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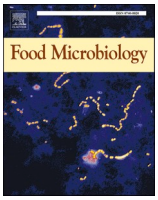

Determination and quantification of microbial communities and antimicrobial resistance on food through host DNA-depleted metagenomics

Samuel J. Bloomfield [a], Aldert L. Zomer [b], Justin O'Grady [a,1], Gemma L. Kay [a,1], John Wain [a,c], Nicol Janecko [a], Raphaëlle Palau [a], Alison E. Mather [a,c,*]

[a] Quadram Institute Bioscience, Rosalind Franklin Road, Norwich Research Park, Norwich, NR4 7UQ, United Kingdom
[b] Utrecht University, Heidelberglaan 8, 3584 CS, Utrecht, Netherlands
[c] University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, United Kingdom

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ABSTRACT

Food products carry bacteria unless specifically sterilised. These bacteria can be pathogenic, commensal or associated with food spoilage, and may also be resistant to antimicrobials. Current methods for detecting bacteria on food rely on culturing for specific bacteria, a time-consuming process, or 16S rRNA metabarcoding that can identify different taxa but not their genetic content. Directly sequencing metagenomes of food is inefficient as its own DNA vastly outnumbers the bacterial DNA present. We optimised host DNA depletion enabling efficient sequencing of food microbiota, thereby increasing the proportion of non-host DNA sequenced 13-fold (mean; range: 1.3–40-fold) compared to untreated samples. The method performed best on chicken, pork and leafy green samples which had high mean prokaryotic read proportions post-depletion (0.64, 0.74 and 0.74, respectively), with lower mean prokaryotic read proportions in salmon (0.50) and prawn samples (0.19). We show that bacterial compositions and concentrations of antimicrobial resistance (AMR) genes differed by food type, and that salmon metagenomes were influenced by the production/harvesting method. The approach described in this study is an efficient and effective method of identifying and quantifying the predominant bacteria and AMR genes on food.

1. Introduction

All food products, of both animal and plant origin, will carry bacteria unless specifically sterilised. Foods vary in the number and types of microorganism with which they are colonised (Mira Miralles et al., 2019). Some of these microorganisms may be benign, commensal, beneficial or pathogenic to humans (Gruetzke et al., 2019), or associated with food spoilage (Cauchie et al., 2020) and may also be resistant to antimicrobials (de Souza et al., 2019). It is estimated that foodborne pathogens are responsible for 2.4 million infections in the United Kingdom (Holland and Mahmoudzadeh, 2020) and cost the economy £9.1 billion each year (Daniel et al., 2020). Pathogens which are resistant to antimicrobial drugs compound the problem, as these infections may result in treatment failure (WHO, 2020).

The identification of pathogens on food that can cause foodborne disease is most commonly achieved through selective enrichment and culturing followed by either biochemical testing or a MALDI-TOF, a process which can take up to seven days (Elbehiry et al., 2017; Mooijman et al., 2019). In terms of identifying problems and applying interventions, this has several drawbacks; first, the shelf life of foods may be less than the time it takes to selectively enrich and culture the bacteria, and the quantity of pathogens on food may increase over time (Meldrum et al., 2014). Second, this approach will miss bacteria; for example, if a pathogen is present in low numbers, or is not the subject of specific enrichment, it will be missed. Third, both non-pathogenic and pathogenic bacteria can act as reservoirs of antimicrobial resistance (AMR) genes and can transfer AMR genes between them, contributing to the burden of disease in humans (Rolain, 2013). Culture-independent

Abbreviations: AMR, Antimicrobial resistance; µl, Microlitre; bp, Base-pair; BPW, Buffered peptone water; Cq, Quantification cycle; DNA, Deoxyribonucleic acid; Gb, Giga-base-pair; l, Litres; mg, Milligram; $MgCl_2$, Magnesium chloride; ml, Millilitre; NaCl, Sodium chloride; ng, Nanogram; PBS, Phosphate-buffered saline; PERMANOVA, Permutational Multivariate Analysis of Variance; qPCR, Quantitative polymerase chain reaction; rpm, Revolutions per minute; U, Units.

* Corresponding author. Quadram Institute Bioscience, Rosalind Franklin Road, Norwich Research Park, Norwich, NR4 7UQ, United Kingdom.
E-mail address: alison.mather@quadram.ac.uk (A.E. Mather).
[1] Current address: Oxford Nanopore Technologies PLC, Norwich, UK.

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approaches offer an alternative which may be able to overcome these drawbacks.

Multiple culture-free approaches have been developed to detect specific bacteria and genes from food. Immunoassays allow for the rapid detection of specific microbes (Carrell et al., 2019). 16S rRNA metabarcoding has helped describe food bacterial diversity (Zhang et al., 2016), but bacteria have multiple copies of the ribosomal operon, and variation between these copies can be greater than between different bacterial species (Johansen et al., 2017; Vetrovsky and Baldrian, 2013); classifications using 16S rRNA metabarcoding are generally not reliable below the genus (Gruetzke et al., 2019). 16S rRNA metabarcoding is also not quantitative, relying on additional spiking and qPCR for quantification (Zemb et al., 2020). Multiplex PCR methods have helped identify the presence of specific genes of interest, such as AMR genes (de Paula et al., 2018), but novel AMR genes are routinely discovered, requiring very large primer panels and continually revised databases and expansion of PCR methods for their detection. Culture-free approaches can also be performed after selective enrichment to help identify target microorganisms present in low numbers (Lopez-Perez et al., 2016).

Metagenomic sequencing approaches allow for the genomic DNA of microorganisms within a sample to be detected, facilitating an understanding of the breadth and diversity of microorganisms and identifying the virulence and AMR genes (resistome) present on food samples (Yap et al., 2022), provided the proportional abundances of these microorganisms are sufficiently high for detection. However, this involves sequencing all DNA in a sample using shotgun metagenome sequencing which is difficult to apply effectively to food samples of animal or plant origin because the ratio of DNA from the original host to the DNA from microorganisms is very high. For example, the chicken (*Gallus gallus*) genome is 1.2 Gb in length (Bellott et al., 2017), 240-times larger than the 5 Mb *Escherichia coli* genome (Makino et al., 1999). DNA directly extracted and sequenced from foods of animal or plant origin often contains bacterial DNA that is sufficient for population identity and diversity estimates but not for detecting specific strains (Kobus et al., 2020). In addition, a high sequence depth may be required to identify specific bacterial genetic factors (e.g., AMR and virulence genes) to compensate for a low proportion of bacterial reads.

Multiple methods have been developed to increase the efficiency of shotgun metagenome sequencing. Commercial kits such as New England Biolab's NEBNext® Microbiome DNA Enrichment kits take advantage of high CpG methylation in eukaryotic DNA to filter out the DNA using methylated CpG binding proteins. In contrast, Molzym's Molysis™ kits use chaotropic reagents to selectively lyse eukaryotic cells followed by a DNAse treatment to degrade the eukaryotic DNA released (Thoendel et al., 2016). Charalampous et al. (2019) developed a method to deplete human DNA from respiratory samples through the use of a detergent (saponin) to lyse human cells and a DNAse (HL-SAN) to degrade the human DNA released. All three of these methods were developed for clinical samples, but food and clinical samples differ in complexity, composition and the types of microorganisms with which they are colonised. Very few studies have applied any of the depletion methods to food samples (Gruetzke et al., 2021).

In this study we utilised and extended the host DNA depletion method by Charalampous et al. (2019) prior to metagenome sequencing to classify and quantify bacteria and AMR genes present on specific retail food. This extension involved adding a protease step (Neutrase) described by Escobar-Zepeda et al. (2016) to break up protein in food. As this extended method uses Neutrase to degrade protein and saponin to lyse cells, we refer to this method as the Neutrase-saponin method. We compared the method to the Molysis kit for host cell depletion and used qPCR to quantify the AMR genes found on food. We applied the Neutrase-saponin method to mock communities of bacteria and to multiple types of retail food samples to determine its effectiveness with different species of bacteria and different food commodities, respectively. Through doing so, we increased the proportion of sequence data belonging to microorganisms and obtained unbiased estimates of the bacteria and AMR genes to which humans are exposed through food. We also applied the method to a diverse collection of foods to determine their microbial composition, their resistome, and the association of these traits with food commodity.

2. Materials and methods

2.1. Food samples

The food samples utilised in this study consisted of chicken, leafy greens, pork, prawns and salmon collected from retail shops in Norfolk county of the United Kingdom between 17/09/2018 and 25/11/2019, in a survey described in Janecko et al. (2021) (Tables S1–S3). Chicken samples included whole chickens, chicken breasts, chicken drumsticks, chicken fillets and chicken thighs; leafy greens included chard, lettuce, rocket, spinach, and watercress; pork included pork belly, pork chops, pork shoulders, and pork steaks; prawns included whole prawns, headless prawns, and shell-less prawns; and salmon included salmon fillets with and without skin.

2.2. Method development

The host DNA depletion method described by Charalampous et al. (2019) was trialled on food samples. To prepare the food for the method, 100 g of each food sample was placed into a FBAG-03 filter blender bag (Corning, New York, USA) and stomached (Seward stomacher 400C laboratory blender, Worthing, UK) with 225 mL of buffered peptone water (BPW) (Southern Group Laboratory (SGL), Corby, UK) at 100 rpm for 30 s. 1.5 ml of stomached food was centrifuged at 8,000×*g* for 5 min, before the supernatant was discarded and the pellet was resuspended in 250 μl of phosphate-buffered saline (PBS) (Merck, Darmstadt, Germany). 200 μl of 5% saponin (Tokyo Chemical Industry, Tokyo, Japan) in PBS was added and the mixture was incubated for 10 min at 20 °C on a ThermoMixer rotating at 1000 rpm. 350 μl of molecular water (Merck) was added to the mixture, before it was vortexed for 2 s, incubated at room temperature for 30 s, 12 μl of 5M NaCl was added, and vortexed for a further 2 s. The mixture was centrifuged at 8,000×*g* for 5 min, before the supernatant was discarded and the pellet was resuspended in 100 μl of PBS. 100 μl of HL-SAN buffer and 10 μl of 25 U/μl HL-SAN (ArcticZymes Technologies, Tromsø, Norway) were added to the mixture, and was vortexed for 2 s, before being incubated at 37 °C for 15 min rotating at 800 rpm. 800 μl of PBS was added to the solution before it was centrifuged at 6,000×*g* for 3 min, the supernatant was discarded, and the pellet was resuspended in 500 μl of PBS. 500 μl of PBS was added to the mixture, before it was centrifuged at 6,000×*g* for 3 min, the supernatant was discarded, and the pellet was resuspended in 800 μl of PBS.

The method was improved for food by trialling different saponin incubation times (10 versus 20 min), saponin concentrations (0.225%, 2.25% and 5%), the addition of a Neutrase (Novozyme, Bagsværd, Denmark) step, and varying the amount of stomached sample used (1.5 versus 10 ml). The Neutrase method was adapted from Escobar-Zepeda et al. (2016). Here, 27 μl of 1 U/ml Neutrase was added to 1.5 ml of stomached food sample and was incubated at 45 °C for 60 min in a rotating incubator at 1000 rpm. We also trialled different Neutrase incubation times (20, 40 and 60 min). In addition, the commercial Molysis kit (Molzyme GmbH & Co. KG, Bremen, Germany) and an untreated sample were used for comparison.

DNA was extracted from untreated and depleted metagenomes using a Maxwell® RSC PureFood Pathogen Kit (Promega, Madison, Wisconsin, USA) on a Maxwell® RSC 48 automated extraction system according to manufacturer's instructions.

To quantify the effectiveness of different host DNA depletion conditions, the Qubit dsDNA HS assay kit was used to measure DNA concentrations of metagenomic extractions and qPCR was used to measure the amount of host and bacterial DNA present before and after host DNA depletion. Host DNA of the untreated and depleted chicken samples

were measured using qPCR methods specific to the *TGFB3* gene (Cai et al., 2014). No TaqMan method was found that could effectively quantify the host DNA of the other food commodities, therefore a SYBR green method specific for the 18S rRNA gene was used instead (Hadziavdic et al., 2014). Bacterial DNA was quantified using a TaqMan qPCR method specific for the 16S rRNA gene (Liu et al., 2012) (Appendix 1: Tables S4–S9). Primers and probes were created by Integrated DNA technologies UK, Ltd. The PrecisionFAST qPCR Master Mix (PrimerDesign, Southampton, UK) was used for TaqMan methods and QuantiFast SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) was used for SYBR green methods.

For host and bacterial qPCR methods, the difference in quantification cycles (ΔCq) for untreated and host depleted samples were calculated. The difference between the bacterial and host qPCR ΔCq (referred to henceforth as the qPCR difference) was used as a measure of depletion efficiency.

2.3. Neutrase-saponin host DNA depletion method

For the final Neutrase-saponin method, 10 ml of stomached food sample was added to 180 μl of 1 U/ml Neutrase and incubated at 45 °C for 40 min in a rotating incubator at 200 rpm. The mixture was centrifuged for 30 min at 4,000×*g*, before the supernatant was discarded and re-suspended in 250 μl of PBS. A 200 μl volume of 0.225% saponin in PBS was added and the mixture was incubated for 10 min at 20 °C whilst rotating at 1000 rpm. 350 μl of molecular water was added to the mixture, before it was vortexed for 2 s, incubated at room temperature for 30 s, 12 μl of 5M NaCl was added, and vortexed for a further 2 s. The mixture was centrifuged at 8,000×*g* for 5 min, before the supernatant was discarded and the pellet was resuspended in 100 μl of PBS. 100 μl of HL-SAN buffer (5M NaCl, 0.1M $MgCl_2$) and 10 μl of 25 U/μl HL-SAN were added to the mixture, before it was vortexed for 2 s and incubated at 37 °C for 15 min whilst rotating at 800 rpm. 800 μl of PBS was added to the solution before it was centrifuged at 6,000×*g* for 3 min, the supernatant was discarded and the pellet was resuspended in 500 μl of PBS. 500 μl of PBS was added to the mixture, before it was centrifuged at 6,000×*g* for 3 min, the supernatant was discarded and the pellet was resuspended in 800 μl of PBS.

For each food sample, an untreated preparation with no additional targeted approach was prepared alongside the host DNA-depleted preparation from the food sample by centrifuging 10 ml of stomached sample for 30 min at 4,000×*g*. The supernatant was discarded and re-suspended in 800 μl of PBS.

For each sampling trip, a blank was run to monitor reagent contamination (comprising 225 ml of BPW) in parallel.

DNA was extracted from untreated, depleted and blank samples using a Maxwell® RSC PureFood Pathogen Kit as above. Blank samples had undetectable DNA concentrations as assessed with Qubit dsDNA HS assay kit, therefore were spiked with phiX174 RF1 DNA (New England Biolabs, Ipswich, Massachusetts, USA) up to a concentration of 0.5 ng/μl so they could be sequenced.

2.4. Method validation

The Neutrase-saponin host DNA depletion method and the commercial host-depletion Molysis method were trialled on three chicken, three pork, three leafy green, three prawn and three salmon samples. Both methods were also applied to the ZymoBiomics microbial community standard (Zymo Research, Irvine, California USA) in PBS. The Molysis method was performed according to the manufacturer's instructions.

An in-house mock community was assembled by incubating seven bacterial species in BPW overnight and then combining different percentages of these bacterial species: *Escherichia coli* (10%), *Klebsiella pneumoniae* (20%), *Pseudomonas aeruginosa* (10%), *Salmonella enterica* (35%), *Staphylococcus aureus* (5%) and *Vibrio parahaemolyticus* (20%). This community, still in BPW, was divided into four groups that were stored for 72 h at different temperatures: room temperature (20 °C), refrigeration (2–8 °C), freezer (−20 °C), and long-term freezer (−80 °C). For each of the in-house mock communities stored at different temperatures, along with a ZymoBiomics microbial mock community, the mock communities were divided into five samples and PBS was added to each up to 10 ml, two of which underwent the Neutrase-saponin method to test for method variability, one underwent only the Neutrase component of the method and another only the saponin component to evaluate the individual performance of these components on the results, and one was unaltered for comparison (untreated sample).

2.5. Method application

The Neutrase-saponin method was performed on 154 food samples (34 chicken, 33 leafy greens, 31 pork, 28 prawns and 28 salmon) that were collected on fourteen sampling trips conducted on different days.

To determine if the host DNA depletion method had differential efficiency between food types, depleted DNA concentrations and qPCR ΔCq values were modelled using linear regression with food commodity and sampling trip as the explanatory variables. For prawns and salmon, we also investigated if these parameters were affected by how they were produced/harvested (wild caught or aquacultured). Partial-F tests were used to determine if the potential explanatory variables significantly improved the fit of the model.

Host DNA depleted metagenomes with DNA concentrations greater than 0.05 ng/μl and qPCR differences of three or greater were selected for sequencing. Untreated samples were obtained for qPCR comparisons but were not sequenced for these food samples as the Neutrase-saponin method had been validated (Section 2.4) and this would have dramatically increased the sequencing costs.

2.6. Illumina sequencing

For method validation (Section 2.4), libraries were constructed for untreated, depleted and blank metagenomic extractions. For method application (Section 2.5) samples, libraries were constructed for depleted and blank metagenomic extractions. Nextera DNA flex library prep kits (Illumina, San Diego, California, USA) were used to create the libraries. Libraries for method validation were sequenced on an Illumina NextSeq at 1 Gb per metagenome, as 150 bp paired-end reads, to evaluate the effect of depletion on the microbial composition. Libraries for method application were sequenced on an Illumina NovaSeq 6000 at 8 Gb per metagenome, as 150 bp paired-end reads to obtain a higher sequence depth to investigate the presence of genetic factors such as AMR genes. Raw sequence data were uploaded to the Sequence Read Archive under project PRJNA849983.

2.7. Metagenome analysis

BBsplit v38.75 (Bushnell, 2014) was used to align paired reads from blank samples to the phiX174 genome (SAMN04281799); reads mapping to phiX174 were removed from further analysis.

Paired metagenome reads were processed using fastp v0.19.5 (Chen et al., 2018) to trim reads with a minimum quality value of 20 and default other parameters. Trimmed reads were classified using Centrifuge v1.0.4 (Kim et al., 2016) and the Centrifuge NT database. KMA v1.2.3t (Clausen et al., 2018) with the ResFinder v3.2 database (Zankari et al., 2012) was used to identify AMR genes amongst the trimmed reads with a 90% identity and 60% coverage. BBSplit was used to align reads to the ResFinder database to estimate the proportion of reads associated with AMR genes.

To look at differences between metagenomes, taxonomic read data from the food metagenomes were analysed using the vegan v2.5.6 (Oksanen et al., 2019) and phyloseq v1.38.0 (McMurdie and Holmes, 2013) packages of R v3.6.1 (R core team, 2019). Taxonomic data were

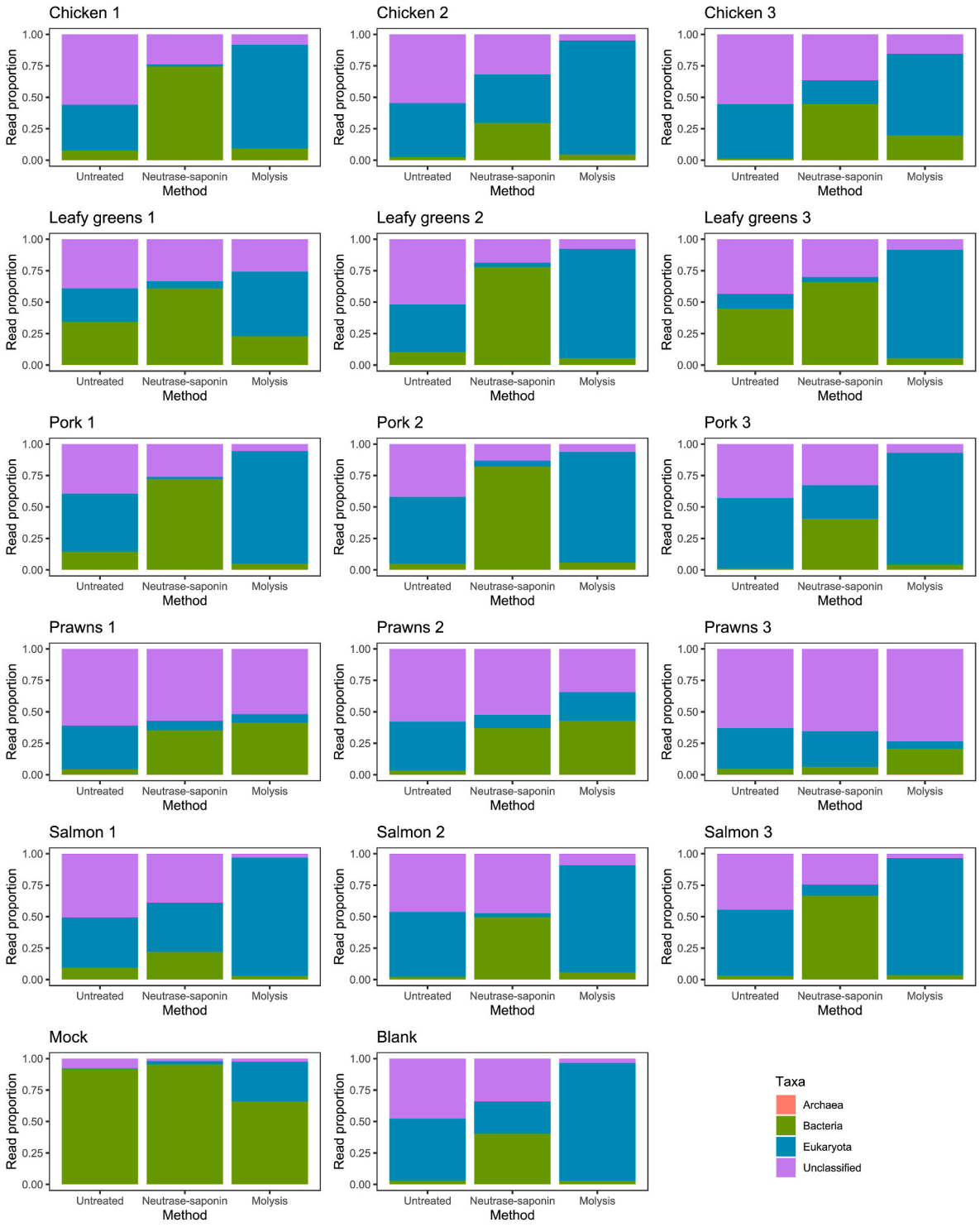

Fig. 1. Proportion of reads that belong to each superkingdom and reads that were unclassified for three chicken samples, three pork samples, three salmon samples, three prawn samples, three leafy green samples, a Zymo mock community, and a blank presented by three metagenome processing methods: untreated (no host DNA depletion), Neutrase-saponin method and Molysis kit. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

rarefied so that dissimilarity between food metagenomes could be measured using Bray-Curtis dissimilarity (Bray and Curtis, 1957) and to ensure that the wide differences in taxonomic variation, between samples, was taken into account. Adonis2 (Oksanen et al., 2019) was used to perform Permutational Multivariate Analysis of Variance (PERMANOVA) on the distance matrix with metadata as the explanatory variables. Non-metric multi-dimensional scaling (nMDS) was performed on the distance matrix to visualise the dissimilarity between metagenomes and coloured by the metadata variables.

The concentration of bacterial pathogens on food was calculated from the proportion of the metagenome made up of the taxon and the proportion of prokaryotic reads as calculated by Centrifuge and the Centrifuge_NT (2018_3_3) database, and the 16S qPCR results (Appendix 2).

To evaluate and quantify the potential human exposure to AMR from food, the AMR gene concentration per gram of food was calculated from the proportion of metagenomes originating from prokaryotes, the proportion of metagenomes made up of AMR genes, and the number of bacteria in the metagenomes as estimated from the 16S qPCR result (Appendix 2). Linear regression was used to model the concentration of AMR genes in samples with metadata as the explanatory variables as above. The European Food Information Resource (Westenbrink et al., 2016) was used to determine the portion size of the food samples (chicken = 195 g, leafy greens = 80 g, pork = 200 g, prawns = 150 g and salmon = 125 g) and calculate the number of AMR genes per portion size.

3. Results

3.1. Method development and optimisation

The method described by Charalampous et al. (2019) was optimised for food by applying it to chicken, pork, prawn, salmon and leafy green samples over three sampling trips and varying the key conditions of the method (Table S1). Initial optimisation involved varying the incubation times with saponin (10 versus 20 min), the introduction of a 60-min long Neutrase step, and comparison to the commercial Molysis kit. The Neutrase step was trialled as it was observed that samples often contained large clumps of protein, preventing mixing with saponin. The saponin method resulted in a median qPCR difference of 4.17 (range: 3.69–15.95) (Appendix 1: Fig. S1). Increased incubation resulted in a similar median qPCR difference of 4.45 (range: 3.71–15.9), but the addition of the Neutrase step resulted in a higher qPCR difference median of 6.03 (range: 3.32–10.6). The Molysis kit resulted in a qPCR difference median of 5.60 (range: −1.98–8.04).

The Neutrase method described by Escobar-Zepeda et al. (2016) was originally optimised for cheese, a food type with high fat and protein content; we investigated whether or not shorter incubation times could sufficiently break down proteins such that there were no clumps of protein in the stomached food products examined in this study after incubation with Neutrase. Protein clumps were only found in the food commodities of animal origin, so these were used to optimise the Neutrase incubation time (20 min, 40 min and 60 min). A 20-min Neutrase step produced a median qPCR difference of 4.49 (range: −2.64–15.6) (Appendix 1: Fig. S2), but there were still many protein clumps left in tubes after incubation, making it difficult to perform subsequent steps. A 40-min Neutrase step produced a slightly higher median qPCR difference of 4.71 (range: −2.11-13.5) and left no clumps of protein in the tube. A 60-min Neutrase step produced the highest median qPCR difference of 5.67 (range: −2.14–13.0) and left no protein clumps in tubes. However, we wanted to shorten this step as much as possible in case this allowed the multiplication of certain bacteria, so a 40-min Neutrase incubation step was used. For leafy green samples, saponin should struggle to lyse plant cells due to the thick cell wall, so we compared the saponin method with just the DNAse step. However, the inclusion of saponin increased the qPCR difference to 7.74 from 6.63 when just the DNAse step was used, indicating that it is contributing to leafy greens host DNA depletion.

The final optimisation step involved the saponin concentration. The original saponin concentration of Charalampous et al. (2019) was 5%, but we trialled 2.25% and 0.225% to see if we could retain more bacterial DNA whilst depleting host DNA. The best median qPCR difference was with a concentration of 0.225% (median: 5.18, range: 0.349–21.7), compared to 2.25% (median: 3.76, range: 1.71–20.7) and 5% (median: 4.68, range: 0.380–16.8) (Appendix 1: Fig. S3).

Initially the optimised host DNA depletion methods produced very low DNA concentrations for some food samples, complicating sequencing attempts. To increase the DNA concentration, the initial volume of stomached food was increased from 1.5 ml to 10 ml, and the amount of Neutrase was increased to match this volume. The rest of the steps remained the same.

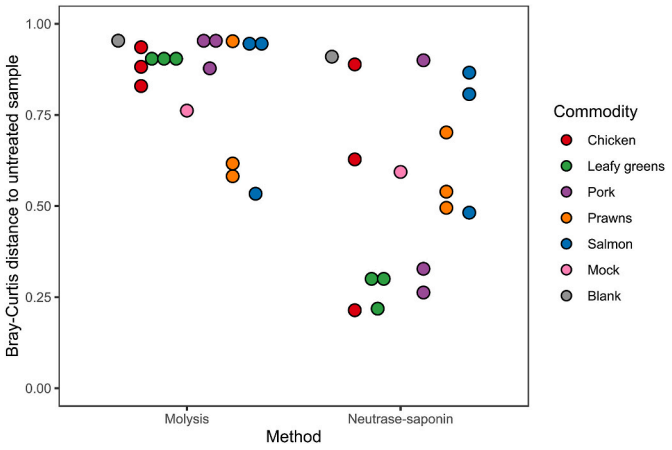

Fig. 2. Bray-Curtis distance between metagenomes depleted using the Molysis kit or Neutrase-saponin method and the untreated sample; coloured by commodity.

3.2. Method validation

The Neutrase-saponin method and Molysis method were trialled on 15 food samples and a commercial mock community to determine variability between different food commodities (Table S2). For the 15 food samples investigated, the Neutrase-saponin method increased the proportion of prokaryotic (bacterial and archaeal) reads compared to the untreated sample 1.3- to 40-fold and produced the highest prokaryotic read proportions for twelve specimens, whilst the Molysis kit changed the proportion compared to the untreated sample by 0.13- to 18-fold and produced the highest proportion for the three prawn specimens (Fig. 1). The Neutrase-saponin method also produced metagenome extracts with higher DNA concentrations compared to the Molysis method (Appendix 1: Fig. S4). Both methods resulted in altered bacterial genus proportions compared to the untreated sample, but metagenomes from the Molysis method were more distant to untreated samples (mean: 0.85) than those using the Neutrase-saponin method (mean: 0.53) (Fig. 2; Appendix 1: Figs. S5 and S6).

To investigate potential differential effects of the Neutrase-saponin method on bacteria, the separate components of the method were applied to an in-house mock community under different conditions as well as the commercial (Zymo) bacterial mock community (Appendix 1: Fig. S7). Comparisons of the untreated samples demonstrated that refrigeration increased the proportion of the metagenome relating to *Salmonella* from 0.35 to 0.56, whilst freezing at −20 °C or −80 °C increased the relative proportion of *Pseudomonas* DNA from 0.1 to 0.34, but the Neutrase-saponin method had little effect on the relative proportions of the different bacterial genera in the in-house mock community (mean difference = 0.058). The Neutrase-saponin method was also very consistent, with similar bacterial genus proportions between repeats (mean difference = 0.012). However, the Neutrase-saponin method affected the relative proportions of bacterial genera sequenced in the commercial mock community (mean difference = 0.12).

3.3. Method application

The Neutrase-saponin method was applied to 154 food samples, 109 of which were chosen for further sequencing based on the selection criteria of DNA concentration and qPCR results (Table S3). These consisted of 28 chicken, 24 leafy green, 15 pork, 18 prawn and 24 salmon samples, along with 14 blanks, one for each sampling trip.

Food commodity was found to be associated with the depleted final DNA concentration ($p = 5.22 \times 10^{-11}$), bacteria qPCR ΔCq ($p = 8.88 \times$

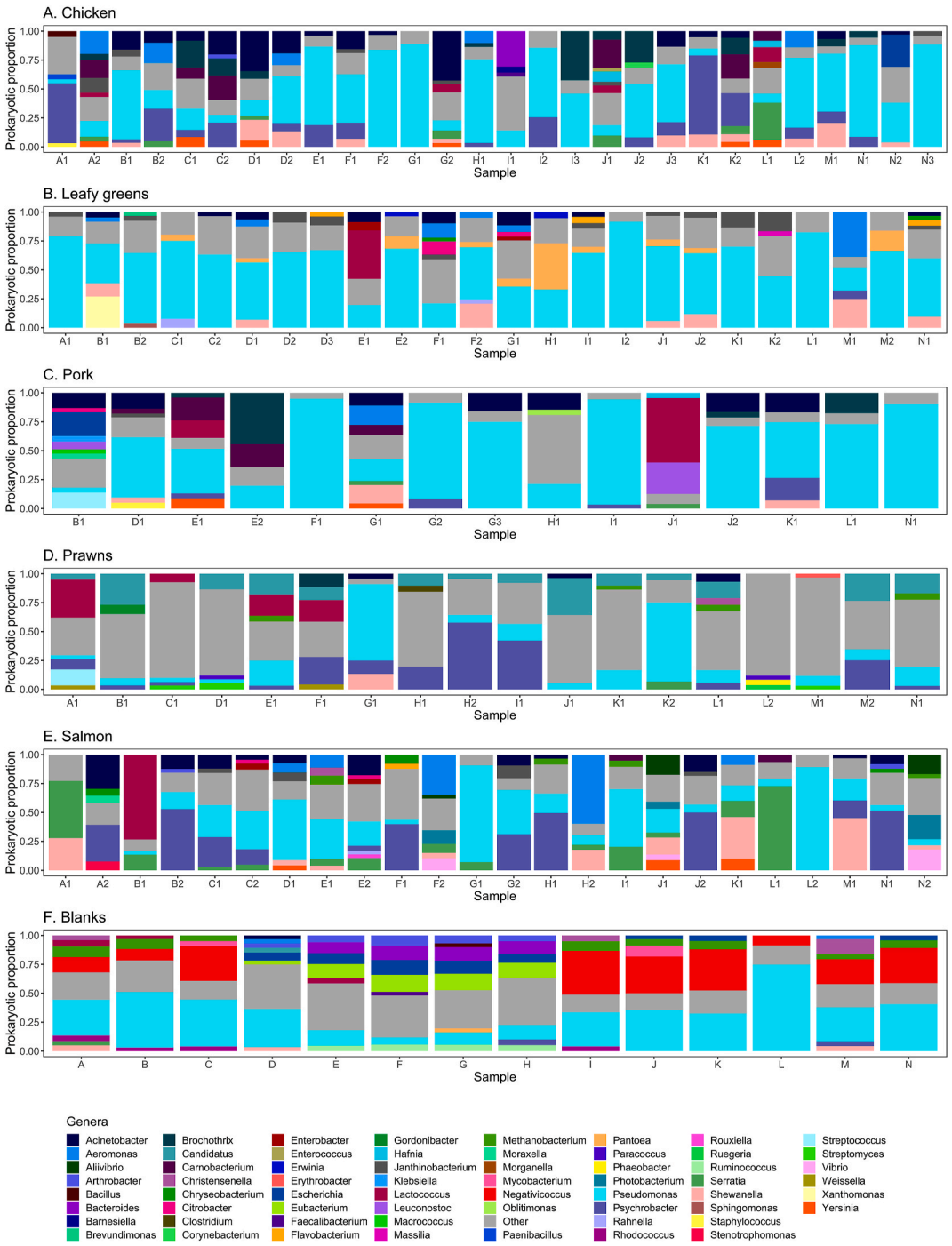

Fig. 3. Proportions of the food and blank metagenomes made up of different prokaryote taxa at the genus level for 109 food metagenomes (A–E) and 14 blanks (F). Taxa that comprised less than 3% of each metagenome were placed into the "Other" category.

10^{-4}) and host qPCR ΔCq ($p < 2.20 \times 10^{-16}$), but not food sampling trip ($p = 0.198$, 0.522 and 0.790, respectively) (Appendix 3: Figs. S9–S11). Aquacultured salmon samples were associated with higher depleted final DNA concentrations compared to those that were wild caught ($p = 0.0114$) (Appendix 3: Fig. S12), but production/harvesting had no significant association with bacterial or host qPCR ΔCq ($p = 0.167$ and 0.145, respectively). For prawns, production/harvesting had no significant association with depleted final DNA concentrations, bacterial or host qPCR ΔCq ($p = 0.107$, 0.517 and 0.308, respectively).

3.4. Metagenome composition

The 109 food metagenomes displayed a large amount of variation in the bacterial taxa present in samples belonging to the same and different food commodities (Fig. 3A–E). The predominant bacterial genus across all commodities was *Pseudomonas* whilst many taxa varied in proportion between the food samples as well as between food commodities, making comparisons difficult. There was also variation in the blanks for each sampling trip (Fig. 3F); this is likely due to different lots of reagents used for each sampling trip, especially for BPW as it was the reagent that has the highest volume used (10 ml per sample). This highlights the need to perform a blank with each sampling trip to control for potential

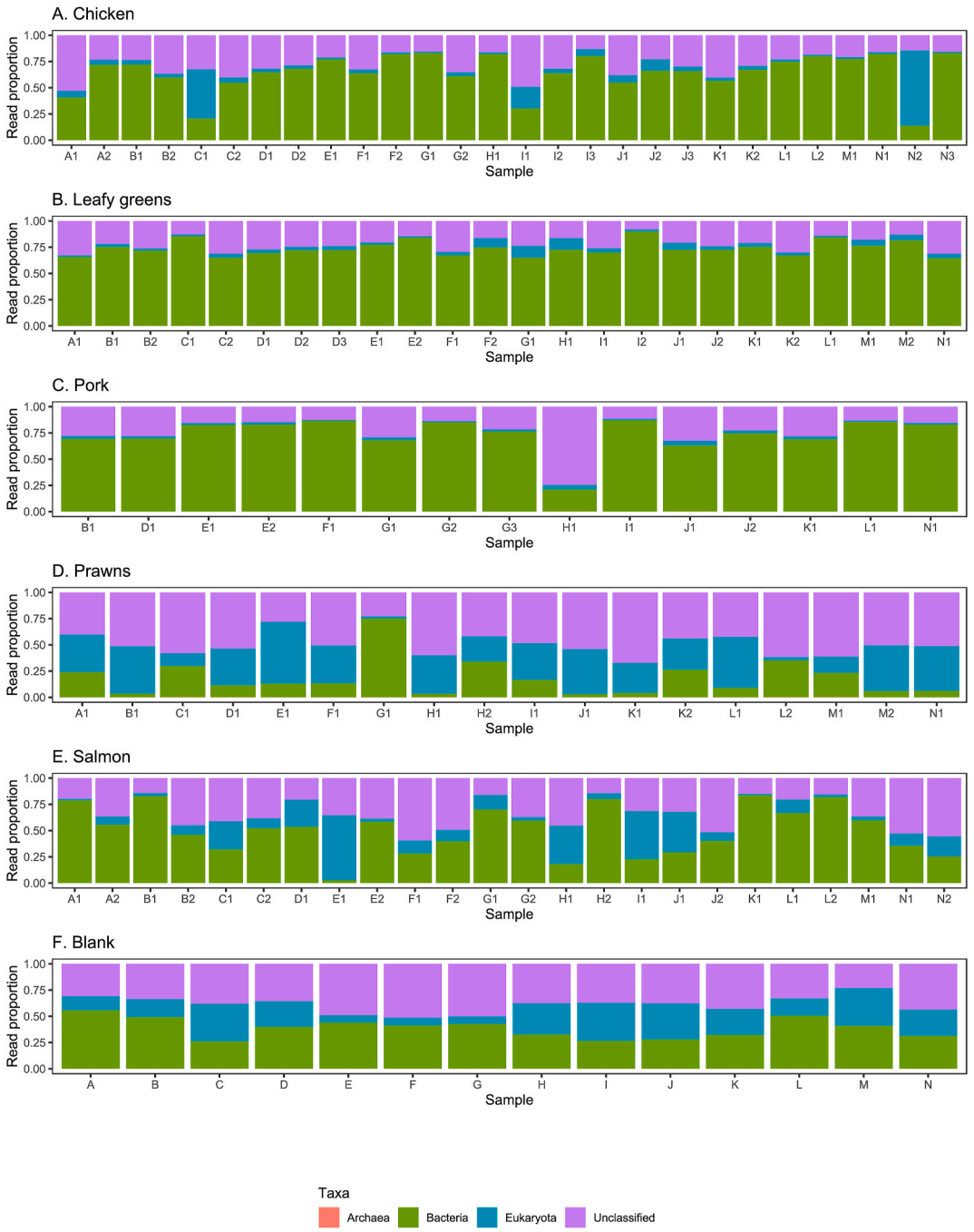

Fig. 4. Proportion of reads that belong to each superkingdom and were unclassified for the 109 food metagenomes (A–E) and 14 blanks (F).

variation in reagent contamination.

The proportion of reads that were prokaryote was found to be associated with food commodity using linear regression ($p = <2.20 \times 10^{-16}$) (Fig. 4), with chicken (mean: 0.64; range: 0.14–0.83), pork (mean: 0.74; range: 0.21–0.87) and leafy green (mean: 0.74; range: 0.64–0.90) metagenomes containing higher proportions than salmon (mean: 0.50; range: 0.028–0.84) and prawns (mean: 0.19; range: 0.031–0.75). There was no significant difference between the 14 different sampling trips ($p = 0.342$). Production/harvesting methods had no significant association with the proportion of reads that were prokaryotic in prawns ($p = 0.215$) or salmon ($p = 0.249$).

Multidimensional scaling and PERMANOVA analysis demonstrated that food metagenomes clustered by food commodity ($p = 0.001$), but not sampling trips ($p = 0.907$) (Fig. 5), providing confidence in the identified association between food commodity and metagenome diversity. Wild caught salmon also clustered separately from aquacultured salmon ($p = 0.001$) (Appendix 3: Fig. S13), but production/harvesting method had no significant association with prawns ($p = 0.248$).

3.5. Pathogens

The concentration of potential pathogens (*Campylobacter*, *Escherichia coli*, *Klebsiella/Raoultella*, *Listeria monocytogenes, Salmonella, Vibrio* and *Yersinia enterocolitica*) were calculated from the taxon read classifications and 16S qPCR results and were compared to the most predominant taxon in the food metagenomes, *Pseudomonas* (Appendix 4: Fig. S14). There was a large amount of variation between the concentrations of bacterial pathogens calculated for different food commodities. However,

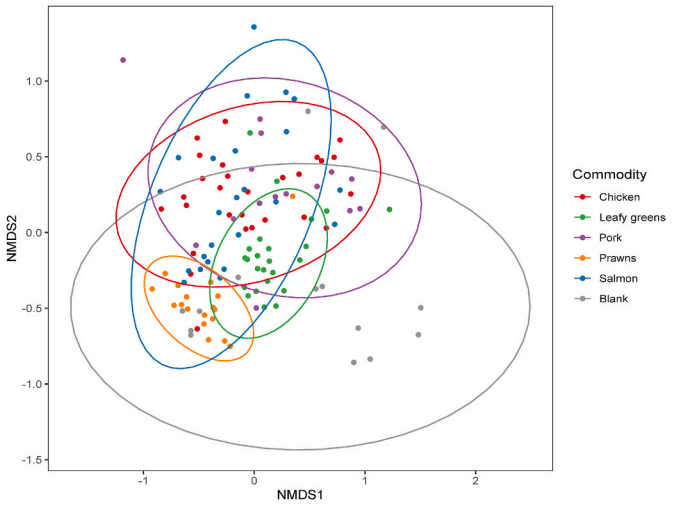

Fig. 5. Non-metric multidimensional scaling of 109 food metagenomes and 14 blanks based on rarefied taxon classifications and coloured by food commodity (stress = 18.5%).

the reads classified as these pathogens made up a small proportion of the prokaryotic reads sequenced (median: 0.0037) and are likely the result of misclassified reads.

3.6. AMR gene concentration

The food samples displayed a large amount of variation in the numbers of different AMR genes comprising their resistome (Fig. 6). Most AMR genes detected encoded resistance to aminoglycoside, beta-lactam or tetracycline antimicrobial agents. The food samples also displayed a large amount of variation in AMR gene concentrations (Fig. 7). Food commodity was associated with different AMR gene concentrations ($p = 4.8 \times 10^{-10}$) but not sampling trip ($p = 0.10$). The salmon and prawn samples demonstrated lower AMR gene concentrations than the chicken, leafy green or pork samples. Aquacultured salmon were associated with higher AMR gene concentrations than those that were wild caught ($p = 0.046$), but production/harvesting had no significant association with prawn AMR gene concentrations ($p = 0.79$) (Appendix 5: Fig. S15). The mean number of AMR gene per food portion was 1.1×10^{11} for chicken, 6.6×10^{10} leafy greens, 5.9×10^{10} for pork, 3.1×10^{9} for prawns and 1.1×10^{10} for salmon.

4. Discussion

Metagenomic approaches confer the ability to investigate the microbial communities on foods, allowing the identification of pathogens, spoilage organisms and AMR genes. Culture-based methods are likely to require multiple isolates to be cultured and sequenced to capture the true diversity of the bacterial population (Worby et al., 2014). Moreover, many prokaryotes are difficult or time consuming to culture (Browne et al., 2016). In this study we examined the microbial communities and AMR genes associated with a range of food products at the closest point in the food chain to the consumer. To increase the resolution with which we can investigate the microbiome, here we describe a method (Neutrase-saponin) to deplete plant and animal (host) DNA from food samples, providing greater sequencing depth of the microorganisms present. The method is effective at preserving the DNA of intact bacteria, but damaged bacteria may be lysed using this method. This was evident with the tested mock communities, where the Neutrase-saponin method had minimal effects on the in-house mock community made up of intact bacteria, but a large effect on the Zymo mock community where preservatives were used to inactivate the constituent bacteria. For this reason, it is important to measure the quantities of bacterial DNA through qPCR prior to and following host DNA depletion, to ensure an unbiased representation of the microorganisms present.

The Neutrase-saponin method resulted in a mean 13-fold increase (range: 1.3- to 40-fold increase) in prokaryotic DNA from food samples. This is not as much as the saponin-based host depletion method that was previously applied to human sputum samples to identify pathogens (median: 352-fold increase) (Charalampous et al., 2019). However, the food samples had higher proportions of prokaryotic DNA in untreated samples (0.011–0.45) than the sputum samples (as low as 0.00001), making a larger-fold increase impossible. The post-depletion prokaryotic read proportion should then also be considered when determining the effectiveness of the method. Based on the mean prokaryotic read proportions post-depletion, the Neutrase-saponin method was effective for chicken (0.64), pork (0.74) and leafy green (0.74) samples, whereas it was less effective with salmon (0.50) and prawn (0.19) samples. However, the method used to identify prokaryotic reads relies on a database of known prokaryotes and eukaryotes, resulting in 0.08–0.74 unclassified reads. This was particularly a problem for the seafood samples examined here and could be because seafood are colonised by a large number of bacteria that have not yet been described. This was evidenced by Collins et al. (2021) who investigated the intestinal metagenome of deep-sea fish and found that of the 111 metagenome assembled genomes (MAGs) constructed, only 39 had at least 75% average nucleotide identity to a reference genome. As the databases used to identify these organisms improve, so will the identification capacity available through metagenome sequencing. In addition, aquacultured salmon samples were associated with higher depleted DNA concentrations to wild caught, but qPCR ΔCq and the proportion of reads that were prokaryotic were not affected, making it unclear as to the cause of this association. The application of the Neutrase-saponin method to more food metagenome samples will help identify these traits leading to greater effectiveness of the method.

In the comparison of the Neutrase-saponin method to the commercial Molysis method, the Neutrase-saponin method resulted in higher prokaryotic read proportions in chicken, leafy green, pork and salmon samples, whilst the Molysis kit resulted in higher prokaryotic read proportions for prawn samples. Both methods rely on the same general process: lysis of eukaryotic cells followed by degradation of the extracellular DNA released, but use different agents to achieve these effects. The Neutrase-saponin method uses saponin to dissolve the membranes of eukaryotic cells, whilst the Molysis method uses a chaotropic agent to disrupt eukaryotic proteins. This suggests that saponin is effective at dissolving the membranes of chicken, leafy greens, pork and salmon cells, but struggles with prawn cell membranes, leaving the chaotropic agent used by Molysis as a better lytic agent for prawn cells. Following treatment, the prokaryotic composition of the Neutrase-saponin and Molysis depleted samples differed to the untreated samples. Both methods involve incubation steps that could allow microorganisms to replicate, particularly the 40-min Neutrase step for the Neutrase-saponin method, and eukaryotic lysis steps that could lyse damaged bacteria. One limitation of this study is that we did not apply the Molysis kit to our in-house mock community and determine if it lysed intact bacteria. However, the bacterial composition of the Neutrase-saponin samples was closer to the untreated samples than those of the Molysis kit, indicating that it is better at capturing the prokaryotic population of foods and efficiently sequencing these populations for the majority of food commodities examined here.

The food microbial communities sequenced in this study showed a large amount of variation in the types and quantity of prokaryotic taxa present. Some of the microorganisms detected could be the result of contaminated reagents, which is why blanks were included for each sampling trip, where the same batches of reagents were used. If a microorganism was found in both a food sample and the associated blank, it would be difficult to determine if it was the result of contamination or was genuinely part of the microbial community on that food sample. However, all blanks in this study had undetectable DNA

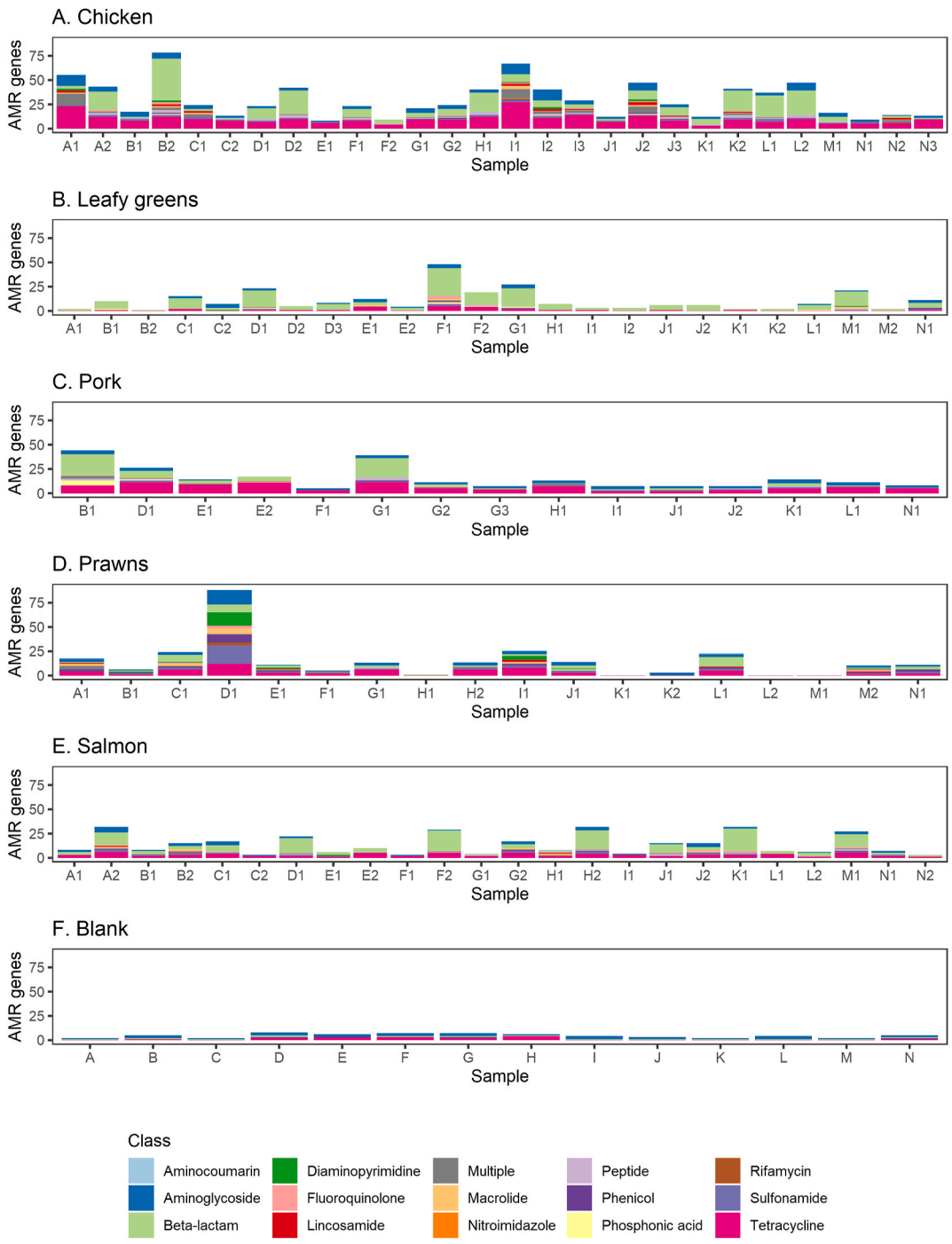

Fig. 6. Numbers of unique AMR genes identified for 109 food metagenome by food commodity (A–E) and the 14 blanks (F), and coloured by the antimicrobial agent class to which the genes confer resistance.

concentrations and had to be spiked with phiX174 RF1 DNA in order to facilitate sequencing, so is unlikely to contribute significantly to the microbial taxa identified on the food.

Pseudomonas was the predominant genus for most of the food samples investigated. These bacteria are ubiquitous in soil, plants and animals, so could have colonised food at multiple stages through the food chain (Crone et al., 2020; Stellato et al., 2017). *Pseudomonas* are also associated with food spoilage, and its quantification using food metagenomics could be used to measure the effectiveness of food processing and preservation techniques (Stellato et al., 2017).

Food metagenomes were shown to cluster by food commodity. For salmon samples, they were also clustered by whether they were obtained from wild-caught or aquacultured salmon. Lorgen-Ritchie et al. (2021) found that the microbiota of salmon was influenced by their environment, possibly explaining the differences in composition between the caught and farmed salmon samples observed in this study. This observation was not seen with aquacultured prawns, but few wild caught prawns were sequenced for comparison. The wider application of this type of approach will provide further insight into the associations of food production and processing methods with the food metagenome.

Reads associated with pathogens and food spoilage organisms were detected in all food metagenomes analysed, which in turn were used to calculate the concentration of the microbes of interest on foods. It is important to note that this does not necessarily represent a food safety

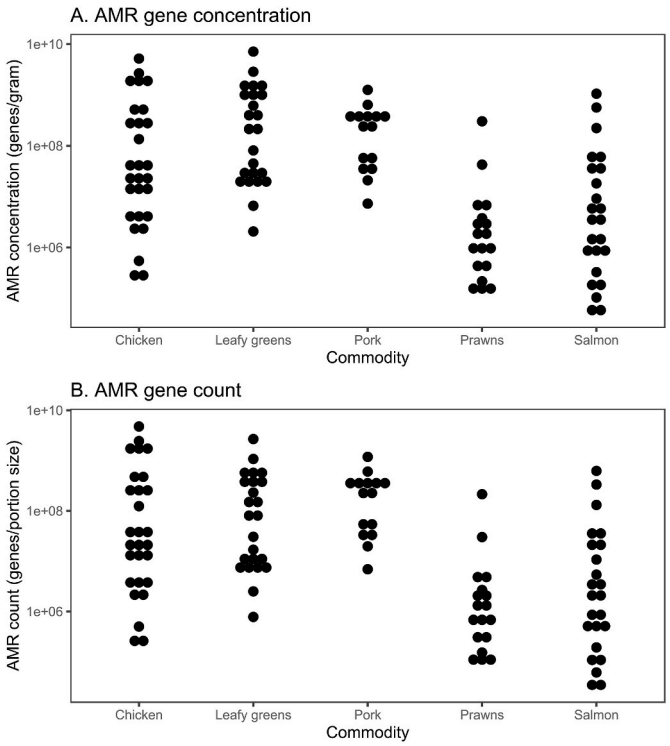

Fig. 7. AMR gene concentration per gram of food (A) and number of AMR genes per portion size (B) for 109 food metagenomes separated by food commodity.

risk; for example, reads associated with *Vibrio*, a marine-associated bacterium were detected on chicken. These unexpected detections are likely the result of analytic algorithms misclassifying reads of closely related bacteria, and as such must be interpreted with caution. Parallel application of the Neutrase-saponin method with pathogen culturing or pathogen-specific qPCR is required to validate the method for this type of analysis. Culturing will also provide validation of the method for the identification of specific genetic elements, such as AMR genes, within specific bacteria.

Antimicrobial resistance is a global problem (IACG, 2019), and food represents one way in which resistant bacteria or AMR genes can be transmitted between the environment, animals, plants and humans. In this study we found a wide variation in the numbers, types and concentration of AMR genes on food. The mean AMR gene concentration was higher in the chicken, pork and leafy green samples than the prawn or salmon samples, indicating that different food commodities are likely to expose us to different quantities of AMR genes. Chicken, pork, salmon and prawns are usually cooked before consumption, likely decreasing the number of AMR genes to which consumers are exposed. The portion sizes varied from 80 g for leafy greens to 200 g for pork, but this variation was small compared to the variation in AMR gene concentrations, resulting in similar distributions in the number of AMR genes per serving size for food commodities compared to AMR gene concentration (10^4-10^{10} AMR genes/gram). The high AMR gene concentration of leafy greens may be because they contain a larger surface area compared to the other food commodities, providing more area for bacteria to colonise. Consumers do not usually cook the types of leafy greens investigated in this study. The evaluation of other ready-to-eat foods, in addition to the leafy greens examined here, will provide a different perspective on the risks of food as a source of AMR.

The AMR concentrations calculated for food (mean 3.90×10^8 genes/gram) is lower than those previously calculated from pig faeces (2.48×10^9 genes/gram) (Pollock et al., 2020) and dewatered sludge from municipal wastewater treatment plants (2.44×10^9 genes/gram) (Gao et al., 2012). However, these concentrations are not directly comparable. People are exposed to food more frequently than these other sources of AMR genes, but in many cases the foods will be cooked before consumption and therefore the AMR gene concentration will likely decrease dramatically. In addition, these other sources were calculated using AMR gene-specific qPCR and different AMR gene targets. The food metagenome selection method may have also selected for food metagenomes with more bacteria and therefore higher AMR gene concentrations. Standardised methods are required for calculating the AMR gene concentrations from different sources so they can be compared with each other.

5. Conclusions

In this study we adapted and extended a host DNA depletion method for food that allows efficient sequencing of food sample metagenomes, increasing the proportion of prokaryotic reads sequenced 13-fold (mean) compared to untreated samples. The method worked best on chicken, pork and leafy green samples which had high mean prokaryotic read proportions post-host DNA depletion, some salmon samples, but few prawn samples. We demonstrated its applicability for identifying the different bacteria and AMR genes present and calculating the AMR concentration of food. Different food commodities were shown to have different AMR gene concentrations and metagenome compositions, and for salmon the metagenomes were influenced by how they were produced/harvested. The method described in this study was useful at identifying the predominant bacteria on food and their associated AMR genes, but further work is required to use metagenomics to identify specific food pathogens.

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Declaration of competing interest

Professor Justin O'Grady and Dr. Gemma Kay are now employed by Oxford Nanopore Technologies plc and Professor O'Grady holds shares and share options in the company; the work presented in this study was performed before their change in affiliation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104162.

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