Assessing the impact of heat treatment on antimicrobial resistance genes and their potential uptake by other 'live' bacteria

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1 Lay Summary

Food plays an important role in the development and spread of antimicrobial resistance (AMR) and is one of many routes by which consumers can be exposed to AMR bacteria. The ingestion of pathogenic AMR bacteria via food may result in human illness which may be difficult to treat with antibiotics. Non-pathogenic AMR bacteria also contribute to the reservoir of AMR within our food chain which may lead to the onward transmission of antimicrobial resistance genes (ARGs) to pathogenic bacteria. The Food Standards Agency (FSA) advises that thorough cooking combined with good hygiene when handling raw meats and foods will help to mitigate consumers' risk of exposure to AMR bacteria and ARGs in food. Whilst thoroughly cooking will 'kill' AMR bacteria, it is unclear whether this will inactivate their ARGs and prevent resistance genes being passed onto other 'live' bacteria. The overall aim of this project was to carry out a broad critical review of the available scientific literature on the impact of heat treatment on ARGs that may be present in heat-killed foodborne bacteria and their potential uptake by surrounding viable bacterial communities, including in the human gut and foods.

Two scientific literature databases (Web of Science, and PubMed) were searched for relevant publications, supplemented by focused Google searches, searching within relevant publications, and through contact with authors. Literature relating to AMR and heat treatments was rare, and while 17 publications were found that had evidence on the heat resistance of AMR bacteria, only four publications were found that had specifically studied the impact of heat treatments on ARGs. Due to the small number of publications identified and different laboratory methodologies used in these studies no statistical analysis was possible, a narrative approach was taken to their review and to the review of supplementary materials.

The literature review showed that there is evidence that AMR bacteria are likely to be no more heat-resistant than non-AMR bacteria and that there is therefore evidence that heat treatments sufficient to kill non-AMR bacteria (such as 70°C for at least 2 min, or the equivalent) will be equally effective in killing AMR bacteria. The

evidence as to whether viable ARGs may persist after such heat treatments is sparse, and also whether these genes can be transferred to other bacteria.

Whilst the published evidence is limited, the studies identified do provide some evidence that ARGs may persist in cooked foods following heat treatments that are effective in killing AMR bacteria. If ARGs are not sufficiently damaged during heat treatment, it is possible that genes could be subsequently taken up by other bacteria. Although the occurrence of this transfer is probably rare. None of the published studies demonstrated whether the ARGs from heat-treated AMR bacteria could be 'taken up' by other 'live' bacteria in the human gut. There is currently not enough evidence to determine if there is a risk to human health and we would recommend that further focused practical research is undertaken to produce clear evidence that can be used to fully assess whether there is a risk of exposure to ARGs in heat-treated/cooked food.

2 Executive summary

Antimicrobial resistance (AMR) is a complex issue driven by a variety of interconnected factors enabling microorganisms to withstand the killing or microstatic effects of antimicrobial treatments, such as antibiotics, antifungals, disinfectants, preservatives. Microorganisms may be inherently resistant to such treatments or can change and adapt to overcome the effects of such treatments. Microorganisms can acquire antimicrobial resistance genes (ARGs) because of mutation or from other microorganisms through a range of mechanisms. The widespread use of antimicrobial treatments is known to result in selection for AMR in microorganisms. AMR and ARGs are a major public health issue worldwide and it is estimated that unless action is taken now to tackle AMR the global impact of AMR could be 10 million deaths annually by 2050 and cost up to US $100 trillion in cumulative lost economic output (O'Neill Report, 2014).

It is recognised that anthropogenic, commensal, and environmental microorganisms all contribute to the reservoir of ARGs, collectively forming the antimicrobial resistome (Wright, 2007). Relatively little is known regarding the role of heat-treated/cooked food in disseminating AMR, and whether heating/cooking is sufficient to inactivate ARGs to the extent that resistance is not passed onto other 'live' bacteria.

This study was undertaken to critically review the available scientific literature for assessing the impact of heat treatment of food on ARGs, and the potential uptake of such ARGs by surrounding viable bacterial communities resident in other foods and the human gut.

For the purpose of this review, heat treatments were regarded as any thermal processes that are undertaken during the processing or prior to consumption of any foods. The review focused particularly, but not exclusively, on what scientific evidence exists that provides an understanding on whether cooking (heating) food to eliminate bacterial contamination can also induce sufficient damage to ARGs to

prevent their uptake by surrounding viable bacteria present in other settings, including other foods and the human gut.

The review question was defined as:

> "Do different heat treatments applied to eliminate bacterial contamination in foods also induce sufficient damage to ARGs to prevent or inhibit their uptake by surrounding viable bacteria present in other settings, including the human gut and other foods?"

Systemic searching of two literature databases (Web of Science, and PubMed) was undertaken, supplemented by additional records identified through other sources. A total of 2681 of publications were identified between 1990 and May 2021, which were reduced to 247 after screening the titles and abstracts. This total was further reduced to 53, from which some data were extracted after appraising the full publications. This clearly indicated that literature relating to AMR bacteria and ARGs and heat treatments was sparse.

Of these 53 publications identified that were considered eligible for some data extraction, only four were found that had studied the impact of heat treatments on ARGs. The majority of publications identified related to the relative heat resistance of various AMR bacteria in comparison to non-AMR strains and serotypes /serovars.

Nine publications were reviews with some mention of the impact of heat on AMR bacteria, while 17 had evidence on the relative heat resistance of AMR bacteria in comparison to non-AMR bacteria. These publications provide evidence that AMR bacteria are likely to be no more heat-resistant than non-AMR bacteria. There is therefore evidence that heat treatments sufficient to kill non-AMR bacteria (such as 70°C for at least 2 min, or the equivalent) will be equally effective in killing AMR bacteria.

Most of these publications have not considered whether ARGs may persist after such heat treatments, and whether these genes could be transferred to other bacteria.

Only four publications were identified that provide some evidence on the fate of ARGs after heat treatments. Due to the small number of publications identified and different laboratory methodologies used in the studies no statistical analysis was possible. Three of the four studies provided evidence that ARGs can at least be identified after heat treatments that are effective at inactivating AMR bacteria, but there is no certainty that such ARGs are intact and functional.

Of the four studies identified, one (Koncan *et al*., 2007) used *in vitro* experiments to mimic cooking processes. Another *in vitro* study (Taher *et al*., 2020a) mimicked commercial milk pasteurisation, whilst the third (Le Devendec *et al*., 2018) was not designed to mimic any particular heat treatment but did use strains originating from animal sources and temperatures and times similar to thermal processes used to treat and cook food. A further study on autoclaving (Masters *et al*., 1998) was considered relevant, but was not applied to food.

The *in vitro* mimic of cooking processes study (Koncan *et al*., 2007) detected the presence of ARGs after cooking but did not demonstrate that these genes were transferable to other bacteria. The other three studies did demonstrate that plasmid-encoded ARGs could be transferred to other bacteria following heat treatments under laboratory conditions.

The ARG considered by Koncan *et al*. (2007) was *aac(6')-Ie-aph(2'')-Ia*, while Taher *et al*. (2020a) considered *blaZ*, *mecC*, *tetK*, and Le Devendec *et al*. (2018) considered $bla_{\text{CTX-M-1}}$, $bla_{\text{CMY-2}}$, *tet*A, *str*A. Masters *et al*. (1998) did not give any details of the gene considered.

These studies did not establish how likely was the occurrence of such transfer in the field. One of the studies (Le Devendec *et al*., 2018) theorised that natural transfer is probably rare.

None of the studies demonstrated whether ARGs from heat-treated AMR bacteria could be taken up by other live bacteria in the human gut after ingestion.

In conclusion, only a small number of studies were identified on the persistence of ARGs in heat-treated foods and their possible uptake by surrounding viable bacteria present in other settings, such as the human gut and other foods. Because of differences in conditions, these studies were not directly comparable.

While the literature suggests that adequate heat treatment / cooking (e.g., cooking until the middle of the food commodity reaches 70°C for at least 2 min, or the equivalent) should be effective in 'killing' AMR bacteria in food, there is very little evidence if intact and functional ARGs are released from AMR bacteria following such heat treatments. Similarly, there does not appear to be any convincing data for the ready transfer of ARGs to the commensal bacteria of the mammalian gastrointestinal tract following cooking.

Evidence to determine if there is a risk of transfer is sparse. We would therefore recommend further focused practical research be undertaken to provide evidence for a full assessment of risk in relation to transfer of ARGs from heat-treated foods to bacteria in other matrices.

3 Glossary and abbreviations

Term/Abbreviation	Definition/Description
Amplification	In the context of this report, amplification tests produce many copies from a target sequence of DNA (amplification). This enables the detection of specific fragments of DNA, for example, ARGs, by incorporating fluorescent labels during amplification or subsequent electrophoresis.
Antibiotic	Antibiotics are a type of antimicrobial. An antibiotic is a drug used to treat bacterial infections in both humans and animals. They have no effect on viral or fungal infections. Examples of antibiotics include penicillin, tetracyclines, methicillin, and colistin.
Antimicrobial	Any substance that kills or stops the growth of microorganism, such as antibiotics, antifungals, disinfectants, and preservatives.
AMR	Antimicrobial resistance. For the interpretation of AMR in this study, the WHO definition was applied (WHO, 2018a): “Antimicrobial resistance is resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it. Resistant microorganisms (including bacteria, fungi, viruses and parasites) are able to withstand attack by antimicrobial drugs, such as antibacterial drugs (e.g., antibiotics), antifungals, antivirals, and antimalarials, so that standard treatments become ineffective and infections persist, increasing the risk of spread to others.”
ARG(s)	Antimicrobial resistance gene. An ARG is a gene implicated in or associated with conferring resistance to one or more antimicrobial. The resistance may

Term/Abbreviation	Definition/Description
	result from the presence or absence of a gene or specific mutations acquired spontaneously or through evolution over time. ARGs confer resistance, however clinical treatment with higher doses of the microbial may still be effective.
Bacteriophage	Often shorted to phage, a bacteriophage is a virus that parasitises a bacterium by infecting it and reproducing inside it. Phages are capable of packaging part of their host's genetic material (including ARGs) either by reproducing within the host cell before lysing the cell (lytic) or through incorporation into the host cell genome (lysogenic). Phages cannot infect human cells.
Conjugation	A mechanism of HGT. A process requiring cell-to-cell contact *via* cell surface pili or adhesins, through which DNA is transferred from the donor cell to the recipient cell.
CCP(s)	Critical Control Point(s). A CCP is a step in food handling where controls can be applied to prevent or reduce any food safety hazard.
CFU(s)	Colony Forming Unit(s). A unit used to estimate the number of viable, cells capable of growing on the growth media used, microbial cells in a sample. Colonies may arise from one cell or a group of cells. May be expressed as $\log_{10}$ per gram (g) or milli-litre (ml) of sample.
CIAs	Critically Important Antimicrobials. There are some differences in the categorisation of CIAs between different organisations. The WHO (WHO, 2018b) categorises CIAs as meeting two criteria:

Term/Abbreviation	Definition/Description
	Criterion 1 (C1): The antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people. Criterion 2 (C2): The antimicrobial class is used to treat infections in people caused by either: (1) bacteria that may be transmitted to humans from non-human sources, or (2) bacteria that may acquire resistance genes from non-human sources.
Commensal	An organism that uses food supplied in the internal or external environment of the host without establishing a close association with the host, for instance by feeding on its tissues.
Dielectric heating	A form of heating in which an electrically insulating material is heated by being subjected to an alternating electromagnetic field, such as in a microwave oven.
DNA	Deoxyribonucleic acid. Deoxyribonucleic acid is a molecule composed of two polynucleotide chains that coil around each other to form a double helix carrying genetic instructions for the development, functioning, growth and reproduction of all known organisms and many viruses.
DSC	Differential Scanning Calorimetry. DSC is a thermo-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. DSC can be used to measure a number of characteristic properties of a sample.

Term/Abbreviation	Definition/Description
D-value	The time required to inactivate or reduce 90% of the initial population of the microorganism, for instance, from 10^7 to 10^6 at a given temperature.
EFSA	European Food Safety Authority.
Electroporation	The application of an electric field to introduce DNA into cells by inducing temporary pores in the cell membrane.
ESKAPE	An acronym comprising the scientific names of six highly virulent and AMR pathogens including: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.
ESBL(s)	Extended spectrum beta-lactamase. ESBLs are enzymes produced by bacteria such as *Escherichia coli* and *Klebsiella.* ESBLs mediate resistance to cephalosporins.
FSA	UK Food Standards Agency.
GFP	Green Fluorescent Protein. The GFP from the jellyfish (*Aequorea Victoria*) enables gene expression in individual cells to be examined non-destructively and in real time by using fluorescence microscopy or flow cytometry.
GTA(s)	Gene Transfer Agents. GTAs are host-cell produced particles that resemble bacteriophage structures and are capable of packaging and transferring part of their host's genetic material (including ARGs) to other cells.
HGT	Horizontal Gene Transfer

Term/Abbreviation	Definition/Description
	Transfer of genetic material (including ARGs), among different bacteria and species, other than by the transmission of DNA from parent to daughter cell. There are a number of mechanisms through which HGT can occur.
Integron	A type of MGE with the ability to capture and disseminate genes (including ARGs). These genes are located on gene cassettes (a term that is changing to integron cassette). Though an integron does not necessarily include any gene cassettes. Integrons can be found in plasmids, chromosomes, and transposons.
Lysis	The breaking down of the membrane of a cell compromising its integrity and the release of its contents.
MDR	Multi-Drug Resistance. Resistance to three or classes of antimicrobial.
MIC	Minimum Inhibitory Concentration. The lowest concentration of an antimicrobial that prevents visible growth of a bacteria.
Microorganisms (microbes)	Organisms that include bacteria, viruses, fungi, and parasites.
MGE(s)	Mobile Genetic Element. MGEs, also known as transposable elements (Tes), are fragments/sequences of DNA that can move around. They can encode a variety of virulence or resistance determinants (such as ARGs) that can change places on a chromosome, and can be transferred between chromosomes, between bacteria, or even between species. Types of MGEs include plasmids, integron gene cassettes, and transposable elements.

Term/Abbreviation	Definition/Description
MRSA	Methicillin-Resistant *Staphylococcus aureus.*
MV	Membrane Vesicle. MVs are proteo-liposomal nanoparticles produced by both Gram-negative and Gram-positive bacteria generally in response to environmental stresses. They have diverse functions, including the transport of virulence factors, DNA transfer (including ARGs), interception of bacteriophages, antibiotics and eukaryotic host defence factors, cell detoxification and bacterial communication.
NAP	National Action Plan UK Government 5-year (2019 to 2024) AMR National Action Plan (NAP) to tackling antimicrobial resistance.
Plasmid	A type of MGE in a cell that can replicate independently of the chromosomes, typically a small circular DNA strand in the cytoplasm of a bacterium. Plasmids can carry and transfer ARGs from the host to other cells, via other MGEs (integron gene cassettes and transposable elements).
PCR	Polymerase Chain Reaction. A technique used to “amplify” small segments of DNA by cycling targeted by complementary sequences of primers (oligonucleotides).
qPCR	Quantitative Polymerase Chain Reaction. The main difference between PCR and qPCR is that PCR is a qualitative technique whereas qPCR is a quantitative technique.
Radio frequency (RF) heating	A dielectric electromagnetic form of heating, similar to microwave heated, but using a different electromagnetic frequency.

Term/Abbreviation	Definition/Description
RTE	Ready-to-eat. Used to describe foods that are ready for consumption without prior preparation or cooking.
Spp.	Species.
STEC	Shiga Toxin-producing *Escherichia coli.*
Transduction	A mechanism of HGT. This is a bacteriophage (and related particles, such as GTAs) mediated transfer process. The transfer of short fragments of DNA (such as ARGs) from one cell into another via a bacteriophage (or related particle).
Transformation	A mechanism of HGT. The uptake of short fragments of naked DNA (such as ARGs) by naturally transformable bacteria.
Transposon	A type of MGE. A chromosomal segment that can undergo transposition, especially a segment of bacterial DNA that can be transferred as a whole between chromosomal, phage, and plasmid DNA in the absence of a complementary sequence in the host DNA.
VBNC	Viable But Non-Culturable. VBNC cells are defined as live bacteria that do not either grow or divide but remain metabolically active. Such bacteria cannot be cultivated on conventional media (they do not form colonies on solid media, they do not change broth appearance), but their existence can be proved using other methods.
WHO	World Health Organisation (of the United Nations).

4 Introduction

The World Health Organization (WHO) definition of AMR is “resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it. Resistant microorganisms (including bacteria, fungi, viruses and parasites) are able to withstand attack by antimicrobial drugs, such as antibacterial drugs (e.g., antibiotics), antifungals, antivirals, and antimalarials, so that standard treatments become ineffective and infections persist, increasing the risk of spread to others” (WHO, 2018a).

AMR microorganisms and ARGs are a major public health issue worldwide. It is estimated that unless action is taken now to tackle AMR the global impact of AMR could be 10 million deaths annually by 2050 and cost up to US $100 trillion in cumulative lost economic output (O’Neill Report, 2014).

Addressing the public health threat posed by AMR is a national strategic priority for the UK and led to the Government publishing both a 20-year vision of AMR and a 5-year (2019 to 2024) AMR National Action Plan (NAP) which sets out actions to slow the development and spread of AMR with a focus on antimicrobials. The NAP used an integrated ‘One-Health’ approach which spanned people, animals, agriculture, and the environment and calls for activities to “identify and assess the sources, pathways, and exposure risks” of AMR. The FSA have and are continuing to contribute to delivery of the NAP through furthering our understanding of the role of the food chain and AMR, conserving the effectiveness of current treatments through the adoption of good hygiene practices, and encouraging the food industry to reduce usage of antimicrobials where possible. ARGs that result in resistance to critically important antimicrobials are of particular concern to the FSA.

It is recognised that anthropogenic, commensal, and environmental microorganisms all contribute to the reservoir of ARGs collectively forming the antimicrobial resistome (Wright, 2007). AMR may be intrinsic or acquired. Intrinsic microorganisms are inherently resistant to certain antimicrobials (Verraes *et al*.,

2013). AMR can also be acquired either because of mutation (e.g., genomic point mutations) or the acquisition of resistance genes by horizontal gene transfer (Verraes *et al.*, 2013; Munita & Arias, 2016). Microorganisms may be resistant to just one antimicrobial or to many (multi-resistant or multi-drug resistant; MDR), with cross resistance depending on which ARGs and other mechanisms of resistance are present (such as, enzymatic, permeability barriers, and efflux pumps). This can make infections caused by these organisms difficult to treat and cause illness to persist, with recognised extra costs and increased morbidity and mortality (Likotrafiti *et al*., 2018).

The transmission of AMR microorganisms and ARGs to food is complex. Food can be contaminated with AMR bacteria and/or ARGs in several ways (Verraes *et al*., 2013; Food Standards Agency, 2016) including (but not exclusively):

1. Through contamination with AMR bacteria in the environment.
2. Through the presence of AMR bacteria on food treated by antimicrobials during agricultural production.
3. The possible presence of ARGs in bacteria that are intentionally added during the processing of food (starter cultures, probiotics, bio-conserving microorganisms and bacteriophages).
4. Through cross-contamination with AMR bacteria and ARGs during food processing.

ARGs in AMR bacteria can be transferred to other surrounding bacteria through Horizontal Gene Transfer (HGT), as will be discussed later. Thus, commensal non-pathogenic AMR bacteria can act a reservoir for ARGs and transfer resistance to non-resistant human pathogenic bacteria (Bengtsson-Palme, 2017). HGT is enhanced by mobile genetic elements (MGEs), such as plasmids, integrons, and transposons, that facilitate the movement, transfer, and integration of genes between cells (Bennett, 2008). ARGs are not always associated with cultivable ‘live’ bacteria (**Error! Reference source not found.**). Non-cellular ARGs, which also cover genes

encapsulated in membrane vesicles (MVs) or bacteriophages, can persist after disinfection, and can transfer to recipient bacteria in the absence of a live donor bacteria (Woegerbauer *et al.*, 2020). The frequency of HGT largely depends on the properties of the MGEs, MVs, or bacteriophages, the characteristics of the donor and recipient populations, and the environment (Verraes *et al*., 2013; Rossi *et al*., 2014).

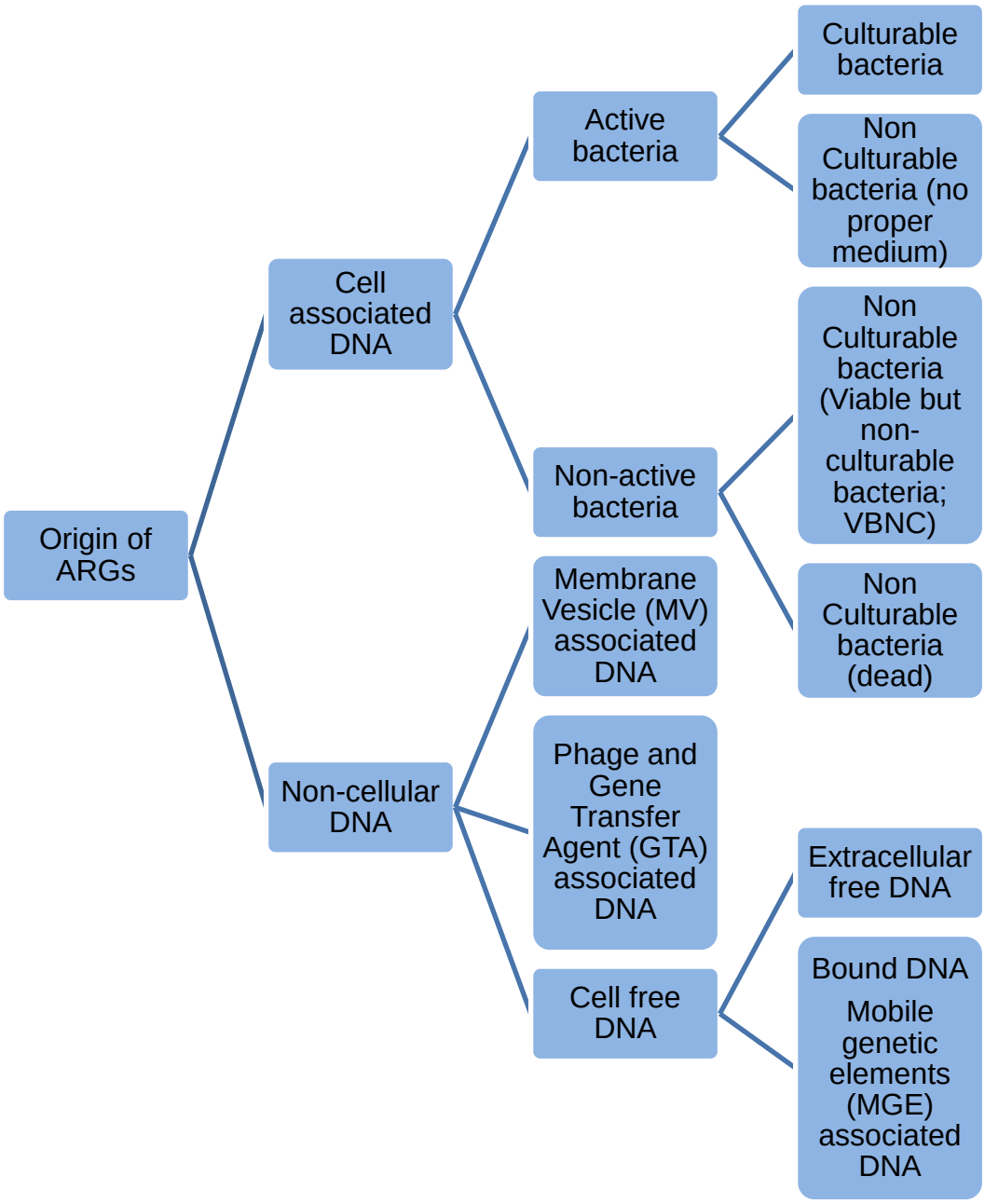


Figure 1. Forms and origins of ARGs quantified by molecular biology approaches (adapted from Woegerbauer *et al*., 2020)

There are three main canonical mechanisms of HGT through which this can occur: (1) conjugation, (2) transformation, or (3) transduction. Though, as noted by Hall *et al*. (2017) and Verraes *et al.* (2020), amongst others, other less well recognized mechanisms of DNA transfer may occur. These processes are described in detail in reviews such as that by Verraes *et al*. (2013). Conjugation occurs between live bacterial cells (Verraes *et al*., 2013) and will not occur if cells are killed by heat, thus this mechanism is not relevant in the context of this review and will not be discussed.

Dead cells cannot use cell-to-cell mechanisms to pass ARGs to other bacteria by conjugation (Verraes *et al*., 2013), but as soon as DNA fragments have been released after death and lysis, ARGs as extracellular free DNA or facilitated by MGEs may, theoretically, be transferred by transformation (McMahon *et al*., 2007; Verraes *et al*., 2013; Le Devendec *et al*., 2018; Pérez-Rodríguez & Taban, 2019). It is reported by Lorenz & Wackernagel (1994) that transformation was first demonstrated by Griffith in 1928, who observed that mice were killed when infected with a mixture of heat-killed pathogenic S-form (“smooth”) and living non-pathogenic R-form (“rough”) *Streptococcus pneumoniae* cells. Pneumococci isolated from the cadavers revealed the S-form colony type. Thus, Griffith concluded that the R-form had undergone transformation by the dead S-form. According to Verraes *et al*. (2013), any bacterial chromosomal or extra-chromosomal DNA can be theoretically transferred by transformation. To be stabilized in the recipient cell, it is reported that the transformed DNA must be available as a plasmid or must recombine with homologous regions in the resident chromosome (Verraes *et al*., 2013). The overall process of natural genetic transformation can be broken down into six major steps, as described by Lorenz & Wackernagel (1994) and Huddleston (2014): (1) DNA is released from donor cells; (2) DNA is dispersed; (3) DNA persists in the environment; (4) recipient cells become competent for DNA uptake; (5) competent cells interact with and take up the DNA, incorporating it into the genome either through homologous or illegitimate recombination; and finally (6) genes encoded on the donor DNA are expressed in the recipient cell.

Natural transformation is known to occur in more than 60 bacterial species, and probably far more (Rossi *et al*., 2014). The general consensus in the literature is that the process of transformation occurs at low frequency and is subject to a large number of requirements mostly observed in very controlled laboratory conditions (Lorenz & Wackernagel, 1994; Verraes *et al*., 2013; von Wintersdorff *et al*., 2016). Few investigations have expressly analysed exogenous DNA uptake by bacteria in food (Rossi *et al*., 2014).

There is evidence that DNA stability is an inverse function of DNA length (Kharazmi *et al*., 2003). Kharazmi *et al*. (2003) carried out an *in vitro* mimic of production processes for soya milk, tofu, corn masa, and cooked potato on the degradation of plant DNA in which genetically modified (GM) potatoes with the AMR *nptII* gene, which encodes kanamycin resistance, as a marker was used. Cooking of potatoes (80°C for 1 h) resulted in a strong degradation of the *nptII* gene.

Studies to transform, by electroporation, *Bacillus subtilis* LTH 5466, using *nptII* gene fragments similar to those measured after cooking of the potatoes (but not using the heat-treated fragments) showed that smaller fragments resulted in lower transformation frequencies, while plasmids containing the fragments resulted in greater transformation frequencies. This study provides evidence that although heat treatments will degrade lysed ARGs, extracellular free DNA fragments may still be of sufficient length to be transformed by other bacteria.

As already mentioned, there is third mechanism of HGT, transduction, which is a transfer process mediated by bacteriophage and similar related particles, called Gene Transfer Agents (GTAs) (Verraes *et al*., 2013). Often shorted to phage, a bacteriophage is a virus that parasitises a bacterium. Phages are capable of packaging part of their host's genetic material (including ARGs) either by reproducing within the host cell before lysing the cell (lytic) or through incorporation into the host cell genome (lysogenic). GTAs are host-cell produced particles that resemble bacteriophage structures. The mechanism of how bacteriophages/GTAs promote the transfer of ARGs is described, amongst others, by Colavecchio *et al*.

(2017) and Jebri *et al*. (2020). The overall process of transduction can be broken down into eight major steps, as described by Bennett *et al*. (2004): (1) phage particle attaches to cell via specific surface receptor; (2) phage genomic DNA injected into bacterial cell; (3) phage DNA replicated and host genome fragmented; (4) new phage particles assembled, including transducing particles containing host cell DNA fragments in place of phage genome; (5) host cell lysed and new phage particles released; (6) transducing particle attaches to new bacterial host; (7) bacterial DNA fragment injected into new host cell; and finally (8) injected DNA sequences rescued by homologous recombination.

There is a realisation that phages/GTAs may be significant vectors in the transmission of ARGs through the transduction mechanism (Colavecchio *et al*., 2017; Jebri *et al*., 2020). Though, as highlighted by Colavecchio *et al*. (2017), there is considerable debate on their importance. Although phages have been believed to be host-specific there is increasing evidence that phages can have broader host ranges (Jebri *et al*., 2020). As noted by Colavecchio *et al*. (2017) and Jebri *et al*. (2020), the occurrence of phages/GTAs harbouring ARGs has been reported in different food and animal matrices.

Notably Gómez-Gómez *et al*. (2019) detected six ARGs (*bla*$_{TEM}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$, and *sul1*) in DNA extracted from phage particles from ready-to-eat (RTE) samples of ham and *sul1* in mortadella purchased from a local retailer. Only five samples of each meat were analysed. *sul1* (which encodes sulfonamide resistance) was only detected in one sample of mortadella, though *bla*$_{CTX-M-1}$ (which encodes resistance to extended-spectrum cephalosporin) was detected in four out of the five samples of ham. Though the meat was cooked it must be stressed that the authors did not associate this presence with survival following thermal processing, but rather post-processing cross-contamination. Nevertheless, the survival of phage containing ARGs following heat treatment cannot be ruled out.

As noted by Sommer *et al*. (2019) there is clear evidence that phages can show a degree of thermal stability and in some cases may survive heat treatments that are

sufficient to kill target bacteria. The survival of thermal-stable lactococcal phages in pasteurised milk is a long-recognised problem (Murphy *et al*., 2013). Another example of a heat-tolerant phage has been reported by Lee *et al*. (2016), who reported that while HY01 (a phage infecting *E*. *coli* O157:H7 and *Shigella flexneri*) was inactivated when incubated at temperatures above 70°C, it did show stability (though reduced in comparison with 50°C) when incubated at 65°C for 12 hours.

Ahmadi *et al*. (2017) reported that while exposure to 71°C for 30 s reduced *Listeria* phages P100 below the limit of detection, and A511 from 10^8 to 10^5 PFU mL^{-1}, the P100 phage partly reconstituted to 10^3 PFU mL^{-1}after cooling.

Jebri *et al*. (2020) theorised that phages surviving processing methods could be “a reservoir of ARGs that might be transduced to host bacteria occurring in [the] human gut once [RTE] food is consumed.”

A further route of transfer that is receiving attention is membrane vesicle (MV) mediated HGT (Woegerbauer *et al*., 2020). MVs are proteo-liposomal nanoparticles produced by both Gram-negative and Gram-positive bacteria generally in response to environmental stresses (Caruana & Walper, 2020). They have diverse functions, including the transport of virulence factors, DNA transfer (including ARGs), interception of bacteriophages, antibiotics and eukaryotic host defence factors, cell detoxification and bacterial communication (Toyofuku *et al*., 2019).

MVs were first found to originate in the outer membrane of Gram-negative bacteria and therefore often called outer-membrane vesicles (OMVs). Recent work has shown that different types of MVs also exist and hence the inclusive term MV is preferred (Toyofuku *et al*., 2019; Woegerbauer *et al*., 2020), and used in this report.

Studies have found the presence of DNA of chromosomal, plasmid, and phage origin incorporated into MVs (Uddin *et al*., 2020). MVs have been found to transfer ARGs between bacterial species (Kulkarni *et al*., 2015; Wagner *et al*., 2018; Uddin *et al*.,

2020). While MV-mediated HGT has been demonstrated according to Woegerbauer *et al*. (2020), its occurrence in the environment has been largely unexplored.

Woegerbauer *et al*. (2020) observed that "(i) MVs are produced by numerous bacteria, (ii) they can transport any kind of DNA (including ARGs), (iii) with a membrane fusion-based mechanisms for MV-assisted DNA transformation, no taxonomic limitation are expected regarding the DNA uptake process, (iv) maintaining the acquired DNA in bacterial cell is likely to meet the same limitations as for natural transformation, and (v) the budding process is favoured in antibiotic-stress environments".

Reviews of ARG exchange in the gut have been carried out (such as Schjørring & Krogfelt, 2011; Broaders *et al*., 2013; Huddleston, 2014; Lerner *et al*., 2017; McInnes *et al*., 2020), but whether ARGs from heat-treated foods can be a source of transfer has not been discussed.

It is fully accepted that for heat treatments such as sterilization, ultra-high temperature (UHT) treatment, and (full) pasteurization under well-defined time/temperature combinations will eradicate /kill vegetative bacterial cells, including those of AMR bacteria. Industrial, food service, domestic or institutional cooking is normally sufficient to eliminate the effect of pathogens from food, but it remains unclear whether thorough cooking destroys all components of AMR bacteria or ARGs. To what extent 'complete' ARG DNA or even fragments of bacterial DNA that survive from chromosomal or plasmid DNA may be capable of transfer to other microbiota in the human gut and be incorporated to become a functional source of a novel bacterial genome is unknown. There is some literature (Aubry-Damon *et al*., 2004; Hart *et al*., 2006; Ramchandani *et al*., 2005) that lends weight to this hypothesis. This is one area that appears to have escaped scrutiny. This review attempts to assess the impact of heat treatments on ARGs that may be present in heat-killed foodborne bacteria and their potential uptake by surrounding viable bacteria in the other foods and the human gut.

4.1 Review question and focus of the review

The review question was:

> "Do different heat treatments applied to eliminate bacterial contamination in foods also induce sufficient damage to ARGs to prevent or inhibit their uptake by surrounding viable bacteria present in other settings, including the human gut and other foods?"

The review was structured and aimed at addressing the following key elements:

- To identify and critically review what scientific evidence is available to clarify whether heat treatments of food at a level which eliminates bacterial contamination induces sufficient damage to ARGs to prevent their uptake by surrounding viable bacteria present in other settings including the human gut and other foods.
- To include not only clinically important ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) and other pathogenic organisms with AMR/industrial relevance (such as *Enterobacter* species, *Enterococcus faecalis*, *Listeria* species, and *Salmonella* species), but also non-pathogenic AMR microbiota (such as commensal *Escherichia coli*).

If evidence was found to the primary review question in the identified literature, then the further questions to be answered were:

- Is there compelling evidence to show that heat completely destroys DNA (particularly ARGs either as extracellular free DNA or within AMR bacteria)? What evidence is there of thermal degradation of DNA in various contexts.
- Can heat-treated or damaged DNA (ARGs being the focus) originating from dead bacteria be taken up by live bacteria? General information relating to whether heat treatment of DNA and particularly ARGs (including those on MGEs – e.g., plasmids/transposons, integrons) affects its ability to be taken up by viable bacteria will be obtained, ideally within a food context.

Information relating to transformation frequencies of heat-treated/damaged DNA versus intact DNA will also be considered.

- What is the impact of different heat treatments on ARG uptake by viable bacteria? Literature will be considered on the effects of bacterial DNA (with a focus on ARGs) exposure to different heat treatments and subsequent effects on uptake of this DNA by viable bacterial cells. MGEs will also be considered. If the literature does not contain such detailed information (e.g., time/temperature combinations), any information obtained will be contextualised in terms of food, e.g., cooking conditions, where possible. Milder heat treatments such as low temperature sous vide, flash frying, slow cooker, and rare or light cooking, will also be considered.
- Is there any evidence of uptake of heat damaged DNA (particularly ARGs) by pathogenic and non-pathogenic bacteria? If this evidence is not directly related to the food/gut environment it will be contextualised where possible. The review will focus on the resistance genes relating to the Critically Important Antimicrobials (CIAs), where possible.
- If the literature provides some indication that heat-damaged DNA (particularly ARGs) can be taken up by naturally or induced competent viable bacteria in the laboratory, then is there any information to suggest that this can also occur in complex environments (e.g., in the presence of large, diverse microbial communities such as the human gut, or complex media such as food (including combined foods) or on food contact materials or biofilms)?
- Is there any evidence to suggest that the behaviour of chromosomal DNA and plasmid DNA in response to heat differs? ARGs of most concern are likely to be the transferable, plasmid-encoded genes.
- Is there any evidence on the role of proteins in the transfer of ARGs from AMR bacteria subjected to heat treatments? Proteins are more vulnerable to heat than DNA but have multiple functions and most of the transfer functions will not occur if they have degraded, such as due to heat damage.
- Is there any evidence on the role of MVs in the survival and transfer of ARGs from AMR bacteria subjected to heat treatments? There is some evidence that they could be an important route of survival of plasmids and/or

chromosomal DNA either naturally or following stress conditions (exposure to antimicrobials).

5 Materials and methods

A systematic review approach was taken to the literature search. Because of the paucity of specific published studies on this topic a narrative critical review approach was taken to the review of the publications identified.

The review question was:

> "Do different heat treatments applied to eliminate bacterial contamination in foods also induce sufficient damage to ARGs to prevent or inhibit their uptake by surrounding viable bacteria present in other settings, including the human gut and other foods?"

The key elements of the question (PIO): Population (P), Intervention (I), and Outcome (O), were:

- The **population** of interest include pathogenic and non-pathogenic AMR bacteria (such as *Acinetobacter baumannii*, *Campylobacter*, *Enterobacter*, *Enterococcus faecium* and *faecalis*, commensal *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria*, *Salmonella*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) and specifically their ARGs.
- Any heat treatment **interventions** applied to foods are considered relevant, such as pasteurisation, sterilisation, cooking treatments (e.g., hot air (oven), steam, hot water (boiling, blanching), hot fat or oil (shallow or deep frying), grilling, radiant, dielectric (microwave), extrusion, pressure, retort), and mild heat treatments (e.g., low temperature sous vide, flash frying, slow cooker, rare or light cooking, hot smoking).
- Relevant **outcome measures** for interventions are: does the intervention induce sufficient damage to ARGs to prevent or inhibit their uptake by surrounding viable bacteria present in other settings including the human gut and other foods.

All ARGs of immediate or emerging concern, especially in clinical contexts, were considered.

The review adopted a comprehensive search strategy considering all available evidence in the public domain, including peer-reviewed articles, grey literature (e.g., government and industry reports), relevant government reports (e.g., FSA published studies, ACMSF reports), European and International literature (e.g., the EFSA Scientific Opinions, WHO reports) up to May 2021. This included previously published systematic and critical reviews, and risk assessments, as well as primary research.

The primary source databases searched were Web of Science, and PubMed. The searches were restricted to records published from 1990 to end of May 2021. Finalised keywords were agreed with the Agency and were:

antimicrobial resistance OR antimicrobial resistant OR antibiotic resistance OR antibiotic resistant OR antibacterial resistance OR antibacterial resistant OR drug resistant OR multi resistance OR multi resistant OR multidrug resistance OR multidrug resistant OR multi-drug resistance OR multi-drug resistant OR multiantibiotic resistance OR multiantibiotic resistant OR AMR OR MDR OR MAR OR AR OR AMRG
AND
Acinetobacter OR Campylobacter OR commensal OR Enterobacter OR Enterococcus OR Escherichia coli OR E. coli OR Klebsiella OR Listeria OR Salmonella OR Staphylococcus OR pathogen OR Pseudomonas
AND
Blanch* OR boil* OR canning OR cook* OR fried OR fry* OR griddle OR grill* OR heat OR “high temperature” OR HTST OR “hot fat” OR “hot fat” OR “hot oil” OR “hot water” OR microwave* OR oven OR pasteuri* OR “pressure cook*” OR retorting OR roast OR “sous vide” OR steam OR steili* OR thermal OR UHT

Focused Google searches were used to identify relevant grey literature. In total 2446 citations were initially identified in Web of Science and 937 were identified in PubMed. There was some overlap between the databases with 737 duplicates. An additional 35 records were identified through Google searches, other references, and through contact with authors. For all searches, citations and abstracts were uploaded from each of the electronic databases into Covidence (an online tool for systematic reviewing). The following exclusion criteria were applied:

(1) The publication did not address the impact of heat treatments on AMR bacteria or genes;

(2) The publication was in a language other than English;

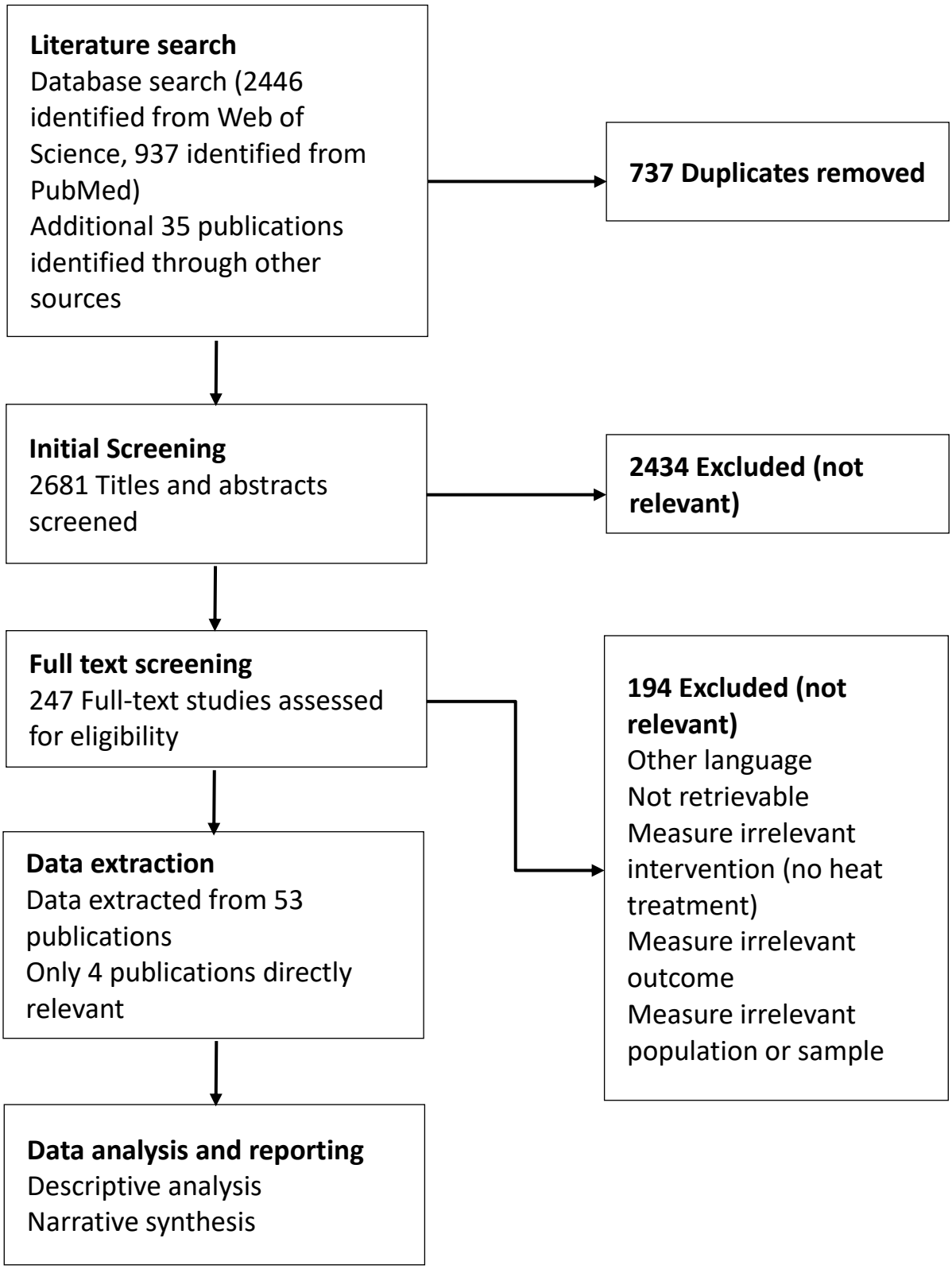
(3) The publication measured irrelevant interventions (no heat treatment), outcomes, or populations or samples.

The criteria were independently applied to the abstract of each paper by at least two members of the five-member project team. For each citation, a consensus was reached that the citation is relevant for inclusion. Arbitration by a third member of the project team was used to settle conflicting appraisals. 2,681 abstracts were screened and 2434 references excluded. Full texts were obtained for all abstracts that passed the inclusion criteria.

A total of 247 publications were considered relevant by title and abstract and full texts collected for second screening. This number was reduced to 53 publications from which some data were extracted, with 194 references being excluded because they were not relevant. Of these publications, nine were reviews with some mention of the impact of heat on AMR bacteria, while 17 had evidence on the relative heat resistance of AMR bacteria in comparison to non-AMR bacteria. Only four publications were identified that were considered to fully meet the search criteria, i.e., had considered the impact of heat treatments on the persistence of ARGs after such treatments. There was an intention to carry out a systematic analysis of publications, but following discussion between reviewers, due to the small number of

publications and differences in experimental approach between the publications it was considered more useful to fully present what these studies reported. Similarly, due to the lack of necessary statistical descriptors making a comparison of studies not suited to a meta-analysis approach it was considered best to discuss these findings narratively rather than quantitatively.

5.1 Flowchart of knowledge synthesis



6 Review of published literature on the impact of heat treatment of food on ARGs

The literature search identified nine publications in the last decade that in part reviewed aspects of the thermal resistance of AMR bacteria in foods subjected to heat treatments (Verraes *et al*., 2013; Friedman, 2015; Report of the Scientific Committee of the Food Safety Authority of Ireland, 2015; Zhang & Wang, 2018; Pérez-Rodríguez & Taban, 2019; Liao *et al*., 2020; Bennani *et al*., 2020; Hutchison *et al*., 2020; Woode *et al*., 2020).

The survival of AMR bacteria in insufficiently heat-treated foods, and whether AMR bacteria are more heat resistant than non-AMR bacteria are discussed in part in some of these reviews (Zhang & Wang, 2018; Liao *et al*., 2020).

Three of these reviews (Verraes *et al*., 2013; Report of the Scientific Committee of the Food Safety Authority of Ireland, 2015; Pérez-Rodríguez & Taban, 2019) mention the theoretical persistence of ARGs after heat treatment. The origin of this theory appears to be Verraes *et al*. (2013) who note, in the context of the transfer of AMR in the food processing environment, that "food processing and/or preservation techniques can kill or inactivate the bacteria. Those dead bacterial cells can stay intact or can be lysed due to cell wall damage. As a consequence, the bacterial DNA, including the eventual present ARGs, are liberated in the environment." Although none of these publications provide any citations that have demonstrated this.

Liao *et al*. (2020) provide a fairly comprehensive review of the heat tolerance of AMR bacteria, but does not consider the impact on, or persistence of, ARGs after any heat treatment. Overall, the reviews all find consensus that:

(1) Heat treatments capable of reducing non-AMR bacteria are equally effective in reducing AMR bacteria.

(2) The presence of AMR bacteria or genes in cooked food after cooking is likely to be the result of insufficient heat treatment or contamination after cooking.

A comprehensive review of the impact of food processing on AMR bacteria in secondary processed meats and meat products found no specific publications describing the fate of AMR bacteria after thermal processing (i.e., cooking) (Hutchison *et al*., 2020). The authors note that there are reports of cooked meats contaminated with AMR bacteria (and we would also note ARGs), primarily from China (Jiang *et al*. 2014; Li *et al*. 2016; Yu *et al*. 2016; Jiang *et al*. 2017; Yu *et al*. 2017).

Hutchison *et al*. (2020) also noted that these surveys were collected at retail and in foodservice and were unable to determine if the AMR bacteria/genes were detected after effective cooking or cross-contamination. We would also note that similar surveys of pasteurised and sterilised milk report the presence of AMR bacteria/genes, but again may also be post treatment contamination.

The recent work by Taher *et al*. (2020a, is one of the few studies that have reported that a standard and globally accepted milk pasteurisation treatment (63.5°C for 30 min) may not be sufficient to inactivate plasmid-mediated ARGs (this is discussed in detail in Section 6.2). The study reveals concerns about inducing a viable but non-culturable (VBNC) state in bacteria.

In addition, as previously discussed in the introduction, Gómez-Gómez *et al*. (2019) detected ARGs in DNA extracted from phage particles extracted from cooked ham and mortadella. The authors attributed this presence to post treatment contamination, noting that the thermal processing that the ham and mortadella would have eliminated microorganisms. They do not appear to have considered the possibility that ARGs could have persisted in the meat following thermal processing,

however in our opinion the possibility of persistence after effective cooking cannot be ruled out.

6.1 Are AMR bacteria more heat-resistant than non-AMR bacteria?

It is accepted in the literature that heat treatments such as sterilization, ultra-high temperature (UHT) treatment, and (full, traditional) pasteurization under well-defined time/temperature combinations will eradicate/kill vegetative bacterial cells, including AMR bacteria. Industrial, food service, domestic or institutional cooking undertaken correctly is normally sufficient to eliminate bacterial pathogens from food.

Risk assessments of the impact on human health of cooking, such as Anderson *et al*. (2001), assume “proper handling and cooking of food” can almost entirely eliminate the risk from many pathogens. Such assessments do not address whether ARGs may persist after cooking in a detectable and functional form.

Studies have indicated that foodborne AMR bacteria (such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* species, *Staphylococcus aureus*, *Yersinia enterocolitica*) do not exhibit enhanced thermal resistance characteristics (see Table 1). Studies on serovars of *Salmonella* spp. that were AMR or non-AMR by Stopforth *et al*. (2008) and Bacon *et al*. (2003) concluded that there was no evidence of any association between antimicrobial susceptibility and the ability of specific serovars to survive or repair damage associated with heat stress. On the other hand, as noted also by Liao *et al*. (2020), some studies (Doherty *et al*., 1998; Dombroski *et al*., 1999; Duffy *et al*., 2006) provide evidence that AMR may impair thermal tolerance in bacteria. McKay (2008) found that D-values of *mecA*-positive *Staphylococcus* spp. at 56°C in whole milk were substantially lower than those of methicillin-susceptible *S. aureus* (MSSA) (max 2.82 min vs 20.1 min) and thus concluded that heat treatments intended to control *S. aureus* should be equally effective at controlling MRSA.

The majority of published reviews and studies conclude that there is no evidence to suggest that AMR bacteria are more heat-tolerant than non-AMR bacteria, with the exception of one publication by Walsh *et al*. (2005), which is often cited by reviews and other publications as a contradiction. Although other recent publications such as by Sarjit *et al*. (2020) and Yehia *et al*. (2020) also provide evidence that some strains of AMR bacteria may be more thermally-resistant than non-AMR strains. Walsh *et al*. (2005) reported that an AMR strain, *Salmonella enterica* serovar Typhimurium definitive phage type (DT) 104 (= *S*. Typhimurium DT 104) had enhanced thermal tolerance characteristics. This study also found that *S*. Typhimurium DT 104 subjected to a sub-lethal heat shock (48°C for 30 min) was significantly more heat-resistant than non-heat-shocked *S*. Typhimurium DT 104, indicating that heat shocking conferred thermotolerance could be incited in this strain. It must be stressed conferred thermotolerance is not unique to this strain or is related to AMR.

Sarjit *et al*. (2021a) investigated the impact of a dry heat treatment at 70°C for up to an hour on inoculated strains of *Salmonella* spp. on beef, lamb, and goat meat in the context of a processing CCP intervention step. This study found that a 70°C for 1 h dry heat treatment was less effective than a 70°C for 5 min treatment on the same *Salmonella* strains in a model meat juice system (Sarjit *et al*., 2021b). Of the *Salmonella* serovars used, an AMR strain of *S.* Typhimurium 2470 on beef and lamb, and *S.* Heidelberg (329 and 2581) on lamb were reported as displaying a significant ($p \leq 0.05$) lower decline in numbers. The authors speculated that AMR "in *Salmonella* [spp.] may influence its thermal resistance depending on the environmental influence such as a meat matrix and its composition". In our opinion, further trials are required to clearly establish whether there is any association. Yehia *et al*. (2020) found a high proportion (10%) of what were identified as heat resistant MRSA isolated from pasteurised camel milk to be more heat resistant than a reference strain *S. aureus* ATCC 29737, which was used as a control. While heat treatments of 85°C and 90°C were sufficient to inactivate *S. aureus* ATCC 29737, the heat resistant MRSA had a mean D_{85} of 111 s, with three isolates being heat resistant even at 90°C for 60 or 90 s. This is in contrast to the findings of McKay (2008), as previously reported.

Few publications have compared the efficacy of heat treatments used as interventions during the processing of red meat and poultry (as used in the USA and other countries) on AMR bacteria. Hughes *et al*. (2010) reported AMR *Salmonella* strains to be no more heat-resistant than non-AMR *Salmonella* strains in heat-treated beef, although as previously reported, Sarjit *et al*. (2021a) found that AMR strains of *Salmonella* spp. were more heat-resistant than non-AMR strains to a dry heat treatment.

The literature search identified few publications on the impact of commercial thermal processing on AMR bacteria or genes in foods. The survival of MRSA during the thermal processing of frankfurters, summer sausage, and boneless ham was investigated by Campbell *et al*. (2014). Heating the products to an internal temperature of 70°C, followed by rapid cooling reduced inoculated MRSA (levels approximately 7.76 to 7.73 $\log_{10}$ CFU g^{-1}) in frankfurters, summer sausage, and boneless ham by 5.53, 6.75, 7.28 $\log_{10}$ CFU g^{-1}, respectively. Since the level of MRSA contamination is reported to be low in these meats, the authors considered the heat treatments to be adequate. There is no mention of consideration of the survival of ARGs or whether the resistance of MRSA is any different to non-AMR bacteria. Conversely, as previously reported Yehia *et al*. (2020) isolated a high proportion (10%) of what were identified as heat resistant MRSA from pasteurised camel milk in Saudi Arabia. The authors recommended that heat treatments such as 93.8°C for 0.1 s, 96.2°C for 0.05 s, or 100°C for 0.01 s, be used to destroy the contaminating heat resistance MRSA. The literature search only identified one publication on the impact of dielectric heating (microwave or radio frequency [RF]). Rincón & Singh (2016) evaluated the use of nalidixic acid-resistant strains of three major Shiga toxin-producing *E. coli* (STEC) and non-pathogenic *E. coli* for use as marker organisms to challenge test the effectiveness of RF heat treatments. They concluded that the heat resistance of nalidixic acid-resistant strains were not significantly different to nalidixic acid-sensitive strains at the endpoint temperatures investigated (55, 60, and 65°C).

While there are many publications on the thermal inactivation of bacteria (particularly pathogens) during a wide variety of cooking operations, especially regarding the gridling of burgers (patties) and steaks, the literature search identified no publications on the possible impact of different domestic or foodservice cooking methods specifically on AMR bacteria.

As noted by Walsh *et al*. (2005) laboratory-acquired AMR strains of bacteria are often used as “marker” strains for use in process validation experiments. Prior to their use their heat resistance is usually compared with non-AMR strains to establish their fitness for such purpose and that their heat resistance is similar to but not greater than the target organism (examples being Foeging & Stanley, 1991; Shen *et al*., 2011; Luchansky *et al*., 2014).

We would agree with comments of Lianou & Koutsoumanis (2013) that in addition to bacterial species and strains, many other factors may have a significant impact on the heat resistance of bacteria, whether AMR or non-AMR. Such factors include the growth conditions, growth phase, and lineage of the cultures to be tested, the type and composition of the medium used (food matrix or various laboratory medium), the challenge conditions (e.g., temperature and pH of challenge media, type of acidulant), the presence of competing microflora, and the procedures and recovery media used for determination of bacterial survival.

Overall, the publications identified in the literature search provide evidence that there are differences in thermal tolerance between different bacteria species, serotypes, or strains, and different substrates. None of these publications on the comparative heat resistance of AMR bacteria provide evidence of whether ARGs may survive such treatments or even express a concern on whether ARGs survive the heat processes applied. The few publications that have been identified that have addressed the survival of ARGs following heat treatments are discussed in detail in the next section of this report.

A number of reviews note that increased use of sub-lethal, rather than lethal, food preservation heat treatments may be more important than was previously considered for the development and dissemination of AMR bacteria and genes (Verraes *et al*., 2013; Capita & Alonso-Calleja, 2013; Ferri *et al*., 2017; Pérez-Rodríguez & Mercanoglu Taban, 2019; Liao *et al*., 2020). They note that mild heat treatments (45-60°C) may be ineffective in inactivating both AMR and non-AMR microorganisms and could trigger bacterial stress responses. None of these reviews cite published evidence of what impact "mild heat treatments" could have on ARGs and the literature search did not identify further clear evidence on this risk.

There is evidence that stress conditions (such as heat stress) may trigger several mechanisms in bacterial cells, e.g., stress adaptation, cellular repair, application of response mechanisms and enhanced virulence (Wesche *et al*., 2009). In their review of sub-lethal injury Wesche *et al*. (2009) noted that thermal treatments that included an extended "come-up phase", such as slow roasting of meats, or certain sous-vide processes, might cause sub-lethal injury to microorganisms. The literature search identified only two studies on the impact of sub-lethal heat treatment on AMR bacteria. McMahon *et al*. (2007) reported that incubation at a sub-lethal temperature (45°C; no time was stated) increased the antimicrobial susceptibility, determined using minimum inhibitory concentration (MIC) tests, of strains of *E. coli* (ec1, ec2, ec3, and ec5) to amikacin, ceftriaxone, and nalidixic acid, *S.* Typhimurium DT 104 (st11, st12, st 16, and st17) to amikacin, ceftriaxone, and trimethoprim, and *S. aureus* (sa3, sa4, sa5, and sa6) to amikacin, ceftriaxone, trimethoprim. Although on the other hand incubation under increased salt (>4.5%) or reduced pH (<5.0) conditions increased resistance. Some of the bacteria subjected to low-pH and high salt stress also continued to show higher levels of resistance after removal of the stress, indicating stable increases in AMR. As previously noted, in contrast the study by Walsh *et al*. (2005) found that heat shocking (48°C for 30 min) conferred thermotolerance in *S*. Typhimurium DT 104.

Table 1. A summary of studies that have compared the heat resistance of Antimicrobial resistance (AMR) and non-AMR bacteria (adapted from Liao *et al*., 2020 and expanded)

Evaluation temperature (°C)	Medium	Species and strains	Enhanced thermal resistance	Stated antimicrobial resistance profiles (antimicrobial or class)	Antimicrobial Resistance Genes (ARGs) present	Reference
50-60	Minced beef and potato	*Y. enterocolitica* *L. monocytogenes*	No	Nalidixic acid	NS	Doherty *et al*. (1998)
47	Oysters	*Vibrio vulnificus*	No	Nalidixic acid	NS	Dombroski *et al*. (1999)
54, 82	Egg white powder	*S.* Typhimurium DT104 Non-DT104 *S.* Typhimurium	No	NS	NS	Jung & Beuchat (1999)
51, 53, 55, 57, 59, 61	Liquid whole egg, egg yolk, egg white, whole egg	*S.* Typhimurium DT104 Non-DT104 *S.* Typhimurium	No	NS, but strains of DT104 quoted as being resistant to ampicillin, chloramphenicol streptomycin,	NS	Jung & Beuchat (2000)

Evaluation temperature (°C)	Medium	Species and strains	Enhanced thermal resistance	Stated antimicrobial resistance profiles (antimicrobial or class)	Antimicrobial Resistance Genes (ARGs) present	Reference
	+ 10% salt, egg yolk + 10% salt			sulphonamides, tetracyclines		
55	Minced beef and potato	*L. monocytogenes*	No	Streptomycin	NS	Walsh *et al*. (2001)
55, 57, 59, 61	Tryptic soy broth (TSB)	Salmonella *spp* serovars Saint-Paul, Anatum, Mbandaka, Agona, Reading, Typhimurium (DT104)	No	Ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracycline, amoxicillin-clavulanic acid, ampicillin-sulbactam, gentamicin, trimethoprim-sulfamethoxazole	NS	Bacon *et al*. (2003)

Evaluation temperature (°C)	Medium	Species and strains	Enhanced thermal resistance	Stated antimicrobial resistance profiles (antimicrobial or class)	Antimicrobial Resistance Genes (ARGs) present	Reference
				Depending on serotype or strain		
55	Chicken pieces	*S.* Typhimurium DT104	Yes	Ampicillin, streptomycin, sulphonamides, chloramphenicol, tetracyclines	NS	Walsh *et al*. (2005)
55	Chicken pieces	*S.* Enteritidis *S.* Typhimurium	No	Nalidixic acid, streptomycin	NS	Walsh *et al*. (2005)
55	Minced beef	*E. coli* O157:H7, O26	No	Ampicillin, kanamycin, streptomycin, trimethoprim, nalidixic acid, rifampicin, sulphonamides, chloramphenicol, tetracycline, minocycline, doxycycline	NS	Duffy *et al*. (2006)

Evaluation temperature (°C)	Medium	Species and strains	Enhanced thermal resistance	Stated antimicrobial resistance profiles (antimicrobial or class)	Antimicrobial Resistance Genes (ARGs) present	Reference
				Depending on serotype or strain		
55, 60, 65, 70	Tryptic soy broth (TSB)	*Salmonella* spp. serovars Montevideo Typhimurium, Anatum, Muenster, Newport, Mbandaka, Dublin Reading, Agona, Give	No	Ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracycline, amoxicillin–clavulanic acid, kanamycin, sulphamethoxazole-trimethoprim, gentamicin	NS	Stopforth *et al*. (2008)
56	Whole milk	*mecA*- carrying *Staphylococcus*	No	Tetracycline, kanamycin, spectinomycin,	*mecA*	McKay (2008)

Evaluation temperature (°C)	Medium	Species and strains	Enhanced thermal resistance	Stated antimicrobial resistance profiles (antimicrobial or class)	Antimicrobial Resistance Genes (ARGs) present	Reference
		spp. strains (*S. epidermidis*, *haemolyticus, lentus*)		erythromycin, trimethoprim, sulphamethoxazole-trimethoprim Depending on serotype or strain		
57	Tryptic soy broth (TSB-G)	60 *Salmonella* spp. *serovars including:* Typhimurium (18 strains), Enteritidis (10 strains), Newport (9 strains), Heidelberg (8 strains), Montevideo (4	No	NS	NS	Lianou & Koutsoumanis (2013)

Evaluation temperature (°C)	Medium	Species and strains	Enhanced thermal resistance	Stated antimicrobial resistance profiles (antimicrobial or class)	Antimicrobial Resistance Genes (ARGs) present	Reference
		strains), Senftenberg (4 strains), Agona (3 strains), Infantis (3 strains) and Derby (1 strain).				
60, 61, 62.5	Tryptic soy broth (TSB)	*E. coli* (STEC) serotypes O26 and O103	No	Ampicillin, penicillin, ceftiofur, spectinomycin, oxytetracycline, clindamycin, sulphadimethoxime, tiamulin, tilmicosin, tetracycline Depending on serotype or strain	*stx*1, *stx*2, *eae*A, *hyl*A *	Akhtar *et al*. (2016)

Evaluation temperature (°C)	**Medium**	**Species and strains**	**Enhanced thermal resistance**	**Stated antimicrobial resistance profiles (antimicrobial or class)**	**Antimicrobial Resistance Genes (ARGs) present**	**Reference**
55, 60, 65 (Radio Frequency heating)	Phosphate buffer saline (PBS)	*E. coli* (STEC) serotypes O157:H7, O26:H11, O11	No	Nalidixic acid	NS	Rincon & Singh (2016)
58	Ringer's solution	*L. monocytogenes*	No	Erythromycin, ciprofloxacin, nitrofurantoin *	NS	Komora *et al*. (2017)
63	Saline solution	*S. aureus*	No	Ciprofloxacin, chloramphenicol, erythromycin, penicillin, sulfamethoxazole, clindamycin, tetracycline, oxacillin, cefoxitin, gentamicin ciprofloxacin Depending on serotype or strain	*sul1, sul2, sul3, tetA, tetB, tetC, tetM, aac(6')/aph (2"), mecA femA, ermA, ermC, norA, cat1, cmlA, flor* *	Ma *et al*. (2019)

Evaluation temperature (°C)	**Medium**	**Species and strains**	**Enhanced thermal resistance**	**Stated antimicrobial resistance profiles (antimicrobial or class)**	**Antimicrobial Resistance Genes (ARGs) present**	**Reference**
55, 60, 65	Minced chicken	Extraintestinal pathogenic *E. coli* (ExPEC)	No	Aminoglycosides, macrolides, sulfonamides, trimethoprim, tetracycline, beta-lactams, cefotaxime, phenicol, aminoglycosides, streptomycin Depending on serotype or strain	*aadA1*, *aadA5*, *mph(A)*, *sul1*, *sul2*, *dfrA17*, *dfrA1*, *tet(A)*, *tet(B)*, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{TEM-1C}}$, *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CMY-2}}$, *catA1*, *aac(3)-Via*, *Aph(3')*, *strB*, *strA* *	Xu *et al*. (2019)
85, 95	BHI medium	*MRSA* *S. aureus (ATCC 29737, control)*	Yes	Cefoxitin, cefadroxil, cephalothin, colistin, polymyxin, aminoglycosides, streptomycin, amikacin, kanamycin:cyclic	*mecA*	Yehia *et al*. (2020)

Evaluation temperature (°C)	Medium	Species and strains	Enhanced thermal resistance	Stated antimicrobial resistance profiles (antimicrobial or class)	Antimicrobial Resistance Genes (ARGs) present	Reference
				peptides, bacitracin, tetracycline: sulfonamide, sulfamethoxazole, nalidixic acid:fluoroquinolone, ciprofloxacin:oxazolidone, linezolid:macrobid		

Table abbreviations: ARGs, antimicrobial resistance gene; AMR, Antimicrobial resistance; NS = Not stated

6.2 Is there evidence that ARGs can transfer from heat-treated AMR bacteria to other bacteria?

Very few publications were identified in the literature search that have directly addressed the fate of ARGs in heat-treated foods. Only four relevant publications were identified (

Table 2). Of these only one (Koncan *et al*., 2007) specifically looked at the fate of ARGs in conventionally cooked food in an *in vitro* mimic of cooking processes. Of the other publications; one *in vitro* mimic of commercial milk pasteurisation (Taher *et al*., 2020a); another was *in vitro* and not designed to mimic any particular heat treatment but did use strains originating from animal sources and temperatures and time similar to thermal processes used to treat and cook food (Le Devendec *et al*., 2018). A further publication that was considered relevant, but not applied to food, was an *in vitro* mimic of autoclaving (Masters *et al*., 1998).

Table 2. Summary of the four studies identified by the literature search that address the impact of heat on the transfer of Antimicrobial Resistance Genes (ARGs)

Publication	**Koncan *et al*., 2007**	**Le Devendec *et al*., 2018**	**Taher *et al*., 2020a**	**Masters *et al*., 1998**
Mimic	Cooking	General heat treatments	Milk pasteurisation (sterilisation)	Non-food autoclaving
Evaluation temperatures (°C)	NS	40, 50,60, 70, 80, 90, 100	63.5, 121	121, 135
Species	*E. faecalis*	*E. coli*	*S. aureus*, *S. sciuri*	Plasmid (pUC18)
Antimicrobial Resistance Genes (ARGs) present	*aac(6')-Ie-aph(2'')-Ia*	*bla*$_{CTX-M-1}$, *bla*$_{CMY-2}$, *tet*A, *str*A	*blaZ*, *mecC*, *tetK*	NS
Stated antimicrobial resistance profiles	Aminoglycosides, except to streptomycin (Predicted profile, not tested)	Cephalosporins, tetracycline, streptomycin	Penicillin, methicillin, tetracycline	Ampicillin

Publication	**Koncan *et al*., 2007**	**Le Devendec *et al*., 2018**	**Taher *et al*., 2020a**	**Masters *et al*., 1998**
Recipient species	*E. faecalis*	*E. coli*	*S. aureus*	*E. coli*
Transformation demonstrated	NO	YES	YES	YES

Table abbreviations: ARGs, antimicrobial resistance gene; AMR, Antimicrobial resistance; NS, Not stated.

Koncan *et al*. (2007) evaluated the possibility of detecting the *aac*(6')-*aph*(2') modifying aminoglycoside resistance gene in meat (chicken, pork, and beef) after conventional cooking procedures. This gene is reported to be encoded by plasmids and transposons, to be widely spread in *Enterococcus faecalis*, and confers resistance to most available aminoglycosides, except to streptomycin (Sparo *et al*., 2018). There are no details reported in the study as to whether the gene was on a plasmid in the *E. faecalis* studied.

Samples (25 g samples cut in the same shape, no dimensions given) of chicken, pork, and beef were inoculated with different dilutions (10^9 to 10^3) of *E. faecalis* (Delaware strain), carrying the bi-functional gene ***aac(6')-Ie-aph(2'')-Ia***. The samples were either boiled (20 min), grilled on a cooking plate (10 min), microwaved (5 min, 900W), or autoclaved for 20 minutes at 1 atmosphere and 121°C. No details of the number of replicates, whether temperatures were recorded, and what end point temperatures were achieved are provided. After the heat treatments no bacteria were detected but positive polymerase chain reaction (PCR) results for the bifunctional gene were observed in all samples. A direct correlation between the density of bacterial inoculum and the intensity of amplified DNA was also observed. Differences between medium were also found, with higher amounts of the bifunctional gene recovered in the beef samples, than in the pork or chicken. Transformation experiments to recipient *E. faecalis* JH2-2 with total DNA from samples were negative in all cases.

This study (Koncan *et al*., 2007), having been presented as a poster, is unfortunately lacking in detail. Though, it is the only study that has been identified that has addressed the fate of ARGs in conventionally cooked food. This study does suggest that heat treatments that are capable of destroying bacteria may not fully destroy ARGs. It also suggests that the survival of ARGs in foods subjected to the same heat treatment is different in different food matrices (which may be expected as the type of food matrix is known to contribute to the heat resistance of bacteria, as reported by Lewis *et al*. (2006) and de Jonge (2019) amongst others; it is likely that

this is due to differences in the thermophysical and structural properties of different foods). This study did not provide any evidence that the bifunctional gene that was detected following heat treatment was a functional gene. DNA remaining after heat treatment is likely to be highly fragmented. A PCR test will still detect highly fragmented DNA remaining after heat treatment and produce a PCR positive amplicon. That the study was unable to transfer genes to a competent recipient strain may indicate that the genes were indeed not functional.

In our opinion, sequencing this gene would have provided a greater understanding the fragmentation, completeness, and structure of the gene. An alternative method of establishing whether the DNA was functional may have been to investigate the physical properties of the gene for authenticity and/or to clone the DNA into a plasmid vector and sequence this.

Le Devendec *et al*. (2018) evaluated the impact of heat treatment, using a traditional water bath method, on the possibility of ARGs being transferred from extended spectrum beta-lactamase (ESBL) *E. coli*. Five strains (2, 40, 174, and 241 details of which can be found in Chauvin *et al.* (2013); and 05-M63-1 details of which can be found in Fleury *et al*., 2015) were used that had previously been shown to carry either a $bla_{\text{CTX-M-1}}$ or a $bla_{\text{CMY-2}}$ gene, which encode resistance to cephalosporins, on a plasmid (*IncI1*) and also carried *tetA* and *strA* genes, which encode resistance to tetracycline and streptomycin. Suspensions in saline were subjected in a water bath to temperatures of 40, 50, 60, 70, 80, 90 or 100°C for 5, 10, 20, 30, or 60 min.

Heat treatments at 40 and 50°C (for 5, 10, 20, 30, and 60 minutes) had little effect on bacterial numbers whereas at 60°C and above reduced bacterial numbers. Treatment at 60°C for 20 min and longer reduced bacterial numbers to below the limit of detection (20 CFU mL^{-1}). All treatments at 70°C and above reduced bacterial numbers below the limit of detection (20 CFU mL^{-1}). All the tested colonies obtained from the suspensions heated to the highest temperatures for the longest time were, after treatment, found by PCR to yield the *E. coli* amplicon and included the amplicons of the $bla_{\text{CTX-M}}$, $bla_{\text{CMY-2}}$, *tetA* or *strA* ARGs initially present in the strains.

The authors therefore concluded that heat stress did not appear to result in the loss of either the ARGs or plasmids.

The authors theorised that AMR *E. coli* that survived a moderate heat treatment were capable of transferring their ARGs to other bacteria present. To evaluate this risk, they sought conjugative plasmids in representative heat-treated suspensions (suspensions heated to 60°C for 10 min and longer in which few culturable bacteria could be detected). These suspensions were mixed with a rifampicin- and kanamycin-resistant recipient strain of *E. coli* K-12 CV601gfp. It is not clear from the publication at what temperature this test was carried out at, but it was carried out after the temperature treatment, implying that the conjugation experiments were at a different (lower) testing temperature. No conjugation could be observed in any of the nine heated suspensions. Suspensions heated to between 40°C and 70°C for 1 h were also tested by PCR assays, and all were positive for ARGs. We note that although detectable by PCR, beyond the conjugation test, no other test of gene functionality was carried out, or sequencing to examine fragmentation, completeness, and structure of the gene.

Further trials were carried out to assess whether ARGs from heated suspensions could be transferred to a recipient *E. coli* DH5α strain by transformation. Three transformation assays were carried out.

In the first trial, suspensions from which no bacteria could be isolated (i.e., strains 2 and 40, heated to 70 °C for 30 min; strain 174, heated to 80 °C for 30 min; and strains 241 and M63, heated to 90 °C for 10 min) were used. No transformant colonies could be detected after plating, indicating to the authors that the heat-treated bacteria had been inactivated, though they could not exclude the possibility that a few viable cells were present. While the plasmid extracts were positive for the different expected genes, transformation assays yielded colonies from only from one strain (strain 2). The resulting colonies shared the phylogenetic group (A) and PFGE profile of the recipient DH5α strain and were found to have acquired one of the

ARGs*, tetA*, from the heat-treated bacteria. They also contained *IncI1* and *IncF* plasmids like the *E. coli* strain 2, suggesting that transformation had taken place.

In the second trial, cell lysates after heat treatment for strains 2 and 40 contained 5.76 and 1.85 ng μL^{-1} of plasmid DNA, respectively, and 0.86 and 1.76 ng μL^{-1} of total DNA, respectively, thus confirming the cellular lysates contained ARGs detectable by PCR. After electroporation with plasmid or total DNA, no transformant colonies could be obtained on the different supplemented media.

In the third assay, DNA concentration of the plasmid preparations ranged from 7 to 14 ng μL^{-1} and the concentrations for total DNA from 2.5 to 4.0 ng μL^{-1}. After electroporation, colonies were obtained only from the transformation performed with one of the five ESBL *E. coli* strains (05-M63-1) on cefotaxime-supplemented media. The colonies were tested and found to belong to the phylogenetic group of the recipient strain, but were found to contained only the *IncI1* plasmid, whereas the donor strain contained plasmids I1, FIB, FIC, and F. The colonies contained the $bla_{CTX\text{-}M\text{-}1}$ and *sul2* genes, but not the *tetA* gene. These results suggest that recipient bacteria had acquired several ARGs, borne by an *IncI1* plasmid, from bacteria that had been subjected to a heat treatment, 70°C for 30 min, that would be considered effective in eliminating bacterial contamination in foods.

This somewhat limited study provides evidence that transformation from 'heat-inactivated bacteria' is possible, although only a limited number of positive results were obtained, indicating that its occurrence is probably rare. Furthermore, as pointed out by the authors, the original suspensions contained more than 10^6 CFU. Thus, indicating that a great number of AMR bacteria may need to be present as contaminants for sufficient ARGs to persist after heat treatment in order for any transfer to other bacteria to take place.

In their study, Le Devendec *et al*. (2018) concluded that the heat-treated bacteria had been inactivated since transformant colonies could be detected after plating.

Though the authors highlighted that they could not exclude the possibility that suspensions still contained a few viable cells. We would suggest that another possibility was that heat treated bacteria could have been in a viable but non-culturable (VBNC) state. Taher *et al*.'s (2020a) study, as discussed below, suggests that AMR bacteria in a VBNC state are metabolically active and able to transcribe and translate genes.

Taher *et al*. (2020a) reported that a standard milk pasteurisation treatment (63.5°C for 30 min) was not sufficient to inactivate plasmid-mediated ARGs *blaZ*, *mecC* and *tetK*, conferring resistance to beta-lactams, methicillin, and tetracyclines, respectively, of staphylococci (*S. aureus* and *S. sciuri*) and, in addition, would induce a VBNC state in these bacteria. In this study, milk and elution buffer were spiked at levels of 10^5 and 10^6 organisms, pasteurised (63.5°C for 30 min) or sterilized (121°C for 15 min), and then stored for up to 21 days at 4°C. Copy numbers of the genes were quantified through PCR and qPCR after the heat treatments and during storage. Copy numbers of *blaZ*, and *tetK* genes remained similar after pasteurisation. While numbers of the *mecC* genes were lower after treatment. However, all genes increased in numbers over the 21-day storage time. Cultivability tests were negative, however use of the BacLight LIVE/DEAD stain showed a significant number of 'live' (green fluorescent) microorganisms in the pasteurised samples, qPCR of 16S ribosomal DNA was also used to quantify VBNC. To assess whether the tested genes were still active, expressed and if resistance was still transferable to another microorganism, detection of the transmissibility of the tested genes was conducted in vitro using the electro-competent *S. aureus* RN42200 strain. The recipient cells showed resistance to methicillin and tetracycline after transformation using electroporation, thus indicating that both *mecC* and t*etK* genes were still functional and expressed.

Overall, this study (Taher *et al*., 2020a) provides some evidence that AMR bacteria may persist in a VBNC state in heat-treated foods and that ARGs from these heat-treated bacteria may be still expressed and transferable. A focused literature search for evidence of any other publications on VBNC of AMR bacteria and heat

treatments did not identify any other similar publications that ARGs may still be expressed by VBNC AMR bacteria. Though, as noted by Taher et al. (2020a), the occurrence of gene expression by VBNC bacteria after milk pasteurisation has also been reported by Gunasekera *et al.* (2002). Gunasekera *et al.* (2002) used *E. coli* and *P. putida* marked with the Green Fluorescent Protein (GFP) gene. They reported that a substantial portion of the GFP-marked *E. coli* and *P. putida*, inoculated in UHT milk, were rendered incapable of forming colonies by a heat treatment but were metabolically active after heat treatment. The heat treatment was 63.5°C for 30 min, the same treatment that Taher *et al*. (2020a) used. The heat treatment reduced CFUs of both *E. coli* and *P. putida* by >4 Log_{10} CFU mL^{-1}, but GFP-based counts measured by epifluorescence microscopy were reduced by only <2.5 Log_{10} CFU mL^{-1} for both of the organisms. While demonstrating that heat-treated bacteria may be rendered incapable of forming colonies whilst remaining metabolically active this study does not provide any evidence on whether viable ARGs may be expressed or transfer to other bacteria from heat-treated bacteria.

A further study by Taher *et al*. (2020b) surveyed the prevalence of ARGs in commercially produced Australian milk. One hundred milk samples at farm level and 152 commercial milk samples (pasteurised and UHT milk) were surveyed, and samples tested by PCR. Their results showed that *sul2*, which encodes sulfonamide resistance, was the most prevalent plasmid-mediated gene in pasteurized and UHT milks (68% and 43%, respectively). In contrast, *mecA*, which encodes for methicillin resistance, was not detected in any sample. Other plasmid-mediated ARGs, t*etA* (54.8 and 27.9%), *tetM* (31 and 26.5%), and *bla*$_{TEM-1B}$ (42.9 and 32.4%), were detected in pasteurized and UHT milks, respectively. Whether these ARGs were functional and could be transferred to recipient cells was not tested. The authors report that examination of cultures of pasteurised and UHT milk samples with light microscopy screening, the BacLight LIVE/DEAD assay, and scanning electron microscopy indicated that some of the bacteria may have lost their culturability but remained viable, i.e., were in a VBNC state. But the authors stated that further studies to identify, differentiate, and quantify those microorganisms.

The only other publication identified as partially relevant but which was not applied to food, was a study by Masters *et al*. (1998) that demonstrated that a small laboratory constructed plasmid (pUC18) heated in distilled water at 121°C for 15 min in the presence of 0.5-2.0 ml L^{-1} sodium chloride was still capable of transforming ampicillin resistance to *E. coli* (DH5a) by electroporation. No transformable activity was detected when a plasmid preparation was autoclaved at 135°C for 20 min. No further studies appear to have been undertaken on this subject by the researchers. The implications of these findings in relation to the persistence of functional ARGs in heat-treated foods do not appear to have been further studied by other researchers and this publication has not been cited by any other publication on this specific topic (i.e., heat resistance of ARGs). It is difficult to assess whether similar results would apply in more complex food matrices. However, many cooked foods contain sodium chloride, and receive a far less severe heat treatment, thus this study provides some limited supportive evidence that functional ARGs in plasmids could persist in foods after heat treatments.

It must be noted that all four studies used electroporation to assess transformability. While Le Devendec *et al*. (2018) considered that while the possibility of ARGs being transferred from heat-inactivated via natural transformation during food preparation could not be excluded it was likely to be infrequent. Only two of the studies used food matrices (Koncan *et al*., 2007; Taher *et al*., 2020a), the other two (Le Devendec *et al*., 2018; Masters *et al*., 1998) used simple saline matrices. As Le Devendec *et al*. (2018) admit, it is highly likely that the heat resistance of bacteria and ARGs in complex media or food matrices will differ from that in simple matrices.

None of the four studies identified addressed whether these genes can be acquired by gut microbiota. Though Le Devendec *et al*. (2018) considered that the probability of functional ARGs reaching the lower intestinal tract was low as was the chance of natural transformation in the gut. Reviews of the transfer of ARGs and exchange in the gut have been carried out (Schjørring & Krogfelt, 2011; Broaders *et al*., 2013; Huddleston, 2014; Lerner *et al*., 2017) but whether ARGs from heat-treated foods can be a source of transfer is not discussed in these reviews nor has the literature

search identified any publication that provide evidence that ARGs from heat-treated foods can be a source of transfer.

6.3 Is there compelling evidence to show that heat completely destroys DNA (particularly ARGs either as non-cellular DNA or within AMR bacteria)?

Heat will denature, degrade, and fragment DNA. It is fully accepted that heat treatments such as sterilization, UHT treatment, and pasteurization under well-defined time/temperature combinations will eradicate/kill vegetative bacterial cells and other microorganisms, including AMR bacteria. That in part is due to damage to their DNA, though no single event is responsible for cell death (Russell, 2003).

As previously discussed, a small number of publications have been identified that have addressed the survival of functional ARG after heat treatments. Three of the four publications identified show there is some evidence that some heat treatments will not completely destroy ARGs either as non-cellular DNA or within AMR bacteria. There is also some evidence that heat treatments may induce a VBNC state in AMR bacteria and they may continue to express ARGs after what may appear to be an effective heat treatment (Taher *et al*., 2020a).

There is evidence that bacterial DNA is not denatured by some heat treatments that would be expected to be sufficient to kill bacteria. Wang *et al*. (2014) reported that fragments of bacterial DNA (part of the *eaeA* gene of *E. coli* O157:H7) were not denatured when heated at 95°C for up to 30 minutes, as determined by measuring the absorbance of DNA at 260 nm after heat treatment. Ducey *et al*. (2017) also report that microbially-derived DNA (on cubes of beef bone and tissue) was still capable of being amplified by PCR when treated at 100°C for up to 240 minutes. Treatments of 150°C and 200°C were sufficient at eliminating microbial DNA. Examination of the thermal denaturation of bacterial cells by differential scanning

calorimetry (DSC) has shown that higher temperatures are needed to denature DNA than kill bacterial cells (Mackey *et al*., 1991; Mohácsi-Farkas *et al*., 1999).

These studies did not study the functionality of the DNA. As previously noted, while there is evidence that ARGs can be detected using PCR after heat treatments, PCR detection does not prove that such genes are functional, either because the DNA integrity has been breached or regulatory proteins have coagulated.

As previously discussed in the introduction, Kharazmi *et al*. (2003) provides some evidence that although heat treatment does degrade lysed extracellular free DNA, surviving fragments may still be still of sufficient integrity to be transformed by other bacteria.

In conclusion, while there is compelling evidence to show that heat is capable of completely destroying microbial DNA (including ARGs), there is evidence that DNA may not fully denatured by some heat treatments that would be expected to be sufficient to kill bacteria and even if fragmented may remain functional.

6.4 Can ARGs be up taken by surrounding viable bacteria present in other settings, including the human gut?

It is recognised that ARGs can be transferred to other viable bacteria through transformation mechanisms. While dead cells cannot pass ARGs to other bacteria by cell-to-cell mechanisms such as conjugation or transduction (Verraes *et al*., 2013), as soon as DNA fragments have been released, ARGs may, theoretically, be transferred by transformation (McMahon *et al*., 2007; Verraes *et al*., 2013; Le Devendec *et al*., 2018; Pérez-Rodríguez & Taban, 2019). The present understanding is that the process of transformation occurs with low frequency and is subject to a large number of requirements mostly observed in very controlled laboratory conditions (Verraes *et al*., 2013).

Theoretically ARGs that are not destroyed during heat treatment and passing through stomach acid may be capable of transfer to other microbiota in the human gut and be incorporated, thereby becoming a functional source of AMR.

There is some literature (Aubry-Damon *et al*., 2004; Hart *et al*., 2006; Ramchandani *et al*., 2005) that lends weight to this hypothesis, although the literature search identified no evidence of ARGs from heat-treated or cooked food being shown to transfer to other microbiota in the human gut.

It is clear, as highlighted by other studies (Buffie & Pamer, 2013; Taher *et al*., 2020a), that the capacity for the acquisition of ARGs by gut microbiota deserves more intensive study. In addition to the human gut environment, some literature exists to indicate that the food environment could potentially facilitate uptake of DNA by certain bacteria (Hasegawa *et al*., 2018).

As previously noted, while Le Devendec *et al*. (2018) could not completely exclude the possibility of the transfer of ARGs from heat-inactivated *E. coli via* natural transformation during food preparation, they considered that given the “infrequency of natural transformation and low probability of a whole functional resistance gene reaching the lower intestinal tract”, they believed it unlikely for indigenous *Enterobacteriaceae* from the digestive microbiota to be transformed by DNA from heat-inactivated foodborne bacteria.

6.5 What is the impact of different heat treatments on ARG uptake by viable bacteria?

Few publications appear to have directly addressed the impact of different heat treatments on ARG uptake by viable bacteria. Different cooking treatments were compared by Koncan *et al*. (2007), but this study was limited, no time-temperatures were provided, and transformation experiments to recipient *E. faecalis* JH2-2 with

total DNA from samples (irrespective of type of heat treatment) were negative in all cases.

Different heat treatments are highly likely to have an impact on the survival and viability ARGs, whether as cell free DNA or in mobile elements such as phage/GTAs or MVs. It is likely that the most important factors will be the maximum temperature the gene is subjected to, the duration at this temperature and temperature history (come-up and come-down times), and the type of food matrix.

6.6 Is there any evidence of uptake of heat damaged DNA, and particularly ARGs, by pathogenic and non-pathogenic bacteria?

Kharazmi *et al*. (2003) found that although heat treatment does degrade lysed cell free ARGs, surviving fragments may still be of sufficient integrity to be transformed by other bacteria. As previously discussed, four publications have directly addressed the uptake of ARGs to viable bacteria from “heat-treated” bacteria. Of these, one (Koncan *et al*., 2007) did not show that transformation was possible from heat-treated AMR bacteria. In contrast, the other publications (Le Devendec *et al*., 2018; Taher *et al*., 2020a; Masters *et al*., 1998) provide some evidence that the transformation of ARGs from heat-treated bacteria is possible under laboratory conditions. None of these publications examined the degree of fragmentation of any surviving ARGs on functionality.

6.7 Is there any evidence to suggest that the behaviour of chromosomal DNA and plasmid DNA in response to heat differs?

No publications were identified that have directly compared the behaviour of chromosomal DNA and plasmid DNA in response to heat.

The four studies on the potential impact of heat treatments on ARGs that were identified, and reviewed, appear to have considered their survival in plasmid-mediated DNA. Though the resistances could be both plasmid and chromosomal. While one of the four studies (Koncan *et al*., 2007) did not specifically mention plasmids, the gene they investigated, *aac(6')-Ie-aph(2'')-Ia*, is reported to be encoded by plasmids and transposons.

6.8 Is there any evidence to suggest that ARGs can transfer *via* heat-tolerant phages/GTAs?

Phages/GTAs have been identified as a potentially important vectors in the transfer of ARGs. In theory ARGs could remain protected in thermally tolerant phages/GTAs that survive heat treatments that are effective in killing bacteria. There is clear evidence of the persistence of thermally-tolerant phages in heat-treated foods (Sommer *et al*., 2019). As quoted in the introduction of this review, Jebri *et al*. (2020) theorised that phages surviving processing methods could be a reservoir of ARGs in RTE food.

No studies appear to have addressed the impact of heat treatments of food on the survival and transfer of ARGs via phages/GTAs. The mechanisms responsible for phage/GTA transfer of ARGs and their importance and role in the transfer of ARGs do not yet appear to have been fully explored. There appears to be no evidence of whether this is more than a theoretical risk.

6.9 Is there any evidence on the role of membrane vesicles (MVs) in the survival and transfer of ARGs from AMR bacteria subjected to heat treatments?

It is not clear from the literature whether MVs containing ARGs are any more heat tolerant than the bacteria from which they come. The literature search did not

identify any studies that have addressed the impact of heat treatments of food on the survival and transfer of ARGs via MVs.

Lee *et al*. (2013) reported that heat-treated MVs from *S. aureus* (ATCC 14458) containing *BlaZ*, a β-lactamase protein, did not mediate the survival of ampicillin-susceptible bacteria. Their study did not find *BlaZ* genes in MVs from *S. aureus* but did identify MVs containing the Beta-lactamase protein. They found that non-heat-treated MVs containing this protein did enable other ampicillin-susceptible Gram-negative and Gram-positive bacteria to survive in the presence of ampicillin. But MVs that contained this protein that were first heated to 100°C for 20 min did not mediate the survival of ampicillin-susceptible bacteria in the presence of ampicillin. This provides some evidence that high temperature heat treatments may inactivate ARGs in MVs. However, the heat treatment used was at a relatively high temperature and long time in comparison to heat treatments that are equivalent to 70°C for at least 2 min, and the MVs were in a simple saline matrix rather than a complex food matrix.

7 Conclusions and recommendations for further work

Overall, this review has established that there is very limited evidence whether different heat treatments applied to eliminate bacterial contamination in foods also induces sufficient damage to ARGs to prevent or inhibit their uptake by surrounding viable bacteria present in other settings, including the human gut and other foods.

The literature review showed that there is evidence that AMR bacteria are likely to be no more heat-resistant than non-AMR bacteria. It is therefore apparent that heat treatments sufficient to kill non-AMR bacteria (such as 70°C for at least 2 min, or the equivalent) will be equally effective in killing AMR bacteria.

The literature suggests that, theoretically, functional ARGs could potentially survive in heat-treated food either as (1) cell free DNA lysed from heat-treated AMR bacterial cells, (2) within heat-tolerant phage/GTAs, (3) within MVs, (4) within VBNC heat-treated AMR bacterial cells. These genes could subsequently theoretically be transferred to live bacteria through a range of HGT mechanisms.

What limited practical evidence exists does imply that heat treatments that are effective at eliminating bacteria may not be sufficient to destroy ARGs and that it is possible in the laboratory to transfer those genes to other bacteria. We caution that some of the evidence of survival of ARGs after heat treatment is limited to positive results based only on PCR and not supported by culture, and which therefore do not provide proof of gene expression or functionality.

It is clear that there are insufficient numbers of published studies on this subject to undertake any reliable analysis of the data or draw meaningful conclusions regarding the evidence on the impact of different heat treatments on ARG uptake by viable bacteria and a clear knowledge gap.

- We would recommend further focused practical research be undertaken to assess the survival of functional ARGs in food matrices that have undergone heat treatment and whether they can be transferred to other bacteria in other matrices, to provide evidence for a full assessment of risk in relation to the transfer of ARGs from heat-treated foods to bacteria in other matrices.

It is clear that any future work should carefully consider the development and adoption of standardised testing protocols to ensure the value of future research and that results can be compared and analysed against similar studies. Although limited in scope, further studies initially based on the approach and protocols of Le Devendec *et al*. (2018) would enable a direct comparison of results to be made.

Since there is evidence that microbial resistance to heat in different matrices and subject to different (non-isothermal) time/temperature profiles differs, it is important that suitable food matrices and representative time/temperature treatments should be considered in such studies.

There is a theoretical risk that heat tolerant phages/GTAs and MVs may be a reservoir and vector for ARGs in heat-treated foods, there would appear to be no published evidence that has addressed this and hence a gap in the knowledge on their significance.

- We would recommend that there is a need to support appropriate practical studies on the transmission of ARGs by phages/GTAs and MVs, the impact of heat treatments on the survival of ARG carrying phage/GTAs and MVs, and the possible transfer of ARGs between bacteria via these mechanisms.

No evidence has been found that ARGs from heat-treated food may transfer to bacteria in the human gut after ingestion. There is some limited evidence that this potential exists but a gap in the knowledge on if this can occur in practice.

- We would recommend that it would be prudent to support appropriate practical research studies on whether functional ARGs are able to survive the conditions that would be encountered in the human stomach, i.e., stomach acids, following ingestion and are able to transfer to other microbiota that may be found in the human gut.

Overall, this review has determined that there are gaps in the knowledge concerning the persistence of ARGs in heat-treated food. We hope that highlighting these gaps may focus future work on this topic, depending on the priorities and budgets of the relevant funding bodies. We would stress that it is essential in any further work that assumptions are not made regarding the temperature/time response during cooking/heating and that accurate relevant measurements are made to ensure that conditions truly mimic cooking / thermal processes.

Although not a focus of this review, there is clearly a concern that sub-lethal food processing heat treatments may play a role in an increase in AMR. There appears to be little evidence and a gap in the knowledge on the impact of sub-lethal heat treatments on AMR bacteria and genes. In our opinion, there is clearly a need to define lethal parameters and identify “sub-lethal heat treatments” that have been adopted by the industry, and what their impact may be on AMR bacteria and ARGs. In order to assess this, heat treatments need to be fully characterised, i.e., what endpoint temperatures and time/temperature histories would be expected in foods subject to different heat treatment.

- We would recommend the support of appropriate practical research studies to establish the impact of sub-lethal heat treatments on AMR bacteria and ARGs in food matrices to provide evidence for a full assessment of risk in relation to the survival of AMR bacteria and functional ARGs in sub-lethally heat-treated foods.

Also, again while not a focus of this review, while there are many publications on the thermal inactivation of bacteria (particularly pathogens) during a wide variety of cooking operations, especially regarding the gridling of burgers (patties) and steaks, there appears to be gap in the literature specifically on the impact of different domestic or foodservice cooking methods on AMR bacteria (as well as ARGs).

- We would recommend the support of appropriate practical research studies to establish the impact of different domestic or foodservice cooking methods on AMR bacteria and ARGs in food matrices to provide evidence for a full assessment of risk in relation to the survival of AMR bacteria and functional ARGs in such cooked foods.

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