself in these foods. This may appear counterintuitive, but there are many reasons why the presence of ARGs does not mean that there are AMR bacteria present. For example;

- An ARG might encode a gene product which is one component of larger molecular machinery which is essential for the AMR phenotype. If other components are absent, there will be no AMR.
- Even with complete molecular machinery, an AMR phenotype may depend on other factors, such as particular metabolic conditions. That is, for a variety of reasons, the

ARGs may never be expressed; or under most conditions not be expressed to sufficient levels to cause an AMR phenotype.

- Some ARGs may encode a gene product which disables a particular antimicrobial molecule which does not have (or has ever had) any clinical importance, even if it is quite similar to others which do.
- Some ARGs may encode a gene product which breaks down a particular molecule which is not lethal to the bacterium at "natural" environmental levels, but may not be able to cope well with clinical concentrations of the same molecule, which may be lethal.
- The detected ARGs may be present in dead bacteria, or on free DNA, and this is not distinguishable by a metagenomic approach. While this would mean that they weren't generating a resistant phenotype in living bacteria when ingested they are still of interest, as free DNA can be taken up by living bacteria as a form of Horizontal Gene Transfer.

Furthermore, any ARGs that are identified are not necessarily attributable to farming or manufacturing practises increasing the risks of AMR evolution. Many ARG types, including β-lactamases, evolved for reasons entirely unrelated to clinical or veterinary antibiotic use, and have been evolving with their hosts for millions of years (Aminov, 2009). ARGs have consequently been found in pristine habitats, including ancient permafrost (Kashuba et al., 2017). That being said, it is well documented that anthropogenic factors can and do increase the prevalence of ARGs in particular environments (Aminov, 2009). What this project can therefore do is highlight potential areas for future work (see section 6) to better understand selective pressures driving ARG prevalence, to determine the extent to which the identified ARGs relate to phenotypic AMR, and what this might mean for consumers.

4.1 Risk and Uncertainty Estimation

4.1.1 Population Level Consumption

Based on the study design, which was agreed by the FSA in order to obtain an average picture of ARG consumption in the UK, we have insufficient sample numbers to allow comparison of exposure risk between foodstuffs (see section 4.1.3). As a consequence, despite some food groups appearing to contain more ARGs than others (for example, tomatoes appear to contain more ARGs than semi-skimmed milk in Figure 12), the sample numbers are relatively small, and the difference in ability to detect ARGs on these sample types may be very large. So, we deliberately and of necessity make no comment in this discussion on the different ARG levels present on different food products. However, considering the total sample set as a whole, the estimated proportion of individual diets containing the most frequently identified ARGs is extremely high. For example, *adeF*, a gene encoding the membrane fusion protein of the multidrug efflux complex AdeFGH (Coyne et al., 2010), is found in approximately 97% of individual diets in the UK. Indeed, the three ARG families found in the highest proportion of diets are all efflux pump families (RND, MFS and ABC pumps).

Such high apparent consumption may be driven by at least two different factors. The first is the fact that this metagenomic approach has the potential to detect ARGs in any bacterium on the food sample, and indeed to detect ARGs present on free DNA. Furthermore, DNA sequences generated in this project can be compared against databases containing many hundreds or thousands of different ARG sequences. Very few metagenomic studies of RTE foods have been undertaken to date (Li et al., 2020), with the project presented here being by far the largest. This approach differs markedly from the more traditional, phenotypic or PCR-based techniques (as seen in the papers reviewed for this project, section 2.1.1). In these approaches, a bacterium or range of bacteria of interest are usually isolated from the foodstuffs in question, and assayed phenotypically or by PCR for the presence of antimicrobial resistance or ARGs to a specified, by necessity restricted, range of antibiotics. It is therefore not surprising that a metagenomic approach should yield apparently high AMR burden estimates.

The second is that the burden estimates employed in this study use incidence of ARGs as their measures. That is to say, once an ARG is detected on a foodstuff it is considered

incident on that foodstuff and assumed that it will at some point be consumed by any consumer who eats that food type. This is because we did not assume any consumer brand loyalty to a particular sub-type or manufacturer brand, nor did we have sufficient data to capture brand-level variations to link to the NDNS consumption diaries. The result was conservative in the sense that any ARG detected in our samples were assumed to be in the diet of all consumers of a given food type.

At the other end of the scale, many of the ARG families which are consumed in the lower proportion of UK diets are the more unusual beta-lactamase families such as OKP, PER, AER and CAU beta-lactamases. It would be interesting to investigate further which food items these were linked to, and whether particular dietary consumption patterns (for example vegetarians) would be consuming different classes of ARGs. Furthermore, if there were scientific reasons to believe that the number of eating events associated with a particular ARG would lead to greater AMR burden overall, then the model could be extended to estimate the number of individual eating events rather than just individuals. Both these points would likely require a modified sampling regime (see section 5).

4.1.2 Antibiotics of Particular Concern

Resistance to a number of antibiotics, or groups of antibiotics, were flagged by FSA as being of particular concern. These were colistin resistance, methicillin resistance, carbapenem resistance, vancomycin resistance, fluoroquinolone resistance and ESBL activity. The numbers of ARGs within each group are shown in Figure 11 and Table 12. Due to the complexities around assigning ESBL status to particular beta-lactamase alleles, any identification of beta-lactamase genes with known ESBL alleles (for example SHV, TEM) were flagged as potential ESBL activity. Of these six types of AMR activity, two were very infrequent. Only two colistin resistance ARGs were identified among all samples. ICR-Mo, was found in three food types (section 3.6.5). ICR-Mo is chromosomally encoded (Wei et al., 2018) and as such is likely of less concern than a mobile colistin resistance gene, as it cannot be easily transmitted to other, more virulent bacteria. Another colistin resistance ARG, MCR-5.2 was identified in one cherry tomato sample. MCR-5.2 is likely of more

importance and interest, as it is a mobile (plasmid-borne) colistin resistance gene. MCR-5.2 was first identified in 2011 in the intestinal contents of a pig, in Germany (Hammerl et al., 2018), however the mcr-5-carrying plasmids identified in by Hammerl et al (2018) did not themselves carry transfer genes involved in plasmid conjugation, meaning other conjugative elements must be present for plasmid transfer. As the current study identified ARGs from short reads, the genomic context of the MCR-5.2 detected here is not known. The MCR-5.2 ARG in this study was found in a sample of cherry tomatoes from Morocco, and mcr genes have been previously identified in Morocco (and many other countries) (Nang et al., 2019), but without detailed information about the production processes involved from field to retail sale it is unknown at what point in production this ARG was introduced.

Methicillin resistance ARGs were also very infrequently found with only two different ARGs found in three food groups: *abcA* in pears and raisins, and *mecA* in cucumber (section 3.6.6). Both *abcA* and *mecA* are found in *S. aureus*. *abcA* is a multidrug efflux pump that confers resistance to a range of beta-lactam antibiotics (Villet et al., 2014). *mecA* encodes a penicillin-binding protein, PBP2', which is resistant to methicillin (Ubukata et al., 1989). *mecA* is part of the mobile SCC*mec* cassette (Deurenberg and Stobberingh, 2008), and therefore has the potential to be transferred to methicillin-sensitive *S. aureus*. However, its detection is perhaps not surprising, as since its discovery in the 1960s it has gone on to become globally distributed (Deurenberg and Stobberingh, 2008).

The other four antibiotic resistance types of concern (vancomycin, fluoroquinolone and carbapenem resistance and potential ESBL activity) were much more frequently detected. Fluoroquinolone resistance ARGs were found in a wide range of foods, particularly fresh produce, including in all samples tested for 37 different food types. More than 50 different fluoroquinolone resistance ARGs were found in apples, cucumbers and white onions, and more than 60 in tomatoes. The proportion of diets containing fluoroquinolone resistance genes was also very high – 99%. This is almost entirely due to various kinds of efflux pumps and transporter (RND, MFS, MATE etc.). In fact, the only widely consumed fluoroquinolone resistance ARG family that isn't an efflux pump is the quinolone resistance

protein (qnr). qnr genes were first identified in 1998 and have become increasingly widespread in a range of gram-negative bacteria. They are plasmid-borne, and encode pentapeptide repeat proteins that protect the DNA/DNA gyrase or DNA/topoisomerase IV complexes from the action of quinolones (Strahilevitz et al., 2009).

Carbapenem resistance and potential ESBL beta-lactamases are also found in a high proportion of individual diets. The most common carbapenem resistance gene families, as with fluoroquinolone resistance, are efflux pumps and porins. Although efflux pumps can be plasmid-borne, for example (Lv et al., 2020), chromosomally encoded efflux pumps are common in gram-negative bacteria (Auda et al., 2020). Indeed, the high frequency with which efflux pumps were identified highlights another limitation around the techniques deployed here, and a reason why these measures are likely overestimates of resistance. Many of the efflux pumps identified are present in all members of a bacterial species and do not of themselves indicate resistance.

A number of different *van* genes were identified in the samples analysed. These are found frequently, with all diets from the UK general population containing vancomycin resistance genes, mainly driven by *vanSA* which was found on products across dairy, produce and cooked meats. Vancomycin-resistant enterococci are increasingly isolated from clinical samples in Europe and around the world (Ahmed and Baptiste, 2018), and other organisms such as *S. aureus* demonstrate vancomycin-resistance which may be due to the presence of *van* genes (Shariati et al., 2020). However, it must be noted that, like many of the genes identified here for numerous antibiotics, *van* genes work as part of operons (Stogios and Savchenko, 2020, Ahmed and Baptiste, 2018), and the presence of one gene in the operon or gene cluster does not guarantee that the other genes are present, or that a vancomycin-resistant phenotype would be observed. This is a general consideration when working with sequence data rather than phenotypic screening.

Also present in around 70% of diets are carbapenem-active beta-lactamases (SHV and ACT), with SHV genes also potentially conferring ESBL activity. Other beta-lactamase families present in >60% of individual diets which could confer ESBL activity are CTX-M,

OXA and TEM beta-lactamases. On the one hand it is perhaps not surprising that beta-lactamase genes should be responsible for both carbapenem-resistance and ESBL activity, as carbapenems are themselves a group of beta-lactam antibiotics. However, there is no single definition of ESBL, and one published, commonly used definition specifically excludes carbapenem-resistance due to the different molecular structure of carbapenem and ESBL antibiotics: “A commonly used working definition is that the ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid,” (Paterson and Bonomo, 2005). This perhaps explains the fact that, despite the prevalence of beta-lactamase families, there is relatively little overlap in those that contribute to carbapenem resistance and potential ESBL activity (only SHV in our list). The lack of a consensus definition for ESBL activity, as well as the fact that ESBL and non-ES beta-lactamases are present in the same gene family, make it difficult to confidently assign ESBL activity to metagenomically identified sequences. Furthermore, many alleles of these genes may have been first identified based on their sequence similarity to known beta-lactamase genes, without experimental confirmation of their ability to cause an ESBL phenotype. The absence of robust and clearly accessible ESBL phenotype information in resistance gene ontologies is a limitation of our ability to predict the presence of ESBL genes from sequence data.

4.1.3 Background and limitations

The aim of the study was to estimate the burden of AMR genes in UK diets at the time of sampling i.e. across the population at a single instant in time. The information about diets used to make this estimate were collected in nine 4-day surveys over nine years. Burden was defined as the incidence of AMR genes in in these diets. In order to achieve an unbiased estimate of incidence we attempted to take and test a body of samples such that the test results would provide a *representative* sample of AMR genes in UK diets. *Representative* does not mean that all AMR genes present in UK diets appear in the body of samples, and test results, but it does mean that the probability of an AMR gene

appearing in the body of samples should be determined *only* by the true proportion of UK diets in which it appears (International Seed Testing Association, 2013): two genes present in the same proportion of UK diets should have the same probability of appearing in the body of samples irrespective of where in the UK and in what ready-to-eat food they occur. And that AMR genes which are present in a larger proportion of UK diets should have a higher probability of appearing in the body of samples, and in the results reported here, than AMR genes which only appear in a lower proportion of UK diets. Or to put it another way: those AMR genes which are reported in the study should, *on average*, make a larger contribution to the burden of exposure in UK diets than AMR genes that do not appear in the results. By focusing on the overall population burden, our results do not necessarily capture information about ready-to-eat items consumed by relatively few individuals (for example ready-to-eat chicken) even if those items contained many AMR genes and some individuals were consuming large quantities. Similar, future studies could be conducted to assess particular food types of concern.

In order to achieve a representative body of samples, the number of samples of each food group was in proportion to their consumption in UK diets. In addition, the number of samples of each food group from each UK region was approximately in proportion to the population in the region. Hence, if both the distribution of AMR genes across portions of food, and the ease of extraction and detection of AMR genes is the same across food types then the AMR genes found in these samples should be a representative sample of AMR genes in the diet at the time of sampling. However differences in the factors that affect the probability of inclusion between AMR genes in different food groups: for example the extent of clustering of prevalence of AMR genes in food items (for example between locations and over time), and differences between dietary portion size and analytical sample size may lead to differences in the probability of detecting different AMR genes which are associated with different foods.

Ideally, all of the AMR genes in a sample should be detected and identified when each sample is tested, however, the probability of detection may vary between different AMR genes due to a number of factors such as whether AMR genes are present in high or low

quantities of DNA, the presence or absence of substances that inhibit amplification of DNA, or the presence of different quantities of DNA from other sources such as the food matrix. Finally, the identification of an AMR gene rests on the presence of the appropriate sequence in a database.

Given that we can expect that many of the factors that affect whether an AMR gene which is incident in UK diets is both present in the body of samples and detected in those samples in which it is present to be strongly correlated with food type, it is likely that the results may provide

1) a representative sample of the AMR burden in each food type;
2) a somewhat representative sample across groups of similar food types;
3) but one that is biased towards AMR genes in those food types in which it is easier to extract and amplify bacterial DNA (for example produce such as apples) and away from those food types in which it is more difficult (notably milk).

So, while we do find a higher diversity of ARGs in produce types than in milk, this may be an artefact of the fact that it is easier to detect ARGs metagenomically in produce rather than milk. Given the non-quantitative nature of ARG detection, we also can't say anything about the number of ARG copies per mass of fruit vs milk.

The design of the sampling strategy deliberately focused on those food types which are highly consumed (for example semi-skimmed milk, bananas), and as a consequence few, sometimes only single samples were analysed metagenomically for low-consumed product types. This approach allowed a more confident estimation of burden based on a limited set of food samples. However, combined with the observations above, it means that it is difficult to draw conclusions about the relative contribution of different food items to this burden. The selected samples are heavily skewed towards a few food types based on the overall UK consumptions. These food types are therefore driving the overall burden calculation. The variations/uncertainties associated with the ARGs detected per food type are large, so the overall uncertainties are also large. To produce estimates of AMR presence in different individual food types we would need a sampling scheme designed for

that purpose. For comparing AMR presence between food types, better quantification of the detection uncertainty is required. Where observations have been made about the underlying sources of exposure to particular ARGs, for example colistin resistance genes in apples, this is based on our ability to measure ARGs, taking into account everything discussed above, and we cannot definitively say that apples contain more colistin resistance genes than other food types. This project raises the possibility of higher presence of colistin resistance genes in apples compared with other products as a hypothesis that can be tested in a study designed for that purpose.

Potential sources of unquantified uncertainties are present throughout all stages of the analysis. A number of potential sources of uncertainty are highlighted in Table 16.

Table 16. Potential sources of uncertainty in ARG detection.

Step	Potential sources of uncertainty
Sample selection in supermarket	AMR genes may be present or absent in individual items of nominally the same product. Hence they may be present or absent on randomly selected samples analysed in this project. This is a common feature of representative sampling. If there are temporal variations (within year) these may be missing from the data, because dairy samples were collected in the first half of year and produce in the second half.
Couriering sample to Fera	The microbiome may change over time leading to different prevalences of AMR and non-AMR genes. If storage time and conditions for samples taken in this

Step	Potential sources of uncertainty
	project is representative of storage between purchase and consumption of RTE foods in the UK then these changes should be representative of changes in the diet.
Receipt at Fera	Some samples were rejected, due to incorrect or damaged/out-of-date products. These were resampled.
Subsampling – either rinsing exterior, peeling and rinsing, dissecting and rinsing, centrifuging (juice), total subsample for extraction (milk and cheese)	Different subsampling methods may cause different ratios of bacteria:host cells for different sample types, for example we would expect more bacteria for externally rinsed types, such as apples, than for total DNA extraction samples, such as milk. This means that AMR genes present at the same level in milk and produce (at the same quantity per mass, in the same proportion of samples) may be less likely to be detected in the milk samples. Hence, while the AMRs found in produce and dairy may each be representative of AMRs in each of those product types, we do not expect the between-product relationship in AMRs that we detect to be representative of the between-product relationship in AMR presence.

Step	Potential sources of uncertainty
	Different subsample types may not reflect as accurately the actual risk of consumption, for example some bacteria may have been internalised in exterior-rinsed fruit, some external bacteria may be ingested by the consumer while eating peeled fruit, or some bacteria may be washed away by consumers prior to consumption.
DNA extraction – either pellet extraction, cheese extraction or milk extraction	Different DNA extraction methods might be more or less effective at extracting DNA from bacterial cells than from host cells. DNA-extraction is not completely without taxonomic bias: some bacterial species' DNA is harder to extract than others. However, the methods employed are regarded as relatively unbiased.
Sample selection (n=256) for metagenomic sequencing, based on the results of the 16S metagenomic sequencing for bacterial communities/organelles (n=1000)	These samples were selected on the basis of that would provide a relatively large bacterial signal compared with samples that were not selected. Given that our endpoint is an estimate of AMR incidence rather than prevalence this non-random sample selection is unlikely to affect representativeness, unless there are particular AMR genes that are

Step	Potential sources of uncertainty
	associated with lower bacterial loads which are not found in higher bacterial loads
Nextera XT library prep (all 256 samples prepared as a single, multiplexed library, which was then run across two NovaSeq flowcells)	Possibly extracts from different sample types may have different inhibitors, which may affect DNA library preparation
Sequencing on NovaSeq	Different flowcells may stochastically produce more or fewer DNA sequences (which was mitigated by sequencing all samples on both flowcells).
Comparison of reads sequence data with reference ARG sequences database	Although the reference sequence database we used is a principal standard in the field, it (like all alternatives) is unlikely to be completely comprehensive or error-free. However, we have confidence that the most clinically important ARG classes, as well as many other classes, will be very well represented and curated.
Precision of matches with ARG sequences	Some types of ARG encode molecular machinery with resistance functions and whose gene/protein sequences are relatively straightforward to identify.

Step	Potential sources of uncertainty
	More problematic are genes whose general forms are not ARGs, but only particular sequence variants confer ARG status. For these types, we have taken care to identify exact matches, to avoid false positives - whose presence would have greatly increased the risk of our ARG table not reflecting actual prevalence. The downside is that some particular sequence variants which are ARGs, but not yet present in the database, will be missed.
Bioinformatics detection of ARGs: general	Our methods involved comparison of short-read data with the reference sequences (due to some of the samples having unsatisfactory sequence assemblies). All sequence-matching to identify sequence entities have some level of false positives and false negatives, but these are unlikely to have affected particular ARGs systematically, and so high-prevalence ARGs will not have had their presence in our table compromised for this reason. Our expectation is that this table is overall an accurate reflection of the presence of

Step	Potential sources of uncertainty
	ARGs known in the reference database, but is unlikely to be a perfect reflection.
Length of surveys and linking consumption data to ARG presence data	The dietary consumption survey is an estimate of the UK diet based on the diet of each surveyed individual over a 4-day period. This may affect estimates of AMR incidence. For example, the proportion of UK individuals consuming apples during the 3-day survey was 41%, leading to an estimate of 41% of diets containing rpoB2. However, it is likely a large proportion of other individuals will include apples in their diet at some time. This issue will apply to all food types, to varying degrees, therefore the impact on the relative ranking of the burden of different ARGS should be limited. The selected foods only cover 90% of total consumption of ready to eat foods. For some foods, with relatively low consumption, only 1 or 2 samples were analysed. Absence of AMR genes reported for these foods is less reliable than in other foods.

4.2 Metagenomic Analysis of short-read data

4.2.1 A priori considerations of the food microbiomes

The microbial communities (microbiomes) of each sample are likely to differ greatly both between and within food categories, in terms of not only types but also relative abundances of the microbes. The collective genomes of these communities (metagenomes) can accordingly be expected to have very different characteristics.

Hypothetically, if a metagenome were sequenced to a sufficient depth and accuracy then the complete genomes of all organisms present could be assembled. However, this required depth (usually infeasible) will be very variable depending on the number of different organisms present and their relative abundance. In the simplest case, equivalent to a single isolate, if only one species is present (assume one strain) and has a small genome (such as a bacterium) then the required depth will be relatively low. However, even with two species/strains present, the ideal depth depends hugely on the relative abundance. If one organism is present in vastly greater abundance than the other, then a vastly greater sequencing depth would be required to sample all of the minority genome sequence.

In reality, almost all metagenomes are much more complex than this, consisting of many species and with very different relative abundance distributions from one metagenome to the next. These characteristics are the subject of a branch of microbial ecology, and in brief, the distributions can be considered as "even" (flat) or very uneven (different organisms have huge differences in relative abundance), or something between. The more that a metagenome is dominated by a small number of organisms (and thus, few genomes and therefore sequence fragments thereof), the nearer to complete it should be when assembled, and the more long assembled (”contig”) sequences should result. In extreme cases, contigs representing an entire bacterial chromosome may result, and / or there may be a small number of very long, sub-chromosomal sequences.

However, even those metagenomes can differ greatly in their contig-length distribution, because some may have a long "tail" of many very low abundance species leading to very

many, diverse short contigs; while others may have a very short tail, with few other taxa besides the dominant one(s).

A further consideration is that many bacterial genomes consist of one or more plasmid(s) as well as a chromosome. The number of plasmids varies a great deal between species and can also be variable within one species but is essentially invariant for some. There is also a very broad range of plasmid sizes, typically spanning from 1% to 10% of the chromosome (Clark et al., 2019); but at extremes, plasmids may be very small encoding no genes, or can be larger than many chromosomes (Smillie et al., 2010). Some bacterial genomes always consist of what are in effect two chromosomes, with one having a plasmid origin (Clark et al., 2019). Therefore, even in a metagenome dominated by a very few species, hypothetically-complete sequencing may result in multiple contigs of a range of sizes.

We briefly address plasmid-located ARGs here, since this does have a particular significance in terms of some forms of AMR proliferation. Some ARGs are by nature plasmid-associated, and this is reflected in the formal annotation of the gene in the AMR databases; in some cases, "plasmid" is indeed part of the name itself (for example "plasmid-encoded cat (pp-cat)"). It is also possible that some plasmids would be completely sequenced and recognised as such, forming circular assemblies (depending on the assembly software used). More generally, the plasmid origin versus chromosome origin of particular contigs could in many cases be resolved by standard sequence similarity searches of appropriate databases.

4.2.2 Metagenome assemblies obtained

Despite the overall success of the NovaSeq sequencing, a significant number of samples generated assembled metagenomes that were deemed unsuitable for ARG analysis. In some cases, these comprised relatively few assembled sequences (contigs) of non-trivial length, either as a result of the low number of short reads remaining after host DNA-removal, or otherwise failure to incorporate many of the reads into the assembly. In other

cases, more impressive assemblies were created in terms of longer contigs, but some of these appeared to also have problems with quality, as assessed by the number of reads from each sample that mapped back to the contigs assembled from those reads. For example, the presence of distinct but very similar sequences in the genomic DNA can lead to chimeric contigs if the sequencing depth is insufficient to resolve them. These various considerations suggest that in essence we were not generating enough reads for those samples to effectively sample the DNA sequence that was present.

4.2.3 Implications of the metagenome assembly results

Crucially, the above assessment necessitated a move from an assembly-based approach to a read-by-read ARG detection approach. This preserves the sequence information in the short reads which would be lost in a poor assembly, and means that we can have a more uniform high confidence in our ARG predictions for all samples. However, it does mean that we might lose some information about which specific ARG allele of a particular gene is present. It also means that all genomic context information is lost, as the analysis of individual reads prevents the identification of the genetic element on which the ARG sits, or the identification of flanking genes.

The fact that suitable assemblies for analysis were not obtained from all samples also has implications for the depth of sequencing required. In this project we aimed for an average depth of 25 million reads per sample, and in the end an average depth of 33 million reads per sample was achieved. For many samples, this was sufficient to provide a good assembly, but for others it did not. By the back-mapping assessment at least 100 of the 256 samples can be viewed as having an unsuitable assembly. This was usually, but notably not always, caused by a low number of input reads for the assembly process.

4.2.4 Metagenome assemblies in relation to types of food-organism

Interestingly, and predictably, this varied to some extent with food type. Produce samples generally showed a higher number of reads remaining after host-derived reads were removed, which meant that the samples that had the highest number of reads going into assembly were also produce samples. There are a number of potential reasons for this.

On the one hand, the large number of reads left over after removal of host-derived reads could be an artefact of the host read removal process. To remove host reads, all reads from a sample are mapped against a host reference genome, and those that do map are removed. Produce sample types represent a greater variety of host organisms than meat or dairy types, which in this project are only cow (dairy foods, and corned beef) and pig (ham). Some of these produce organisms did not have a reference genome available, so a close relative was used, for example nectarine sequences were mapped against peach, small citrus sequences were mapped against orange. Further, for some organisms where a genome sequence was available, it may have been in an early state of release or were incomplete. As a consequence of having a lower quality or less closely related reference genome, the host sequence removal step could have been less effective.

An alternative reason for higher read counts after host-DNA removal in produce samples could be due to the sample treatment themselves. The majority of produce samples underwent rinsing of the surface, to sample bacteria but avoid sampling host DNA and cells. This rinsing was not possible for any dairy samples, and a total DNA extraction was necessary. The higher read counts for some produce samples after host sequence removal could therefore reflect the fact that there is genuinely a higher ratio of bacteria:host present in produce rinsate than in total dairy samples. The fact that produce samples can be seen to cluster into both high and low host contamination sets may be reflective of the fact that some produce types were surface rinsed (presume high bacteria), and some were peeled and the peeled surface rinsed (presume low bacteria), in an effort to sample the parts of the foodstuff that are actually consumed.

4.2.5 Implications of short reads for ARG-identification

For identifying genes of a particular function, in normal circumstances relatively low sequence identity thresholds are sufficient. At the protein sequence level, the same function would normally be inferred from around 40% sequence identity or greater, with functional classes inferable from somewhat lower identity levels (Wilson et al., 2000). Further, due to the redundancy of the genetic code, gene sequences are generally less

conserved (still lower sequence identity) than the proteins they encode. However, such thresholds apply to full-length sequences, and rather higher identity levels would be ideal for inferences made from short fragment matches, such as the metagenomics data.

We designed a post-processing screen to the ARG matches, which for the metagenomics reads in general did not involve an explicit percentage identity threshold but applied a number of rational filters. We did however also apply an identity-based additional screen on a particular category of ARGs as addressed below. As expected, the full procedure did result in a profile of generally high sequence identities in the screened data overall. 96% of all of the matches between the metagenomic reads and the reference ARG sequences had a sequence identity of 75% or higher. 31% of the matches had an identity of at least 97%, and 20% of all matches were exact. Therefore in general, we regard the matches of high confidence regarding the likely gene function. This also is in the light of the special treatment we applied to matches to ARGs of a particular character, as follows.

For the purpose of this discussion, ARGs can be considered broadly as those which are (relatively) "straightforward" to identify by sequence similarity, and those which require great care because their resistance phenotype is conferred by a very small number of mutations (usually point mutations). An advantageous feature of the CARD database and RGI software is that these "mutant variants" are curated and categorised as such. This meant that we were able to treat these differently, insisting on 100% sequence identity between the read segments and the references with which they matched. As a result, one kind of false positives will be avoided, involving a very high similarity match which nonetheless is non-identical and lacks the resistance mutations; but this will be recognisable only where the gene segment sequenced in the short-read pair encompasses at least one of the mutations. If the reads' sequences span only a region that is identical between the susceptible and resistant forms, then a false positive can still result. Conversely, discarding such matched regions would risk false negatives for these resistant variants. This is an inherent drawback of using short-read data.

However, we assessed the difference that was made to our results by this additional screen for "mutant/variant" type of ARGs, in terms of the numbers of positive samples. A key result was that in the screening without the additional special screen, there were only 6 (of almost 800 remaining at that stage of the analysis) named ARGs of this type, i.e. where the AMR function arises from the mutation(s) relative to a non-AMR counterpart gene. Between them, these 6 were positive in the majority of samples, however. This was unsurprising given that several of these are universal bacterial genes, such as an RNA polymerase subunit, and so would be expected to have a high detection rate. However, our additional screen eliminated 3 of these genes from all samples, while the other 3 remained in 1, 3 and 33 samples respectively (the final read-count stage of filtering reduced these further to 0, 1 and 17). Therefore in the worst-case scenario (assume all matches are false positives in these samples), a small percentage of all samples would be affected by having one or a very few number of spurious ARGs counted as positive.

From these results we conclude that this additional screening approach was worthwhile, as it made a difference to most samples. The counterpoint is that the difference made to these samples was very small in terms of the numbers of additional genes, and so changed the general ARG-incidence profile only a little.

Where curated resistance-conferring mutations are matched exactly, it is still possible for these to be false positives, as potentially these could arise by sequencing errors (which would not affect the identification of various other kinds of ARGs in the same way). In theory it is possible to assess the probabilities of such errors, by examining the quality score of the relevant base-call(s) as well as the context of other overlapping reads. However, given that we require 100% identity for these point mutation-dependent ARGs, it is very unlikely for any given read pair that the error(s) occur in - and only in - the relevant mutation site(s). In a data set as large as this, such situations might well occur, but with low frequency.

For reference, we note the error rates of the sequencing platform. This is not straightforward to evaluate, but for the Illumina platforms generally the rate of incorrect

basecalls is often stated as around 1 in 1,000, i.e. 0.1% per-base error rate (Shendure et al., 2017), with some variation between reports; for example 1 in 417 on average (0.24%) by a more recent study (Pfeiffer et al., 2018).Our quality-control removed all bases with a quality-score indicating a rate of 1% or worse probability of error.

In summary, the overall success rates of ARG-detection, in terms of avoiding false positives (and false negatives), cannot be known without the ground truth of which ARGs were actually present in each sample. Although some false positives will inevitable be present, the results of our analyses indicate no evidence of large-scale over-prediction of ARGs.

4.3 Metagenomic Analysis of long-read data

The primary factor affecting the effectiveness of the long-read sequencing was the volume of data output by the sequencing itself. The two PromethION flowcells used in this experiment produced 2.8Gb and 3.3Gb of sequence data, compared with 100-300Gb per-flowcell estimate from the PromethION specifications (Oxford Nanopore Technologies, 2021). This represents 1-3% of expected sequence output and is therefore an extreme limiting factor on the utility of this dataset.

Despite the low output of the Nanopore data, for 14 out of the 24 samples sufficient data was obtained to produce an assembly. The primary predicted benefit of using the long nanopore reads was the assumption that they would provide an improved ability to identify co-located ARGs. These ARGs would be present on the same genetic element (chromosome, plasmid, insertion sequence etc.), and would therefore be likely to be co-inherited, either vertically (through bacterial replication) or horizontally (through horizontal gene transfer). These might then represent a higher risk to the consumer, if bacteria that are resistant to multiple antibiotics are considered riskier. As this work was not used for burden analysis, we were free to use DeepARG (as opposed to CARD) to identify ARGs. This was both faster, and was an opportunity to utilise its more experimental, deep-learning nature. For the 14 samples with sufficient data, ARGs were detected by

DeepARG in both the Meta (short read) and Hybrid (long read) assemblies. Interestingly, very similar overall ARG numbers were seen between Hybrid and Meta assemblies, but more ARGs identified as co-occurring in the hybrid assembly, i.e. when the long reads are used. This suggests that the long reads are indeed useful for identifying co-located ARGs, a trait which would probably be more impressive with greater depth of sequencing. However, in the absence of a strict definition of co-located ARGs, ARGs are reported as co-located if they are present on the same contig. Given the low sequencing depth for Nanopore data in this study, combined with the higher error rates, the co-located ARGs should be inspected manually to avoid false positives.

The long-read data also enabled the assembly of a MAG ("metagenome-assembled genome") identified as *Acinetobacter* sp., in a single contig of 2.9Mb, which was not identifiable in the Illumina data for this sample. Indeed, the Illumina data resulted in only three contigs of 500 kb length of bacteria identifiable with the Progenome classifier (these were between 670 and 841kb). Generally however, there was a dearth of assemblies, both from the Nanopore and Illumina data, which contained contigs which were both of sufficient length and contained sufficient marker genes (universal genes present in all bacteria, whose sequence variation aids species-identification) to achieve such a result.

We note that the per-base error rate of raw Nanopore reads is estimated at 5%-25% (Wick et al., 2018), with rapid improvements seen in the last few years. A rate of 3% was claimed in 2020 (Oxford Nanopore Technologies, 2020).

4.4 Amplicon Sequencing

16S rRNA metabarcoding was performed for the dual purpose of identifying samples rich in bacterial DNA and also for screening for evidence of the presence of some foodborne pathogens; necessarily it can provide no direct information about ARGs.

4.4.1 Limitations of 16S V4 metabarcoding

Metabarcoding of microbiomes using 16S rRNA gene amplicons is in general of limited use for discriminating between bacterial species, but can have much more resolving power

in some branches of the bacterial taxonomy than others. The amplicons we have used (V4 region) are a standard approach for general bacterial 16S rRNA metabarcoding, but if such a technique were to be used for systematic detection of priority taxa of concern (foodborne pathogens in this case), then a more taxonomically directed approach using different primer pairs (perhaps several, directed at different taxa) would be necessary. The limitations of using the 16s rRNA gene - even when sequenced in its entirety - for resolving between some closely-related species, are well established (Fox et al., 1992, Stackebrandt and Goebel, 1994). When using the shorter V4 region, the situation is worse, with difficulties in distinguishing bacteria from closely related genera such as those in the Enterobacteriaceae, which have identical V4 sequences in some cases. Conversely, some particular V4 sequences can uniquely identify the species; indeed, this region can vary between different strains of the same species (including in some of those same Enterobacteriaceae).

There are further difficulties regarding the establishment of ground truth for reference sequences. One approach would be to use only the sequences of type strains, the strains which have been used to describe a bacterial species, but this means that the reference data could be relatively sparse, with many of the experimentally observed query sequences absent. The approach we have taken is to use a database where some of those gaps are filled, by the incorporation of non-type strains and also some environmentally-derived samples (for example sequence data from other metabarcoding experiments), which have been expertly curated, but taxonomy not usually assigned at species-level, for the above reasons. In those cases, judicious use of the source annotations at species level (if any) can identify reference sequences which are very likely to represent particular species. However, any resulting identifications of priority taxa by these methods must be examined carefully before a positive can be declared at the *in silico* level. Furthermore, the presence of a DNA sequence consistent with or even diagnostic of a bacterial species does not guarantee that the bacterium is alive in the sample, and results should be interpreted as with other molecular assays such as targeted PCR.

4.4.2 Metabarcoding to inform selection of samples for metagenomic sequencing

To maximise the usefulness of the metagenomic sequencing data generated for detecting ARGs, we wanted to focus sequencing effort on those samples with higher bacterial DNA levels, and lower amounts of host DNA. This was one of the reasons behind employing a 16S metabarcoding screen of all 1001 samples. This data was then used, in conjunction with consumption data, to select the subset of 256 samples for metagenomic sequencing The factors considered in the screen were i) whether the sample had sufficient bacterial DNA to successfully undergo 16S sequencing, and ii) the proportions of bacterial DNA (as opposed to organellar DNA) that were generated in 16S sequencing. To assess the relevance of point i) for deciding on samples for metagenomic sequencing, ten randomly selected failed metabarcoding samples were taken forward for metagenomic sequencing. When sequenced metagenomically, these ten samples produced read numbers distributed throughout the total range of read numbers, indicating that 16S failure is not a good indicator that a sample will fail metagenomic sequencing. This should perhaps not be surprising, as there could be a number of reasons for PCR failure that might not affect the metagenomic library preparation process (for example inhibition of 16S PCR reaction). Fortunately, in this project only 8% of samples failed 16S sequencing so this is not considered to be problematic. To address point ii) the food types classified as having low bacterial read numbers by 16S sequencing were examined after metagenomic sequencing, and the median percentage of bacterial reads was 5.6%, compared to 38.9% in non-low bacterial samples. The remaining reads were attributed to host (plant or mammalian) DNA or potentially from non-bacterial microbes, for example fungi. This indicates that the 16S metabarcoding may be a useful indicator of the proportion of bacterial reads a samples will produce in metagenomic sequencing. Given the importance of bacterial reads for assembly and ARG detection discussed above, this may be an important area of further study. Furthermore, 16S results could be used to look at the complexity of a microbiome for particular sample types. For example, cheese and probiotic yoghurt drinks were dominated by bacteria from the *Lactobacillus* genus, suggesting that microbiomes from these samples will be less diverse. With further work, the diversity of the

microbiome and the host to bacterial DNA ratio could be used to adjust the concentrations of input DNA in sequencing pools to ensure optimum metagenomic sequencing output across samples.

4.5 Detection and Identification of Antimicrobial Resistance Genes

Even if completely error-free DNA sequence data were available, there is no definitive route to identifying and correctly classifying the antimicrobial resistance genes (ARGs) sampled in that DNA. As in many fields of the biosciences, the complexity of the subject matter and the history of its development means that no single nomenclature, classification scheme, reference catalogue or data analysis methodology is universally utilised.

Crucial to identifying ARGs are the reference sequence databases, and just as with the computational approaches to comparing experimental data with them, there are many alternatives available (Hendriksen et al., 2019). This is partly due to the intrinsic nature of the data. New instances of known gene types are often discovered first at the DNA sequence level in the bacterial genomics field, and frequently will have been annotated automatically. Others are subject to expert curation but will often have not had the precise function confirmed at the experimental level. Sequence-based annotation of newly-discovered sequences can lead to ambiguities, since quite similar sequences can differ significantly in their clinically-relevant function (Antonopoulos et al., 2017). Therefore intensive curation by maintainers of the databases is ideal (McArthur and Wright, 2015), but the ARG databases vary considerably in this degree, with some being substantially reliant on automated computational compilation.

Even if all new sequences were classifiable automatically or by curators to a precise functional level, there are still obstacles regarding differences in nomenclature and classification of those functionally-defined genes. There is no overarching ARG-naming scheme, and nomenclature tends to be steered within the different ARG-classes by recognised groups of experts on those types. In biology generally, issues with gene synonyms and homonyms constitute a long-standing and ongoing problem and this does affect the AMR field, necessitating dedicated rationalisation efforts by expert researchers

(Faltyn et al., 2019 PREPRINT). Further, besides the names of the ARGs themselves, the classification into broad functional types is also far from definitive. For example, there is no consensus of the precise definition of extended-spectrum β-lactamases (ESBLs) (Paterson and Bonomo, 2005).

Consequently, it is unrealistic to expect that analyses of the same experimental data (even using the same methods) using different published ARG reference databases would not differ in some tangible way, both at the functional class level and especially on the level of particular genes. Nonetheless, unless databases or methodologies are particularly deficient or incorrect, broad consensus of incidence of ARGs and classes would be expected.

Indeed, anything approaching unification of the reference sources in the near-future is also an unrealistic expectation to demand of the experts in this field.

The reference databases of the two software systems involved in this study respectively include around 150,000 (CARD, including the sequence variants component of the smaller hand-curated section) and 12,000 (DeepARG) reference sequences. The CARD and DeepARG databases involve around 2,600 and 1,900 different ARG names. The CARD is long-established and widely used and in our view, is the single most suitable to use and also benefits greatly from the associated ARO ontology, with which the ARGs are systematically annotated. We note that due to the above standardisation aspects, it was not tractable to attempt a systematic comparison of the results of two independent analyses on an ARG-by-ARG, or class-by-class basis within the scope of this project.

Even with a single source of reference sequences, there of course exist many different computational approaches and software tools to compare experimental sequencing data with those references (Hendriksen et al., 2019). These differences can include aspects of how both short-read data and assembled sequences are analysed. Short reads are intrinsically limited in that they will almost always represent fragments of genes rather than the whole length. However, the paired-end DNA sequencing approaching effectively doubles the amount of each biological fragment that is sequenced, albeit with a gap in the

middle. A single pair might thus represent two ends of one gene, and should be analysed in that context, rather than treated as two independent fragments. The paired context is observed by the RGI/CARD system but not by DeepARG, although post-processing of the latter can add this context. Nonetheless, depending on the nature of the particular gene involved, it is possible in some cases for very short segments of DNA to uniquely identify an ARG. That is, the incompleteness will sometimes but not always prevent precise identification of a gene.

Related to this are particular problems which apply to only some types of ARG. In the general case, the sampled DNA fragments need not be identical to a reference sequence in order to be identifiable as an instance of that gene, and this applies to many ARG types, where what matters is the ability to recognise the correct functional type (such as an enzyme which breaks down an antibiotic, or an efflux pump). In contrast, some non-AMR genes (which code for molecules which are antibiotic targets, for example) may have near-identical counterpart genes which are ARGs. Crucially, these ARGs may differ in only one or a very few nucleotide positions, which ultimately compromises or prevents the antibiotic from binding to it. For these particular cases, which are recognisable in the RGI/CARD context by particular ARO ontology annotations, it is possible to insist (as we have done) on identical sequence matches with the reference ARG. Using short-read (thus incomplete) sequences only, it is however not possible to discriminate between such ARGs and the associated non-ARG variants if the resistance-conferring mutation(s) are not present in the sequenced fragment. An additional layer of analysis at the individual nucleotide-coordinate level would be possible to at least identify which fragments represent these ambiguous cases and which do not. That would still leave a problem, whichever view was taken of the ambiguities: to discard them all would result in some false negatives, while including them all would result in some false positives. We took the latter approach, which is in the context that these variant/mutant types are a small minority of the ARGs overall.

The availability of more complete DNA data for analysis would of course be ideal for identification of ARGs and any genes in general. As noted in the previous sections, short-

read sequencing at very great depth in order to facilitate very high-quality assemblies, or indeed assemblies of long-read data, would achieve this, but was beyond the scope of this project. We note that for a subset of our samples only, the metagenome assemblies we have obtained should indeed be suitable for more detailed analysis, in the sense that they will contain a reasonable sampling of the full-length or near-complete sequences of the ARGs present in the sample. More complete assemblies will be especially helpful for elucidating the presence of complex AMR operons. For example, *emrB* was found in whole and semi-skimmed milk and is counted as an AMR gene. However, it is only functional and able to confer resistance to carbonyl-cyanide *m*-chlorophenylhydrazone and nalidixic acid when combined with the other two elements of the EmrAB-TolC efflux complex (Yousefian et al., 2021). This highlights a difficulty in inferring phenotypes based on complex systems from short-read data.

4.6 Summary of AMR Gene Findings

In summary, we attempted to provide an estimate of the burden of AMR genes in RTE food in the UK diet that was an unbiased *sample* of the total burden. We defined burden as incidence in the diets of UK consumers. *In general* we expect that observations of presence AMR genes are reliable and that observations of absence may occur because either an AMR gene is not present in the diet or, if it is present that it is at a level (prevalence in samples, copies per mass of food in samples) which is below the limit of detection associated with the sampling plan and analytical methods. We identified a number of factors which may mean that AMR genes in different food types may be over or under-represented among those that we detected, and some features of the analytical method that may lead to false positive observations of AMR genes. Given the caveats identified previously around inferring the phenotype of the bacteria within which these genes are found, especially for individual components of complex operons, it may be more helpful to consider the genes identified as genes which *may* contribute to AMR.

We detected a total of 477 distinct AMR genes from 111 distinct AMR gene families in the samples of ready to eat food analysed in this project. We estimate that all UK consumers

in the general population as defined by the UK NDNS are exposed to AMR genes from ready to eat foods. Looking at the incidence of particular classes of AMR genes in UK diets, with all incidences referring to consumers in the general population as defined by the NDNS:

- Colistin Resistance ARGs: 2 distinct AMR gene detected; estimated incidence 46% of UK diets for ICR-Mo and 7% of UK diets for MCR-5.2.
- Methicillin Resistance ARGs: 2 distinct AMR genes detected; estimated incidence 37% of UK diets for mecA and 17% UK diets for abcA.
- Antibiotic Resistant Gene Variant or Mutant: 2 distinct AMR genes detected; estimated incidence 80% of diets containing Pseudomonas_aeruginosa_soxR and 41% of UK diets containing rpoB2.
- Carbapenem Resistance: 54 distinct AMR genes detected; estimated incidence of at least one Carbapenem ARG is 100% of UK diets. Incidence for individual ARGs was estimated to lie between approximately 100% (Klbsiella_Pneumoniae_KpnH) and less than 1% (mdsB)
- Vancomycin Resistance: 15 distinct AMR genes detected; estimated incidence of at least one Vancomycin ARG is 100% of UK diets. Incidence for individual ARGs was estimated to lie between approximately 94% (vanSO) and less than 3% (vanRC)
- Potential ESBL Activity: 54 distinct AMR genes detected; estimated incidence of at least one Potential ESBL Activity ARG is 100% of UK diets. Incidence for individual ARGs was estimated to lie between approximately 100% (CTX-M-50) and less than 1% (OXA-114a)
- Fluoroquinolone Resistance: 90 distinct AMR genes detected; estimated incidence of at least one Fluoroquinolone resistance ARG is 100% of UK diets. Incidence for individual ARGs was estimated to lie between approximately 100% (adeF) and less than 1% (evgA)

Estimates of the incidence of the individual AMR genes from RTE foods in the UK diet are provided in the report.

We also gained a large amount of information about the numbers of AMR genes in food types and in individual samples. For example, Table 17 shows consumption, the mean number of distinct AMR genes found in each sample, and the number of samples in which each at least one distinct AMR gene was detected. Figure 22 shows the average consumption against the average number of ARGs for each food type. There is considerable between-food variation in both consumption of food, and the number of AMR genes observed in samples. However, because for many foods the number of samples tested was small, and there may be between-food bias in the detection of AMR genes (notably between dairy and fruit and vegetable products), apparent differences between AMR gene presence in different food types found in this study serve more as hypotheses that may be tested in studies designed for examining between-food variation in AMR presence rather than as reliable estimates of difference.

Table 17. For each food type tested, the mean consumption, number of samples tested, samples positive for one or more ARGs, and the mean number of ARGs per sample.

Food	Mean Consumption (g / kg bodyweight)	Samples	Samples with at least 1 ARG	Mean ARGs per sample
Semi skimmed milk	76386	69	30	2.9
Whole milk	45612	42	22	3.3
Bananas	21746	16	15	13.5
Apples	17725	15	15	78.1
Orange juice pasteurised	12156	7	7	31.3
Tomatoes	9431	8	8	63

Food	Mean Consumption (g / kg bodyweight)	Samples	Samples with at least 1 ARG	Mean ARGs per sample
Skimmed milk	8332	7	4	7.6
Cheddar cheese	7080	4	4	5.8
Pears	5998	5	5	82.4
Small citrus	4800	4	4	36.8
Cucumbers	4715	3	3	102.7
Oranges	4838	5	5	27.2
Strawberries	4369	1	1	29
Apple juice pasteurised	4165	2	2	61.5
Ham not smoked	4252	4	1	6
White grapes	3049	2	2	80.5
Melon	2048	1	0	0
Salted butter	1916	2	2	10.5
Soya milk sweetened	1981	2	2	4.5
Carrots	1919	1	1	22
Lettuce	1911	3	3	57.7
Unsweetened yogurt	1684	2	2	6
Vanilla ice cream	1851	2	2	74
Black grapes	1584	2	2	47
Reduced fat spread	1633	2	0	0
Probiotic yogurt drink	1345	1	1	1

Food	Mean Consumption (g / kg bodyweight)	Samples	Samples with at least 1 ARG	Mean ARGs per sample
One percent milk	1325	1	1	1
Low fat unsweetened yogurt	1291	1	1	5
Pineapple	1288	1	1	6
Plums	1170	2	2	68
White onions	1117	3	3	124.7
Orange juice freshly squeezed	1009	1	1	2
Nectarines	1031	1	1	72
Mangoes	819	3	2	2.3
Kiwi fruit	938	4	1	0.8
Cherry tomatoes	762	2	2	131.5
Fat spread	932	1	0	0
Avocado	791	3	1	10.3
Peaches	804	1	1	74
Raspberries	789	1	1	49
Blueberries	605	2	2	41
Red peppers	697	1	1	71
Corned beef	729	2	2	29
Spreadable butter	654	2	2	10.5
Soya milk unsweetened	641	1	1	9
Raisins	645	1	1	23
Iceberg lettuce	616	2	2	32

Food	Mean Consumption (g / kg bodyweight)	Samples	Samples with at least 1 ARG	Mean ARGs per sample
Lactose free semi skimmed milk	563	2	2	23
Watermelon	507	1	0	0
Double cream	539	2	2	1
Grapefruit	458	1	1	119
Olives in brine	445	2	2	58.5

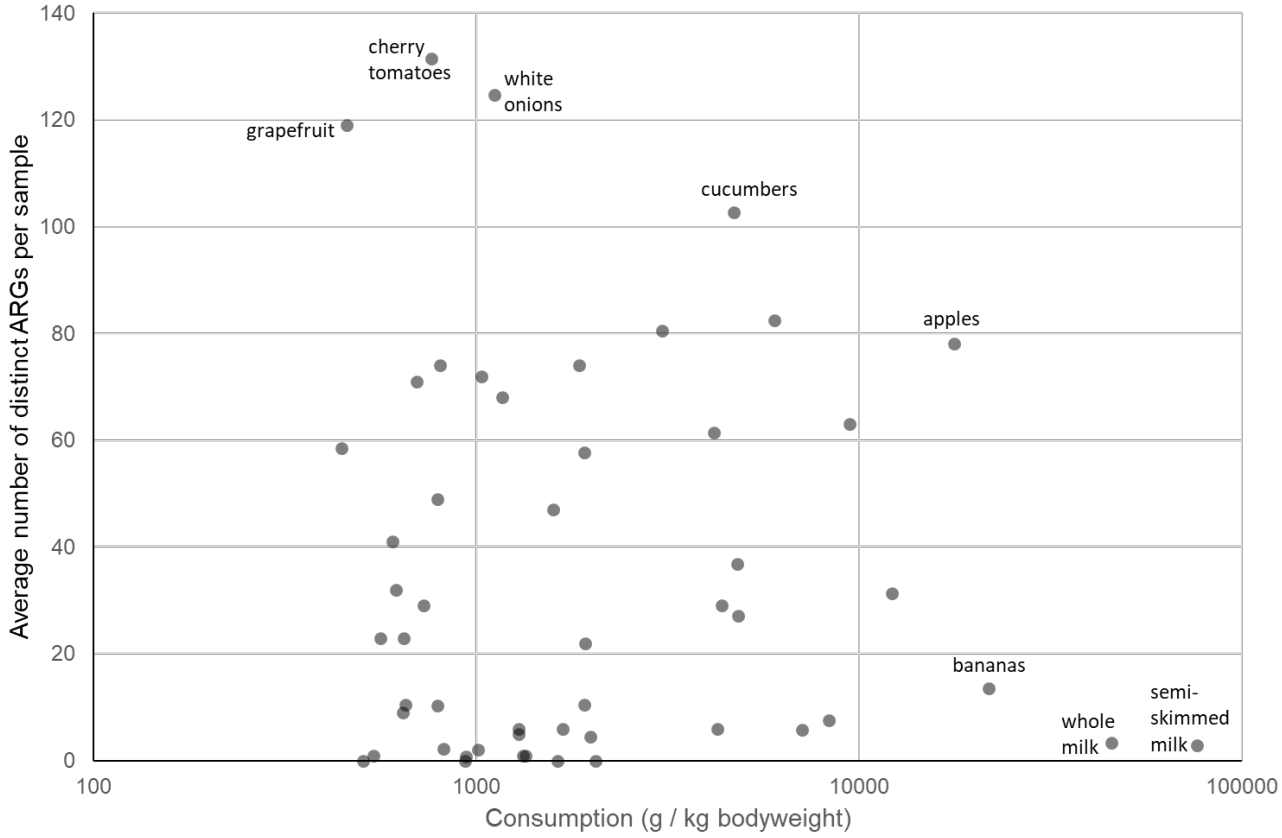


Figure 22. Average consumption against the average number of ARGs for each food type

5. Conclusions

A very high proportion of individual diets contain ARGs from RTE foodstuffs, based on the estimates of burden presented here, with some ARGs, such as those encoding certain efflux pumps being found in approximately 97% of diets of UK consumers in the general population. However, genes that may be involved in conferring resistance to an important last-resort antibiotic, colistin, was found at very low levels. Genes that may confer resistance to methicillin were also found very infrequently. Genes potentially involved in resistance to other import antimicrobials (fluoroquinolones, carbapenems, vancomycin and potential ESBL activity) were much more widely detected, driven by the presence of efflux pumps and beta-lactamases. Across all samples, 477 different ARGs were detected.

However, as noted previously (see section 4) the presence of these genes does not necessarily mean that phenotypically antibiotic resistant bacteria were present.

Technically the methods used in this project were successfully able to detect ARGs in dairy, produce and meat food types. The rinsing methodology appears to have been effective at removing bacterial DNA from the surface of produce type food; for some produce (and dairy) samples, high read numbers post-host filtering, and high-quality assemblies are obtained. For others, lower quality assemblies are produced.

The 16S metabarcoding screen prior to sequencing was effective at identifying samples which had high levels of bacterial DNA relative to host DNA. However, the samples which failed 16S sequencing altogether did not necessarily go on to fail metagenomic sequencing. While this means that some samples may have been screened out unnecessarily, only a low percentage (8%) of samples failed 16S sequencing. Furthermore, these and all other DNA extracts remain available for further sequencing. It is possible that a measure such as DNA yield may be more appropriate for determining the likelihood of a sample failing metagenomic sequencing, although in this instance the DNA yield of many samples was below the limit of quantification of the spectrophotometer used, and as such the maximum input volume of DNA was used.

This overall objective of this project was to provide an overview of the burden of ARGs from RTE foods in average UK diets. This was achieved by sampling according to consumption and sequencing according to consumption weighted by food types which were more likely to generate larger amounts of bacterial DNA sequence. While this means that we have robust estimates for the burden in terms of incidence of ARGs in the diet derived from RTE foods, there are a number of limitations to this approach, outlined below.

The approach used to measure ARGs in each sample in this project was non-quantitative. While measures were taken initially to normalise read counts of ARGs to give within-sample relative abundance, this data was not relevant to exposure expressed as incidence and prevalence in food types and diets, and presence/absence incidence data was used instead.

The lack of quantification also contributes to the difficulty of commenting on the relative contributions of different food types. While for example certain food types (for example apples) display high ARG richness in terms of numbers of different ARGs identified, the lack of quantification data means that it is impossible to distinguish between apples having genuinely higher levels of ARGs than milk, or the techniques applied (for example rinsing) giving a lower limit of detection for ARGs on apples than on milk. Improvements to the DNA extraction methods for non-rinsable food types, and the incorporation of quantitative data suggested above, will improve this situation.

Because the study was designed for the specific purpose of estimating the burden across the UK diet as a whole we don't estimate the relative contribution of different foodstuffs nor the ARG content of specialist diets, for example vegetarian or vegan, whose intakes of food types may differ drastically from the averages in the NDNS. The revised sampling plan (2.1.2) led to increased sample numbers of highly consumed food, but at the expense of less highly consumed food. Many food types had only single samples sequenced metagenomically, and an entire food category (seafood) proposed for sampling was omitted from sampling based on low consumption rates.

6. Recommendations for Further Work

6.1 Understanding the relevance of ARG exposure and associated AMR risk

Given the great diversity of ARGs identified in the samples, and the specialism of AMR experts, it is unlikely that any single expert will be suitably equipped to address the risks that may be associated with all of these ARGs. The high prevalence ARGs/ARG families identified here could form the basis of a review by a specialist(s), on the risks posed by ARGs in RTE foods. If high risk, high consumption ARGs are identified, these would make good targets for both surveillance and reduction measures.

Larger sample numbers for many food items, combined with qPCR-based normalisation, may allow much more to be said about the risk of different food types. A plausible starting

point would be the 745 DNA extracts retained from the current project, which could be metagenomically sequenced and analysed with qPCR (in addition to the 256 already sequenced), to increase sample numbers for many food types. However, given the low sample numbers of some of the least consumed food items (as few as five samples), some additional sampling may be beneficial. Larger sample numbers would also help elucidate the level of variation in ARG content within food types, as we observed significant variation between duplicate pairs of samples for some foods (blueberries and iceberg lettuce in this case).

Additionally, as this project has highlighted, there is a difference between understanding ARG exposure and AMR risk. Future projects could include an element of phenotypic AMR screening of one or more bacterial species to help elucidate the connection between the two.

6.2 Technical improvements to methods

In order to achieve the depths of sequencing required to create assemblies of the completeness required for future ARG analyses, either larger per-sample read numbers should be generated, or a method for removing host cells or DNA prior to sequencing should be developed for dairy and meat type products. An examination of **Error! Reference source not found.** suggests that produce types which were peeled prior to rinsing (being types which are usually eaten peeled, for example bananas, oranges) had in general less bacterial DNA than produce types which were not peeled prior to rinsing (produce types which are generally not peeled prior to consumption for example apples, blueberries). Although the experiment was not structured to assess this directly it would appear a logical trend, with higher levels of bacteria present on the exterior than the interior of the food item. However, future consideration should be given to assessing the exterior surface (prior to peeling) of produce types which can be peeled, as these may still represent a potential reservoir of ARGs. For example, it is difficult to envisage peeling and eating an orange without ingesting at least some microbes originating on the exterior.

Calculating the relative risk posed by interior and exterior ARGs on such produce types would likely be a significant undertaking.

The incorporation of quantitative data sources, for example quantitative PCR (qPCR) assays for selected, common ARGs, would potentially enable normalisation of metagenomic data, allowing quantitative comparison of metagenomically identified ARGs among samples. If a measure of the uncertainty could be attached to the presence of an ARG within a sample (e.g .the probability of presence, given the available data) then this could be combined with the analysis of the NDNS data to provide a more complete probabilistic model of AMR burden per food type and in the UK diet overall. qPCR data could be used to estimate false positive rates for selected ARGs/ARG families.

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