Review of Antibiotic Use in Crops, Associated Risk of Antimicrobial Resistance and Research Gaps

Report to Department for Environment, Food and Rural Affairs (Defra) & The Food Standards Agency (FSA)

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Glossary

ARB- Antimicrobial Resistant Bacteria

ARG- Antimicrobial Resistance Genes

AMR- Antimicrobial Resistance

FAO- Food and Agriculture Organisation of the United Nations

IPPC- International Plant Protection Convention

LMICs- Low- and Middle-Income Countries

LOD- Limit of Detection

LOQ- Limit of Quantification

MGE- Mobile Genetic Element

MIC- Minimum Inhibitory Concentration

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Executive Summary

- Relatively few countries have national legislation in place regulating the use of antibiotics in crop agriculture. Furthermore, in many countries the compounds and volumes applied are not well monitored.
- Where antibiotic use is regulated, there are some specific antibiotics (such as streptomycin, oxytetracycline, and kasugamycin) and crops (such as pome fruit and citrus fruit) where use is repeatedly reported. In general, where information is available, antibiotic use in crop agriculture appears to be at a significantly lower level than in livestock agriculture. However, in many countries official information is unavailable, and use may be considerably higher.
- Soil amendments may constitute a more important source of antibiotics than direct application, especially when these are of livestock (e.g. manure) or human (e.g. sewage sludge) origin. Soil amendments can also introduce antimicrobial resistant organisms or antimicrobial resistance (AMR) genes on to crops.
- Uptake of antibiotics by crops differs widely according to many factors, including antibiotic class, crop type and variety, and soil type.
- Methods exist for the detection of antibiotics in a range of crops, but are limited, certainly in comparison with methods for animal products. Multi-class, multi-residue methods to monitor for a range of residues are available, but further work is required to extend the range of compounds tested for.
- Rapid methods have been developed to test for both AMR genes and phenotypic antimicrobial resistance in bacteria. High throughput sequencing-based techniques, including metagenomics, can be used to simultaneously test for multiple gene targets in multiple organisms and can provide data on the genomic and environmental context of the AMR genes detected.
- The risk of AMR arising in crop agriculture in the UK is likely relatively low, given the lack of antibiotic use on crops in this country. The risk of AMR arising due to co-selection from other agrichemicals is difficult to assess due to a lack of information about the co-selection properties of most pesticides. Some agriculturally important chemicals, such as copper compounds, do co-select for AMR. The possibility of importing AMR bacteria or genes on crops or plant products treated with antibiotics in countries where they are either used or misused is likely higher. Without more quantitative information on antibiotic use in crop agriculture in countries from which the UK sources plant products, or testing of imported products, it is difficult to draw firmer conclusions.

Introduction

Antimicrobial resistance (AMR) is an increasingly important global health problem, with the potential to render antibiotics unusable, and negate medical treatments such as chemotherapy and organ transplant (O'Neill, 2014). In the United Kingdom, concern over AMR has led to large reductions in antibiotic consumption in both humans and animals; a 6% reduction in total antibiotic mass used in human medicine, and a 35% reduction in animal antibiotic use from 2013 to 2017 (Veterinary Medicines Directorate, 2019). This has in turn led to a reduction or plateauing of the majority of the most important indicators of AMR (Veterinary Medicines Directorate, 2019) although certain resistance phenotypes have increased, such as carbapenemase-producing organisms and vancomycin resistant *Enterococcus faecium* in Scotland, and AMR *Escherichia coli* and *Klebsiella pneumonia* in England. This resulted in an estimated 60,788 severe AMR infections in England and 1424 drug resistant bacteraemias in Scotland in 2018 (Health Protection Scotland, 2018, Public Health England, 2019). To date most work on AMR in the agriculture sector has focussed on livestock, as animals are directly treated with antibiotics (e.g. to treat infectious disease). AMR bacteria (including zoonoses and foodborne pathogens) and genes that arise in livestock can be transferred to consumers. However, crop plants also represent a potential pathway for bacteria and genes into the human microbiome, for which in comparison international recognition appears to have been much reduced. Nevertheless, a truly one health approach to combatting AMR must address this transmission pathway as well. This is especially important when considering imports of food and agricultural products from third countries, beyond the scope of UK regulations and surveys.

The objective of this review was to draw upon expert opinion, from within Fera and solicited from international experts, as well as a survey of the published literature, to describe the current state of knowledge of the risk of AMR in crop-based agriculture, and to point towards research gaps and opportunities. This aims to help inform the United Kingdom’s need and approach to safeguarding consumers from the risks of AMR and steer further improvement of our knowledge-stock for crop-based AMR.

This assessment of the risk of AMR arising in crop agriculture starts with a synthesis of expert opinion and available formal and informal literature on direct application of antibiotics for plant health purposes in a range of countries. While direct antibiotic use in crops may be a focus of reduction efforts, an assessment of the major sources of antibiotic inputs into crop systems is also relevant. This identifies manure, sewage and grey water as potentially important sources of antibiotics and gives examples of the prevalence of AMR bacteria and Antimicrobial Resistance Genes (ARGs) in the field (as opposed to on foodstuffs after processing). Further to these direct sources of antibiotics, there are numerous classes of widely used chemicals that can co-select for AMR, or increase the likelihood of AMR evolving, and the most important examples from a crop agriculture point of view are explored. The different techniques available to detect both antibiotics and AMR are discussed, and the most beneficial ways to employ them are highlighted. The results of this assessment are then summarised, and research gaps identified. Investigations are limited to antibiotic resistance in

bacteria (thus excluding biocide tolerance in bacteria, or fungicide resistance in fungi), and excludes post-harvest processing.

Use of Antibiotics in Crops

Information Gathering

To gather more information on the use of antibiotics in plant agriculture, two approaches were taken:

1. Review of existing published reports, and a search for further published information in scientific literature and on the internet;
2. Questionnaires (Supplementary Materials 1) sent to personal plant health contacts and/or International Plant Protection Convention (IPPC) national contact points in a range of countries.

The process of gathering information from the literature involved initial searches of the scientific literature, though due to the paucity of relevant references returned, a citation tracing system was followed. Key references (e.g. Stockwell and Duffy, 2012) were identified, and relevant references within them, and those which cited them, were reviewed. This was supplemented by grey literature and search engine searches, yielding EU, IPPC and Food & Agriculture Organisation of the United Nations (FAO) reports, webinars etc., in addition to references highlighted by contacts and questionnaire respondents. While available literature on antibiotic use in plant agriculture is comparatively scarce, several reports and papers have been written over the last 15-20 years reviewing the use of antibiotics and, in some cases, making an assessment of the potential risks of such use in the development of AMR.

We have also found several more recent reports and news articles published online relating to antibiotic use and, while these are not always impartial and are written from the point of view of the author, they provide further insight into the use and misuse of antibiotics in different countries.

Previous reviews have confirmed that considerable information on antibiotic use is available from a limited number of countries or regions, for instance the USA and the EU, but highlighted the significant gaps in our knowledge and understanding of the extent to which antibiotics are regulated and controlled by many other countries around the world, the degree to which antibiotics are used in these countries, the crops on which they are used, and the diseases they are intended to control.

To try and update the available information, we have carried out further reviews of literature and news articles, and also approached contacts in individual countries with a request to complete a short questionnaire on antibiotic regulation and use in their countries (Supplementary Materials 1). These were emailed to personal plant health contacts and/or IPPC national contact points in the following countries. Countries were selected based on the existence of relevant personal contacts, an assessment of their likely importance to the UK as exporters of crop-based food products, and to ensure a broad geographical spread.

Europe: Switzerland, Germany, France, Portugal, Austria, Russian Federation, Italy, Spain

Asia: Georgia, Kazakhstan, Kyrgyzstan, Korea, China, Japan, Thailand, India, Pakistan, Bangladesh

Oceania: New Zealand, Australia

North and South America: USA, Costa Rica, Brazil, Argentina, Canada

Africa: Kenya, Uganda, Ghana, Rwanda, Gambia, Ethiopia, Nigeria, Zambia, Zimbabwe, Mauritius, South Africa

Other organisations: European and Mediterranean Plant Protection Organisation (EPPO), European Food Safety Authority (EFSA)

The response to our questionnaire was limited. We only received full responses from six countries (New Zealand, Germany, Kazakhstan, Kyrgyzstan, Rwanda and Zambia). We received acknowledgements but no full responses from a further five (Italy, Uganda, Nigeria, Zimbabwe, Gambia).

This level of response is disappointing, although it mirrors the reported experiences of previous researchers.

Summary of Responses

Of the six full responses received, only New Zealand and Kazakhstan have legislation in place regulating the use of antibiotics on food crops, although Rwanda does not include antibiotics on the list of approved agrochemicals. Germany, Kyrgyzstan and Zambia state that they do not have national regulations controlling the use of antibiotics (although in the case of Germany, the EU does not have any antibiotics approved as plant protection products).

Kazakhstan allows the use of streptomycin for scientific research into control of fireblight in apple (laboratory research only).

New Zealand allows the field use of streptomycin on pome fruit, stone fruit, and kiwi fruit; and glasshouse use on tomato seedlings. Field use of kasugamycin on kiwi fruit is also approved in New Zealand.

Of the countries that don't have National regulations controlling the use of antibiotics in food crops, only Zambia provided data on their use, however the compounds listed were not all strictly antibiotics. They reported field application of difenoconazole on tomato and aubergine, dichlorophene on vegetables, copper hydroxide on vegetables, and fungicides on vegetables and cereals. They report that the last two are used indiscriminately.

The respondents had mixed opinions on the potential risks of antibiotic use on crops being a causal factor of AMR, rating them as either Low (1 respondent- that from New Zealand), Medium (2 respondents) or High (2 respondents). Rwanda did not comment. The use of antibiotics as a control measure for bacterial crop diseases is under discussion in three of the responding countries (Germany, Kyrgyzstan and Kazakhstan).

Regulation of Antibiotics

It is difficult to determine which countries permit the use of antibiotics to control bacterial plant diseases. This information is not widely available online, and approaches to individual countries are often unsuccessful. The information we have gathered in this report has been gleaned from several reports and papers, as well as some additional information gathered from a very limited number of responses to our own questionnaire.

Reports suggest that only 30-40 countries have any regulations at all, which includes countries where they are allowed (Rajashekara et al., 2019), but where regulation and oversight of use are strong, the use of antibiotics and the presence of residues on foods of plant origin are minimal.

In the UK, antibiotics, including streptomycin and oxytetracycline, have been used in the past on ornamentals (Young et al., 1999) but presently there are no antibiotics authorised as plant protection products in the UK (or the EU), effectively prohibiting their use for the control of plant diseases (Jon Winfield, Chemical Regulation Division, Health & Safety Executive, personal communication, January 2020). Historically, several EU member states have used antibiotics to control diseases in vegetable and fruit crops but this use has now stopped (Directorate-General XXIV Consumer Policy And Consumer Health Protection, 1999, Health and Food Safety Directorate-General, 2019). Some EU member states (Austria and Hungary) authorise their emergency use to control outbreaks, but the volumes used are negligible and their application is strictly controlled (Stockwell and Duffy, 2012, Health and Food Safety Directorate-General, 2019).

In some countries, although there is some degree of regulation in place, it is not strong and there may be conflicting recommendations for use of antibiotics between different organisations (Khullar et al., 2019).

Antibiotics Used in Plant Agriculture

The main antibiotics authorised for the control of bacterial plant diseases are streptomycin, oxytetracycline, kasugamycin, gentamicin and oxolinic acid. Of these, streptomycin and oxytetracycline are the most widely used (McManus et al., 2002).

Streptomycin

Streptomycin is an aminoglycoside antibiotic first used in commercial agriculture in the USA as early as 1955 (Stockwell and Duffy, 2012), where it has been mostly used for control of fireblight of apple and pear (Stockwell and Duffy, 2012, Sundin, 2018). Other minor usage in the USA is reported in floriculture, potatoes, tobacco, and other vegetable seedlings (Vidaver, 2002), although previous usage on tomato has been discontinued (Sundin, 2018). It has most recently been approved for use across 764,000 acres of citrus in the USA (Jacobs, 2019).

Streptomycin resistance is becoming widespread among bacterial phytopathogens (Vidaver, 2002), and emergence of streptomycin-resistant strains of *Erwinia amylovora*, *Pseudomonas* spp., and *Xanthomonas campestris* has impeded the control of several important diseases (McManus et al., 2002, Sundin and Wang, 2018).

Streptomycin is known to be registered for use in control of fireblight in the USA, Israel, New Zealand, Canada and Mexico. Its use has also been permitted for emergency control of outbreaks in some European countries (Austria, Switzerland and Germany) (Stockwell and Duffy, 2012) although it has been replaced by aluminium potassium sulphate in Germany since 2014 (Health and Food Safety Directorate-General, 2019).

Oxytetracycline

Oxytetracycline is a naturally produced tetracycline antibiotic which is predominantly used in plant agriculture to control fireblight in apple and pears. Its use started in the 1980s in response to streptomycin resistance in *Erwinia amylovora* (Sundin, 2018). In the USA it is also used to control bacterial spot in stone fruit (*Prunus* spp.). In Mexico and Central America, it is used to control a range of bacterial diseases in vegetable crops. While expensive and time-consuming it can also be injected into the trunks of palms and elm trees to control diseases caused by phytoplasmas (Stockwell and Duffy, 2012).

Plant bacterial resistance to oxytetracycline does not yet appear to be a significant issue (Vidaver, 2002).

Kasugamycin

Kasugamycin is another aminoglycoside antibiotic that was originally isolated in 1965. It has been registered for use in crop protection more recently (2015) and is used in the USA, and for emergency outbreak control in Hungary, against fireblight (Sundin, 2018, Health and Food Safety Directorate-General, 2019), and in New Zealand to control kiwi canker (Questionnaire Response). Its development was largely in response to streptomycin resistance.

Gentamicin

Another aminoglycoside antibiotic that is registered for use in Mexico, Central and South America on apple and pear to control fireblight, and in a range of vegetable crops to control diseases caused by *Pectobacterium*, *Pseudomonas*, *Ralstonia* and *Xanthomonas* (McManus et al., 2002, Stockwell and Duffy, 2012).

Oxolinic acid

Oxolinic acid is a synthetic quinolone antibiotic used only in Israel to manage fireblight in pear and related plants, particularly where fireblight is resistant to streptomycin, and is registered in Japan for management of bacterial panicle blight of rice (Stockwell and Duffy, 2012).

"Streptocycline"

Information from India suggests that "streptocycline", a 90:10 mix of streptomycin and tetracycline is recommended for use on 8 crops (Table 2) (Centre for Science and Environment, 2019, Khullar et al., 2019).

Data on Use

Existing data suggests that the USA is the biggest user of antibiotics in plant agriculture (approximately 70,000 kg per annum) however, to put this into the context, this figure represents less than 0.1% or the total antibiotic use in the USA, with use in livestock accounting for more than 75% of total use (Rajashekara et al., 2019).

While the use of antibiotics on crops in the USA is very well documented and up to date information is available for the volumes of different antibiotics used on specific crops, the same cannot be said for other countries. We do not have any figures for antibiotic use in plant agriculture in many of the largest plant producing countries, such as China and India, or other countries from which there is regular trade to the UK in high value fruit and vegetables such as Thailand, Vietnam or Bangladesh. However, Taylor & Reeder (2020) recently showed that antibiotic application on a wide variety of crops may be more commonly recommended than perhaps widely appreciated. They report that from a database of over 400,000 advice records, from an 8-year period, agronomic advisers in Low- and Middle-Income Countries (LMICs) recommended the application of antibiotics on more than 1,600 advice records (0.38% of the total records). There is no guarantee or further confirmation that this advice was followed, and this is likely to represent a small percentage of the total crop area, but there were clear regional and temporal trends within this overview to consider alongside concern for examples where recommendations would represent a misuse of antibiotics.

Irrespective of above, the availability of published information on antibiotic use in many countries is limited or non-existent and this still represents a significant gap in our understanding of antibiotic use in global crop protection.

Crops and Diseases

Antibiotics are primarily used to control plant bacterial diseases in crops. By far and away the greatest use of antibiotics is in the control fireblight in pome fruit (apples and pears) caused by the bacterium *Erwinia amylovora* (Stockwell and Duffy, 2012, Rajashekara et al., 2019). This was the primary driver for the development of antibiotic use as plant protection products and continues to be so to the present day. In the USA, 90% of all streptomycin used in plant agriculture is for fireblight control.

More recently (2019) and potentially very significantly in terms of total use of antibiotics in the USA, the Environmental Protection Agency (EPA) approved oxytetracycline and streptomycin for general use on citrus trees for control of citrus bacterial diseases, most notably citrus greening disease. Previous approval (2016) had only been for emergency use (Donley, 2019, Jacobs, 2019). Given the extent and severity of the disease, and the value to the crop in southern citrus-growing States, this extension of use to citrus leads to a predicted increase of antibiotic use in crop production from 70,000 kg (current), to 510,000 to 900,000 kg per annum (Rajashekara et al., 2019).

In comparison with these two cases, other crop use is comparatively minor, although our research has revealed that in different countries around the world, antibiotics are approved or recommended for use on a diverse range of fruit and vegetable crops.

Records show that antibiotics are used (and approved) to control the following plant bacterial diseases in different countries, although reports indicate wider unauthorised use on a broader range of plants (particularly vegetables). These unauthorised uses are covered in the section on misuse. The plant bacterial diseases reportedly being controlled by antibiotics are listed in Table 1.

Table 1. Plant bacterial diseases reportedly being controlled by antibiotics (S = Streptomycin, O = Oxytetracycline, K = Kasugamycin, OA = Oxolinic acid, G = Gentamycin, SC = Streptocycline).

Plant	Disease	Antibiotics used
Pome fruit (apples and pears) and ornamentals	Fireblight (*Erwinia amylovora*)	S, O, K, G, OA, SC
Citrus	Citrus greening disease (*Candidatus Liberibacter spp*) Citrus canker (*Xanthomonas axonopodis pv. citri*)	S, SC
Stone fruit (Prunus)	Bacterial spot and canker (*X. arboricola* pv. *pruni*) Bacterial blast (*Pseudomonas syringae*)	S, O
Rice	Bacterial panicle blight (*Burkholderia glumae*) Bacterial leaf blight (*X. oryzae* pv. *oryzae*)	OA, SC

Plant	Disease	Antibiotics used
Tomato	Bacterial canker (*Clavibacter michiganensis pv michiganensis*) Bacterial speck (*Pseudomonas syringae* pv tomato) Bacterial spot (*X. campestris pv vesicatoria*)	O, G, SC
Potato	Blackleg (*Pectobacterium atrosepticum*) Bacterial wilt (*Ralstonia solanacearum*) Soft rot (Pectobacterium)	O, G, SC
Capsicum	Bacterial spot (*X. campestris pv vesicatoria*)	S, K, G
Cauliflower and broccoli	Bacterial soft rot (*Erwinia* species)	G
Cabbage	Bacterial black rot (*X. campestris* pv. *campestris*)	G
Agave	Heart rot (*Erwinia* species)	G
Watermelon	Black rot (*Xanthomonas* species)	G
Kiwi fruit	Kiwi canker (*Pseudomonas syringae pv. actinidiae*)	S, K
Bean	Halo blight	SC
Tobacco	Wildfire (*Pseudomonas syringae pv. tabaci*)	S, SC

Reports from India and Thailand support the suspicion that there is extensive unauthorised use of antibiotics on a further range of crops to control bacterial diseases (Wu et al., 2018, Khullar et al., 2019, Centre for Science and Environment, 2019).

Farming Practice

Most applications of antibiotics are by spray treatments in orchards (Vidaver, 2002). Antibiotics used in crop protection are typically formulated as powders with between 17% to 20% active ingredient. The antibiotic is dissolved or suspended in water to concentrations of between 50 to 300 ppm and then sprayed as a fine mist onto the susceptible part of the plant. Due to their expense, their use has generally been limited to high-value crops such as fruit trees, vegetables and ornamentals (McManus et al., 2002).

In some applications, timing of sprays can be critical. For instance, in the case of fireblight control, the antibiotic is only effective during flowering, when the bacterium is spread from overwintering cankers to open flowers by bees, wind or rain. Once the bacterium has migrated from the floral tissue into stems and branches, causing wilting and dieback, the antibiotic is no longer effective. Sprays are usually applied every five days as the antibiotic only remains active for less than a week. However, the need for sprays is also dependent on weather conditions. If temperatures during flowering are too low to support pathogen development, it is not necessary to spray and in countries such as the US, judicious use of disease risk models has significantly reduced the number of sprays and volume of antibiotics used.

A recent FAO report stated that “in countries where regulations and oversight of antibiotic use are strong, the use of antimicrobials and their residues on foods of plant

origin is minimal. However, in LMICs, the quantity and types of antimicrobials being used for agronomic application are undocumented – a problem compounded by challenges of access to quality-assured antimicrobials, including a growing industry of fraudulent and substandard products" (FAO Antimicrobial Resistance Working Group, 2018). In many LMICs there is little if any effective regulation, control and monitoring of antibiotic use.

Table 2 provides a summary of antibiotic use in plant agriculture in the specific countries for which we have information. Whilst Table 2 covers a very limited number of countries for which any kind of information is available, it provides an indication of the types of food crops that may be treated with antibiotics in countries for which we do not have data, and where there may well be inadequate controls over the use of antibiotics on crops.

Table 2. Summary of antibiotic use in plant agriculture in the specific countries for which we have information.

Country	Streptomycin	Oxytetracycline	Kasugamycin	Gentamicin	Oxolinic Acid	"Streptocycline"
USA	Pome fruit (*Erwinia amylovora*) Citrus trees (citrus canker and citrus greening disease) Also used to control diseases of floriculture, potato tubers, tobacco seedlings and vegetable seedlings	Pome fruit (*Erwinia amylovora*) Stone fruit (*Xanthomonas arboricola pv. pruni*)	Apples (*Erwinia amylovora*)	Not approved	Not approved	Not approved
Canada	Fireblight control	Not approved	Not approved	Not approved	Not approved	Not approved
Mexico	Pome fruit (*Erwinia amylovora*)	Apples (*Erwinia amylovora*) Vegetable crops (*Pectobacterium, Pseudomonas* and *Xanthomonas*)	No data	Pome fruit (*Erwinia amylovora*) Vegetable crops (*Pectobacterium, Pseudomonas, Ralstonia* and *Xanthomonas*)	No data	No data

Country	Streptomycin	Oxytetracycline	Kasugamycin	Gentamicin	Oxolinic Acid	"Streptocycline"
Central America (Costa Rica, Honduras, Guatemala, El Salvador)	No data	Apples (*Erwinia amylovora*) Vegetable crops (*Pectobacterium, Pseudomonas* and *Xanthomonas*)	No data	Vegetable crops (*Pectobacterium, Pseudomonas, Ralstonia* and *Xanthomonas*)	No data	No data
Chile	No data	No data	No data	Pears (*Erwinia amylovora*) Tomato (*Clavibacter michiganensis subsp. michiganensis*)	No data	No data
EU/EEA	Not approved Emergency use to control fireblight (Austria, Switzerland) in small quantities	Not approved	Not approved Emergency use to control bacterial diseases on pome fruit, capsicum, tomato and cucumber (Hungary, at least until 2016)	Not approved	Not approved	Not approved
Israel	Fireblight control	No data	No data	No data	Pears (*Erwinia amylovora*)	No data
Japan	No data	No data	No data	No data	Rice seeds and plants (*Burkholderia glumae*)	No data

Country	Streptomycin	Oxytetracycline	Kasugamycin	Gentamicin	Oxolinic Acid	“Streptocycline”
Kazakhstan	Not approved Only allows the use for scientific research for control of *Erwinia amylovora* on apple. (laboratory research only)	Not approved	Not approved	Not approved	Not approved	Not approved
India	No data	No data	No data	No data	No data	Streptocycline (streptomycin (90%)/tetracycline (10%) mix) is recommended for use against bacterial diseases on bean (halo blight), potato (blackleg, soft rot), tea (blister), tobacco (wildfire), tomato (leaf spot), apple (fireblight), citrus (canker), and “paddy” i.e. rice (bacterial leaf blight)

Country	Streptomycin	Oxytetracycline	Kasugamycin	Gentamicin	Oxolinic Acid	"Streptocycline"
New Zealand	Pome fruit (*Erwinia amylovora*) Stone fruit (*X. arboricola pv. pruni, Pseudomonas syringae*) Kiwi fruit (*Pseudomonas syringae pv. actinidiae*) Tomato (Bacterial diseases of seedlings) - glasshouse	Not approved	Kiwi fruit (*Pseudomonas syringae pv. actinidiae*)	Not approved	Not approved	Not approved
Rwanda	Not approved	Not approved	Not approved	Not approved	Not approved	Not approved

Misuse

Examples of misuse have been difficult to pin down. In their response to our questionnaire, the respondent from New Zealand reported minor misuse of streptomycin (unapproved application method of streptomycin on kiwi fruit), but no other responders have provided examples, nor are specific examples provided in official reports and peer-reviewed papers although the FAO report (FAO Antimicrobial Resistance Working Group, 2018) describes problems not so much with misuse by growers, but an expanding industry in fraudulent or sub-standard products on the market. The reliability of any national or international markets for the production and sale of antibiotic products for plant protection was not assessed in this review.

Less official reports of misuse, while still rare, do exist and potentially give an indication of the kind of misuse of antibiotics that may be occurring quite widely. Evidence suggests that regulation in India is not strong, with widespread use or antibiotics on unapproved crops, and conflicting recommendations being provided by different organisations.

For instance, in November 2019, the Indian Centre for Science and Environment reported routine and indiscriminate use of “streptocycline” (a 90:10 mix of streptomycin and tetracycline) in high doses, including in crops for which it is not approved (Centre for Science and Environment, 2019). Farmers were found to be unaware of recommended use and dosages, and applied antibiotics like any other pesticide. In one case, a farmer admitted to mixing streptocycline with a host of other chemicals and spraying it on the plants twice a week throughout the crop season. Another vegetable farmer said that he routinely used streptocycline on cauliflower, cabbage, spinach, bottle gourd, apple gourd, cucumber, mustard, brinjal, fenugreek, radish and coriander, whereas the Central Insecticide Board and Registration Committee (CIBRC), the body that approves the use of pesticides in India, only recommends its use on beans, potatoes and tomatoes. Researchers also found that farmers usually dissolve an entire packet of streptocycline in different volumes of water, depending on the capacity of the spray tank they are using (Khullar et al., 2019). The resulting concentration can be three to four times higher than the recommended rate.

Furthermore, while the CIBRC allows use of streptocycline on 8 crops (bean, potato, tea, tobacco, tomato, apple, citrus and rice (“paddy”)) state agriculture institutes that offer an Agricultural Extension Service (Krishi Vignan Kendra), recommend use on a further 12 crops not recommended by the CIBRC (betel vine, brinjal, cabbage, cauliflower, onion, ginger, banana, mango, pomegranate, watermelon, gram (chickpea), sesame). They in turn are basing their guidance on recommendations from the Indian Council of Agricultural Research (ICAR), suggesting a confused picture of national guidance. Additional, unrecommended use was recorded on apple gourd, bottle gourd, carrot, coriander, cucumber, fenugreek, garlic, lady finger, radish, spinach, grape, mango, pomegranate and mustard.

Another example of misuse was reported online in November 2018 by newspaper The Nation Thailand. A report highlighted the apparent long-standing illegal use of amoxicillin injection into orange trees, three to four times a year, to treat Citrus

greening disease and said that orange farmers did not realise the danger of the practice. The Thai Food and Drug Administration is now working with plant health offices to check on orange orchards for the distribution of amoxicillin to orange farmers and ensure that the sale of antibiotics is made only at pharmacies with pharmacists (The Nation Thailand, 2018).

Risks

With regards the antibiotic itself, research shows that antibiotics breakdown rapidly after application (less than a week) and so remain active for only a short period. Additionally, for fireblight at least, the application of antibiotics is made before fruit formation and so fruit are not directly exposed. Therefore, antibiotics are unlikely to be found either on or in apples and pears.

The use of antibiotics to control high value vegetables in countries that do not have adequate controls in place potentially pose a greater risk. It is very likely that antibiotics are being sprayed directly onto edible parts of the plant and potentially in large quantities. But again, it is likely that the antibiotic itself will have broken down long before the produce arrives in the UK or EU. Antibiotic breakdown products may therefore act as an indicator that produce which has entered the UK has previously been exposed to antibiotics.

Perhaps the greater risks are therefore due to the presence of AMR genes or bacteria on the plant material. AMR evolution in streptomycin-treated apple orchards has been observed in the US in plant pathogens, epiphytic bacteria, and even an opportunistic human pathogen (Rajashekara et al., 2019). In terms of the risk of foodborne AMR pathogens entering the UK, it is also worth considering whether LMICs may have a greater risk of contamination of produce with foodborne pathogens, as well as exposure to antibiotics. Any AMR organisms present may plausibly persist on produce for longer than the original antibiotics themselves, especially if the fitness cost of maintaining AMR mechanisms is low. Even exposed DNA, or DNA in non-viable organisms, is a potential AMR risk if transferred into the consumer's microbiome, as DNA containing AMR genes can be horizontally transferred between bacteria.

If the simple assumption is made that antibiotic treated crops are more risky than untreated, then it would seem prudent to test any such crops from countries where antibiotics are used. However, there is a lack of data available for many countries around the world and so we don't necessarily know whether an import from such a country may have been exposed to antibiotics. There is also evidence that in countries with weaker or non-existent controls over antibiotic use, antibiotics are more likely to be used contrary to any recommended dose, crop or method of application. These countries are likely to be LMICs. Therefore, targeting countries known to use antibiotics, such as the USA and New Zealand, does not necessarily give us the best chance of identifying risky consignments.

Summary

- The primary use of antibiotics in plant agriculture it to control bacterial diseases of pome fruit, stone fruit and vegetables. The largest use by far is in control of fireblight in the USA, though this may be superseded by use on citrus to control of citrus greening disease following recent approval by the US EPA.
- Antibiotic products are in the form of a powder (roughly 17-20% active ingredient) which is then dissolved or suspended in water at around 50-300 ppm and sprayed onto plants. Application of such sprays should be timed carefully to be most effective, and weather conditions considered to avoid unnecessary spraying.
- Antibiotics are approved and used in several countries. There is significant use in the USA where they have been used and regulated for many years. The use of antibiotics in such countries is controlled and closely monitored and no problems have been identified due to their use. Issues have arisen from resistance, particularly to streptomycin, which has led to the approval of other antibiotics such as kasugamycin, and an understanding that antibiotics should be used as part of an integrated approach to disease control.
- Evidence shows that in countries where regulation and control of antibiotic use is not strong, serious cases of malpractice may be likely. For instance, in India there have been recent reports (2019) of rampant misuse of antibiotics, with a mix of antibiotics being applied at very high doses, often in combination with other chemicals, too frequently, and on a wide range of vegetable crops for which use is not approved. It is in LMICs that widespread malpractice is more likely to occur, through lack of regulation and education, and on high value vegetables that may be grown for export.

Prevalence of Antibiotics and Antimicrobial Resistance Genes in Crops

Antibiotics in Crops

Although the use of antibiotics on crops for plant protection is currently limited, antibiotics may also enter the crop agricultural environment due to the use of a) manure or sewage sludge as a soil amendment and/or fertiliser, or b) via the use of (treated) wastewater effluent or similar as irrigation water, or the use of contaminated surface-water as irrigation water. During the initial literature search, it was evident that there had been a wealth of research on the presence of ARGs or similar on food stuffs, particularly where the foods can be eaten raw, i.e. those posing the greatest risk to human health. However, the source of these genes could be from elsewhere along the supply chain so this information is excluded from this section of the review which will focus on antibiotics and/or ARGs present in food stuffs arising from the use of a) manures and similar, and b) irrigation water.

There is substantial evidence that veterinary medicines are widely present in soil due to the use of organic fertilisers (e.g. manure, sewage sludge) and that these compounds can be internalised by plants (Boxall et al., 2006, Dolliver et al., 2007, Dong Hee et al., 2013, Christou et al., 2019). Indeed, the uptake of pesticides by crops (Plant Uptake Factor) is used in regulatory environmental fate models such as Pesticide Emission Assessment at Regional and Local scales (PEARL) or PEsticide Leaching MOdel (PELMO), to represent the proportion of plant protection product absorbed into the plant via the roots, thereby reducing the amount available for leaching into surface or groundwater and reducing the environmental risk to aquatic organisms. As the environmental fate of a compound is driven by its physico-chemical properties rather than its initial use, it could be expected to find antibiotics and other soil-derived xenobiotics in plants.

It is apparent from the literature that the extent of antibiotic uptake is dependent on several factors such as the antibiotic compound, crop type, manure type, soil properties, and the initial concentration in the soil and/or manure (Bassil et al., 2013), although on other occasions this does not impact on uptake (Dolliver et al., 2007). As an organic compound, antibiotics are subject to absorption and degradation depending on a) soil properties such as organic carbon content, pH and clay content, and b) the physico-chemical properties of the soil (Blackwell et al., 2009, Du and Liu, 2012, Wegst-Uhrich et al., 2014, Pan and Chu, 2016, Andriamalala et al., 2018, Shen et al., 2018). However, sorption can vary widely, and, unlike many other industrial compounds and pesticides, this variation cannot be explained easily by hydrophobicity and soil organic carbon content (Boxall et al., 2002). For sulphonamides and macrolides, pH is a significant factor affecting sorption, but the concentration also impacts on their environmental fate (Wegst-Uhrich et al., 2014). This mobility of the antibiotic in the soil influences the bioavailability of the compound to the plant (Yu et al., 2019), hence the processes influencing plant uptake and translocation of antibiotics is highly complex.

Due to the many variables that impact on the concentration of antibiotics in crops, the research conducted is highly varied depending on the particular interest of the researchers, with many different antibiotics being investigated. Many studies quantified antibiotics in different parts of the plants (e.g. roots, leaves, stem) as well as the soil. This section of the review, i.e. antibiotics in crops not resulting from intentional application, has focused on the most-commonly eaten part of a plant (e.g. tomato fruit, cereal grain, leaf – pak choi, lettuce, spinach etc), and it has assumed that radish and carrot root refers to the part normally eaten.

An overview of concentrations of antibiotics found in crops is presented in Table 3. The table includes data from crops grown in manure-amended soil, and soil irrigated with simulated or actual wastewater as the experimental methods to examine the variables were very similar (spiking soil with antibiotic-containing solvent or irrigating with antibiotic-containing water). For studies investigating residues in plants on land that has previously been irrigated with wastewater, the source is effectively the same as manure amended soil, i.e. antibiotics contained in the soil. The data in Table 3 illustrate the wide variability in food crops and antibiotics investigated but, on the whole, antibiotic concentrations in the commonly eaten parts of plants were low < 10 µg/kg. The anomalously high value of sulfamethazine detected in lettuce leaf from Dolliver et al. (2007) is likely to be due to the much larger initial concentrations used (mg *cf* µg in many of the other studies) and the sandy soil.

In addition to the overview table, selected case studies are presented to illustrate the extent to which some of the aforementioned factors can influence antibiotic uptake in crops. Although there are a few exceptions, what is evident is that the majority of crops types investigated are fast-growing (and hence suited to laboratory experiments) and/or crops that can be eaten raw, as these would be likely to pose the highest risk; the amount of antibiotic consumed would be reduced by peeling vegetables. The majority of work has focussed on antibiotics, but as these are metabolised within the plant, so metabolites are produced and a few recent papers have also quantified metabolites (Tian et al., 2019, Tadic et al., 2019).

Table 3. Antibiotic concentrations observed in commonly investigated crop types.

Crop	Antibiotic	µg/kg in plant material	Country	Data source
Carrots (root)	Ciprofloxacin	0	Norway	(Eggen et al., 2011)
	Monensin	< 3.44 - 4*	USA	(Kang et al., 2013)
	Sulfamethazine	< 0.98		
	Tetracycline	< LOQ – 1.33	China	(Pan and Chu, 2017)
	Sulfamethazine	< LOQ – 0.37		
	Norfloxacin	2.52 – 6.54		
	Erythromycin	< LOQ – 0.52		

Crop	Antibiotic	µg/kg in plant material	Country	Data source
	Chloramphenicol	0.96 – 3.99		
Lettuce leaf	Sulfamethazine	1000* – 1500*	USA	(Dolliver et al., 2007)
	Sulfamethazine	1.3* - 1.8*	USA	(Kang et al., 2013)
	Tetracycline	1.35 – 1.85	China	(Pan and Chu, 2017)
	Sulfamethazine	< LOQ		
	Norfloxacin	2.88 – 7.43		
	Erythromycin	< LOQ		
	Chloramphenicol	0.86 – 2.72		
	Ciprofloxacin	< 3.8 – 4	USA	(Sidhu et al., 2019)
	Azithromycin	< 0.8 - 4		
Radish root	Gentamicin	0.051	Lebanon	(Bassil et al., 2013)
	Streptomycin	0.015		
	Chlortetracycline	< LOD	China	(Chung et al., 2017)
	Enrofloxacin	< LOD		
	Sulphathiazole	< LOD		
	Oxytetracycline	8.3	China	(Hu et al., 2010)
	Tetracycline	< LOD		
	Chlortetracycline	< LOD		
	Sulfamethoxazole	< LOD		
	Sulfadoxine	0.1 – 0.4		
	Sulfachloropyridazine	< LOD		
	Chloramphenicol	< LOD		
	Ofloxacin	< LOD		
	Pefloxacin	< LOD		
	Ciprofloxacin	< LOD		
	Lincomycin	0.9 – 3.1		
	Monesin	< LOD	USA	(Kang et al., 2013)
	Sulfamethazine	1.1*		
	Ciprofloxacin	< LOQ	USA	(Sidhu et al., 2019)
	Azithromycin	< LOQ		

* Approximate value – data read from graph

Selected case studies are given below to illustrate the effect of specific factors on antibiotic uptake by plants.

Compound

The extent to which the compound influences the amounts detected in a plant is illustrated by Zhao et al (2019) who investigated the bioaccumulation and translocation of 14 antibiotics in a single species (peanuts; *Arachis hypogaea* L.) in fields that had received pig manure for over 15 years. All the antibiotics investigated were detected in manure and the (manure-amended) soil. Only two of the antibiotics (sulfamethoxazole (SMX) and norfloxacin (NOR)) were not detected in any of the peanut kernel samples whereas sulfamerazine (SMR), sulfamethazine (SMZ),

chlortetracycline (CTC), tetracycline (TC) and ofloxacin (OFL) were detected in over 90% of the samples. The highest mean concentration in peanuts was 20 µg/kg CTC and the TCs were detected at the highest concentrations in the soil. However, there was not a clear relationship between concentration in the soil and concentration in the peanut and ofloxacin (OFL) and Ciprofloxacin (CIP) had higher mean concentrations in the peanuts than the soils. It is evident from Figure 1 that there was substantial intra- and inter-sample variation in the amount of compound detected in the peanut. The authors also analysed the roots, shell, stem and leaves and variation in the plant concentrations between compounds was again evident.

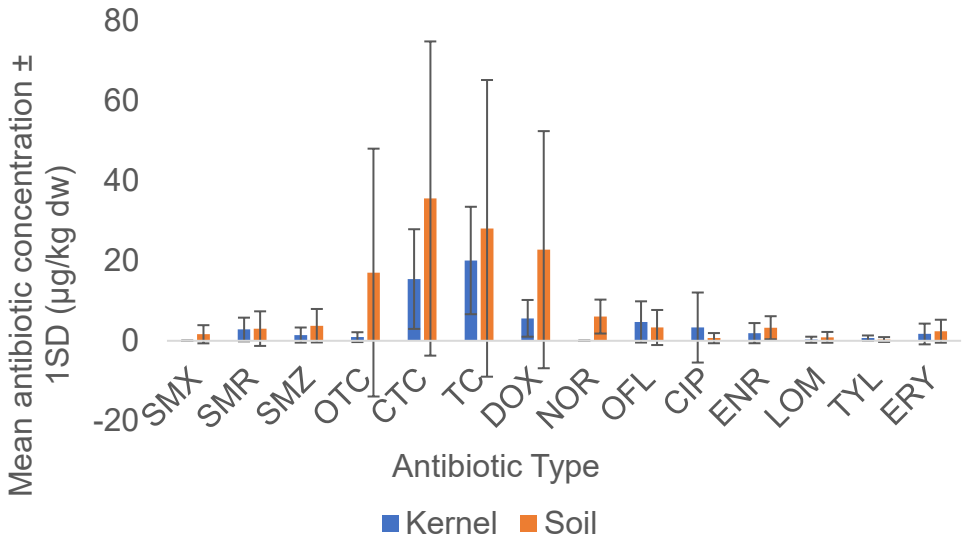


Figure 1. Mean antibiotic concentration in peanut kernels and soil. n= 3 at each of 30 sites. Data from Zhao et al., 2019.

Plant Type

The extent to which plant type influences antibiotic uptake is demonstrated by an investigation using different cultivars of a single crop (pak choi; *Brassica rapa* subsp. *Chinensis;* (Yu et al., 2019)). Their results (Figure 2) illustrate that a) even within a single type of crop, antibiotics are accumulated to different extents by the different cultivars, b) different antibiotics were accumulated to different extents, and c) antibiotics are prevalent in soils - their soil was collected from a vegetable growing area in Beijing and plants grown in the control soil contained all the antibiotics investigated.

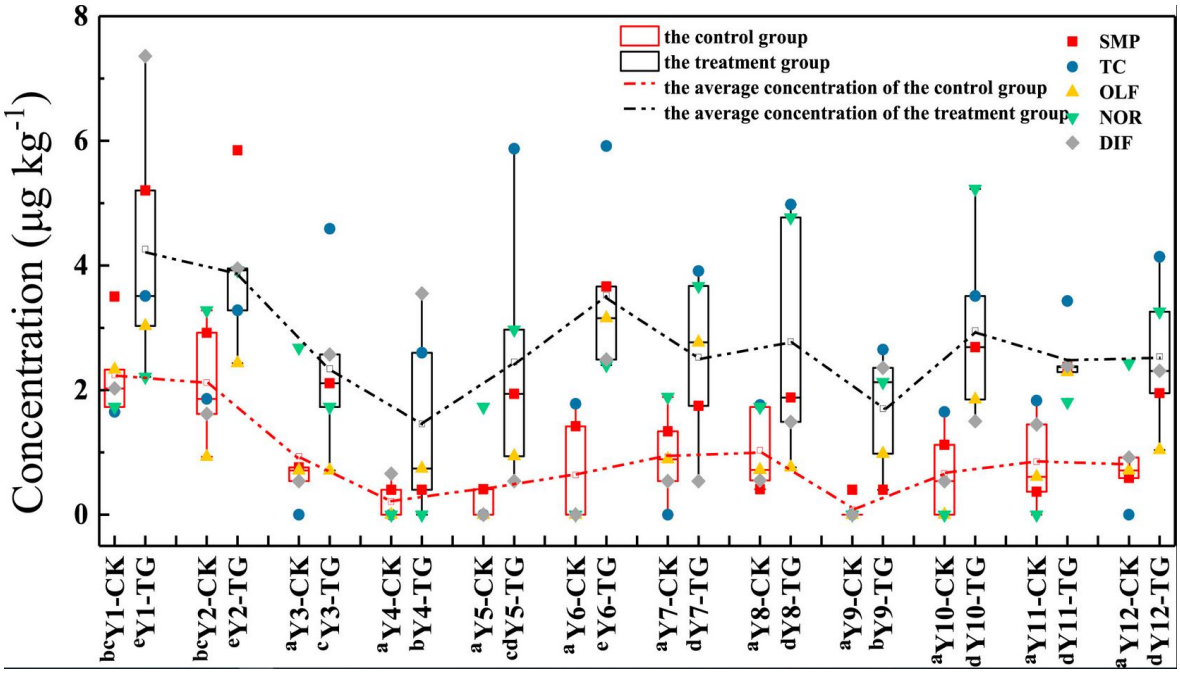


Figure 2. Antibiotic concentrations (fresh weight) in edible parts of 12 cultivars (Y1 – Y12) of pak choi in a control group (CK) and treated group (TG). Dot symbols represent the mean, inner line of box plot is the median and the ends are the upper and lower quartile. Different letters indicate significant difference according to the LSD test ($p<0.05$). Graph from Yu et al., 2019.

Manure Type

The impact of manure type and compositing the manure on antibiotic uptake has not provided any conclusive evidence. Kang et al. (2013) considered the uptake of monensin and sulfamethazine in soil amended with raw and composted hog manure and turkey manure, growing 9 different plant types at 2 different farms in the USA. Overall, there were no detectable levels of antibiotic in the crops. However, there is evidence that composting does reduce the amount of antibiotic and ARGs in manure (Selvam et al., 2013, Youngquist et al., 2016, Chen et al., 2018, Wallace et al., 2018).

Exposure Duration

Yu et al. (2019) demonstrated a positive correlation between uptake and growing time for tetracycline and difloxacin in pak choi, but concentrations of sulfamethoxypyridazine and ofloxacin did not vary significantly with growing time. However Li et al. (2013) also investigating pak choi, detected an initial increase in sulphonamide uptake but after 10 to 12 days, concentrations declined. Indeed, the concentration over time of antibiotics in plants is used to assess their metabolism (e.g. (Tian et al., 2019)). In this review, focus has been given to studies with a full harvest time where possible. The impact of exposure time is also demonstrated in the section on hydroponics.

It is not possible to draw comparisons between the many studies due to experimental differences such as analytical methods/limits of detection, soil type, amounts of antibiotic applied, duration of growth, manure source (i.e. which animal) and application method; in some cases, the soil was spiked directly to emulate antibiotics incorporated into the soil. However, a number of authors have proposed that there is a negative correlation between the size of the molecule and uptake (Kumar et al., 2005, Bassil et al., 2013, Miller et al., 2016, Li et al., 2019c).

Irrigation Water

Irrigation is an essential part of agriculture. Different sources of water can be used for crop irrigation including surface water, groundwater, rainwater stored in reservoirs, untreated wastewater (Uyttendaele et al., 2015) and treated wastewater (Steele and Odumeru, 2004). The pathogenic risk of irrigation water is well documented (Westcot, 1997, Steele and Odumeru, 2004) and in 2006 the Organisation for Economic Co-operation and Development (OECD) produced guidelines for the safe use of wastewater, excreta and greywater. More recently, the EU have developed the OECD guidelines, along with experiences from other countries, to develop minimum quality requirements for water reuse in agricultural irrigation (and aquifer recharge) (Alcalde-Sanz and Gawlik, 2017). This is of increasing relevance given that water reuse has been identified as a solution to address water scarcity (Pistocchi et al., 2018). However, the quality of irrigation water in relation to human health has focussed almost exclusively on microbiological parameters (Uyttendaele et al., 2015, Akinde et al., 2016). However, the EU irrigation water quality standards do include some physico-chemical parameters for monitoring (biological oxygen demand, total suspended solids, turbidity), whereas there is a large body of evidence that the efficacy of wastewater treatment works (WwTW) to remove pharmaceuticals is variable and pharmaceuticals, including antibiotics, are commonly detected in effluent (Nakata et al., 2005, Behera et al., 2011, Gao et al., 2012, Verlicchi et al., 2012, Jiang et al., 2014). In the UK, pharmaceuticals contained in effluent discharging into rivers from as many as 890 WwTW may cause exceedances of estimated riverine predicted no-effect concentrations to the aquatic environment (Comber et al., 2018). The presence of antibiotics in treated wastewater is therefore a reality even in developed countries. The EU minimum quality standards for agricultural irrigation water recognises the potential importance of chemicals of concern, and antimicrobial resistance, but concluded that it was not possible to provide irrigation water quality standards due to the lack of supporting evidence. A recent review on irrigation water quality (Malakar et al., 2019) also identified that the effect of compounds such as pharmaceuticals and antibiotics on food crops and human health due to increased wastewater re-use for agricultural purposes was a major research gap.

The exposure route of crops to antibiotics applied in irrigation water may be direct on contact, and/or indirect via the soil depending on the application method and/or growth stage of the crop. Studies quantifying antibiotic uptake by plants via irrigation re-iterate a) the ease with which antibiotics can be assimilated by plants, and b) the variability both within and between studies.

Franklin et al's (2016) work, from the USA, is one of the very few studies to include a non-vegetable crop and one grown in field conditions. A wheat crop received treated wastewater via spray irrigation every week during the growing season and irrigation was stopped 6 weeks before harvest. Antibiotics were detected, albeit at low concentrations in the wheat grain but this varied with time and compound (Table 4).

Table 4: Mean antibiotic concentration detected in wheat grain 3 weeks before harvest and at harvest, following spray irrigation till 6 weeks before harvest. Data from Franklin et al., 2016.

Antibiotic (and analytical recovery)	Mean concentration µg/kg ± SD	
	3 weeks before harvest	At harvest
Sulfamethoxazole (71%)	< 0.365 (LOQ)	0.64 ± 0.37
Trimethoprim (84%)	< 0.418 (LOD)	<0.418 (LOD)
Ofloxacin (44%)	< 0.600 (LOD)	2.28 ± 0.89

The authors postulated that the higher concentration of sulfamethoxazole at the point of harvest was due to a significant influx of water and nutrients into the grain during the final stages of maturation and, as the grain matured and excess water was removed, it was likely that enzymatic activities decreased allowing the compound to accumulate.

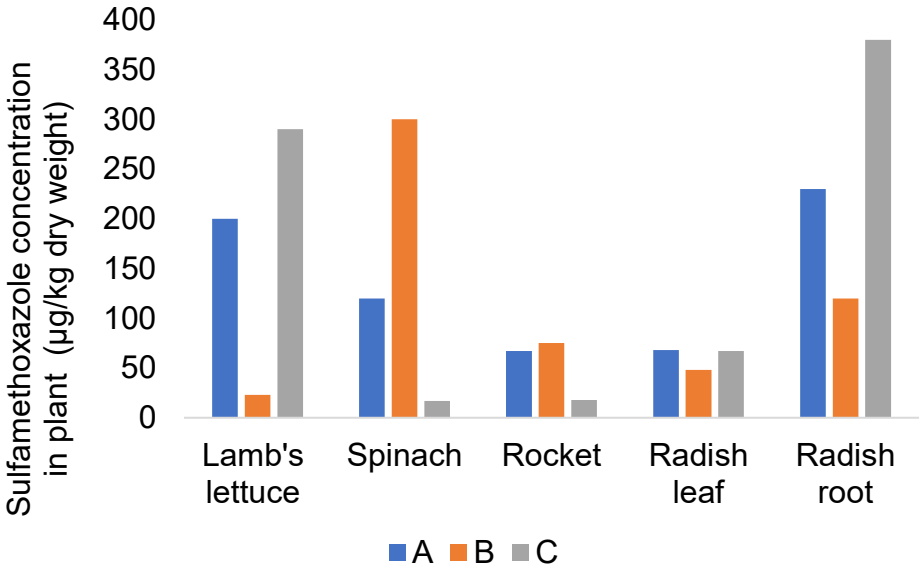
Another study in the United States (Jones-Lepp et al., 2010) was conducted under real field conditions using treated wastewater, but with irrigation via furrows. The vegetables grown were peppers, tomatoes, melons, lettuce, watermelon, spinach and carrots but no antibiotic (azithromycin) was detected in the plants although the analytical method had low recoveries (19-45%) for food stuffs, i.e. excluding root samples on leafy crops with relative standard deviation from 10-32%. It is worth noting that the authors observed lower production in the effluent-treated plots compared to plots receiving well water, so the antibiotics were having an adverse impact on crop growth.

Kodesova et al. (2019) undertook a comprehensive laboratory study to examine the impact of soil type, plant type, and application via a single compound or mixture on the uptake, translocation and plant metabolism of sulfamethoxazole and two other pharmaceuticals (carbamazepine and atenolol). The complexity of the interactions is illustrated by their results shown in Figure 3. It can clearly be seen that antibiotic uptake into plants is affected by all three parameters, and that the interaction is variable. When applied as a mixture, lambs lettuce contained more antibiotic than spinach or rocket, but when applied as a single compound, uptake by lamb's lettuce on soil B was much lower than uptake on soil C, in contrast to application as a mixture. In addition, when sulfamethoxazole was applied as a single compound, uptake by lamb's lettuce on soil B was lower than all the other plant types in contrast to when applied as a mixture. It is possible that in a mixture, the other pharmaceuticals

contribute to the saturation of sorption sites in the plant surface and/or soil, thus there is more sulfamethoxazole available for uptake, but when applied as a single compound a higher proportion of sulfamethoxazole sorbs to the soil and so is unavailable for uptake.

Uptake by radish leaf has been included for comparison with the leafy vegetables; concentrations in the radish root have been included as this is the part most commonly eaten. The quantities of sulfamethoxazole detected in the plants are not unsubstantial, ranging from ~15 µg/kg to 370 µg/kg. However, the authors noted that the concentrations used in their irrigation water were elevated above environmentally relevant concentrations to assist with analytical detection. Nevertheless, the study has highlighted the complex interaction between factors influencing antibiotic uptake by plants following irrigation. In addition, these authors also observed a negative impact on plant growth. As irrigation was guided by plant needs, slightly different quantities of sulfamethoxazole were applied to the different plants (1.24 – 1.56 mg) with rocket and spinach receiving the highest dose. The results indicate that this slight difference in total application did not affect the comparative results. This contrasts to the findings of Pan & Chu (2017) who noted an increase in plant uptake with a ten-fold increase in initial antibiotic concentration in irrigation water in pot experiments (Figure 4).

I. Application as a single compound



II. Application as a mixture

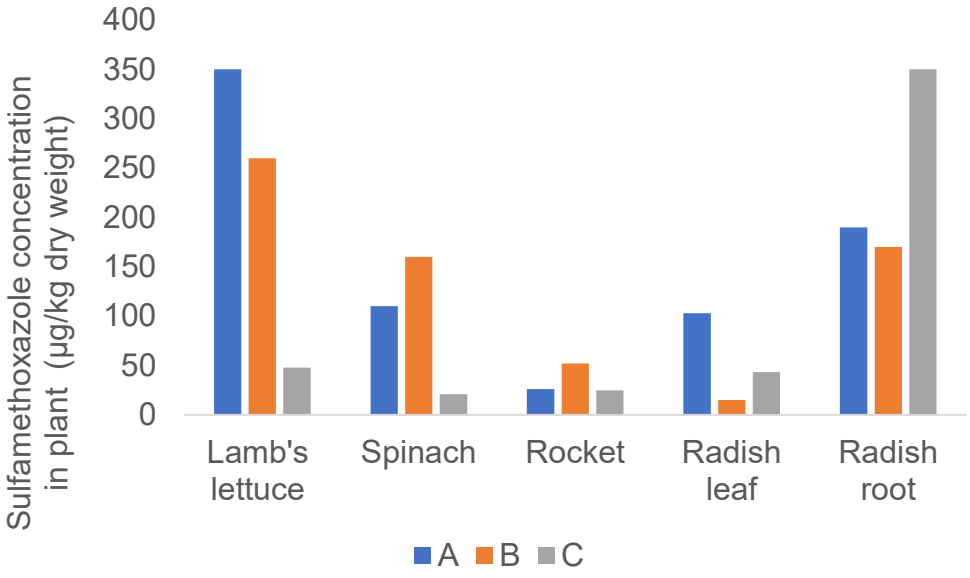


Figure 3. Mean sulfamethoxazole concentrations in plants grown on different soil types (A, B, C) when applied as **i)** a single compound or **ii)** a mixture with two other pharmaceuticals. n=5. Data from Kodesova et al., 2019.

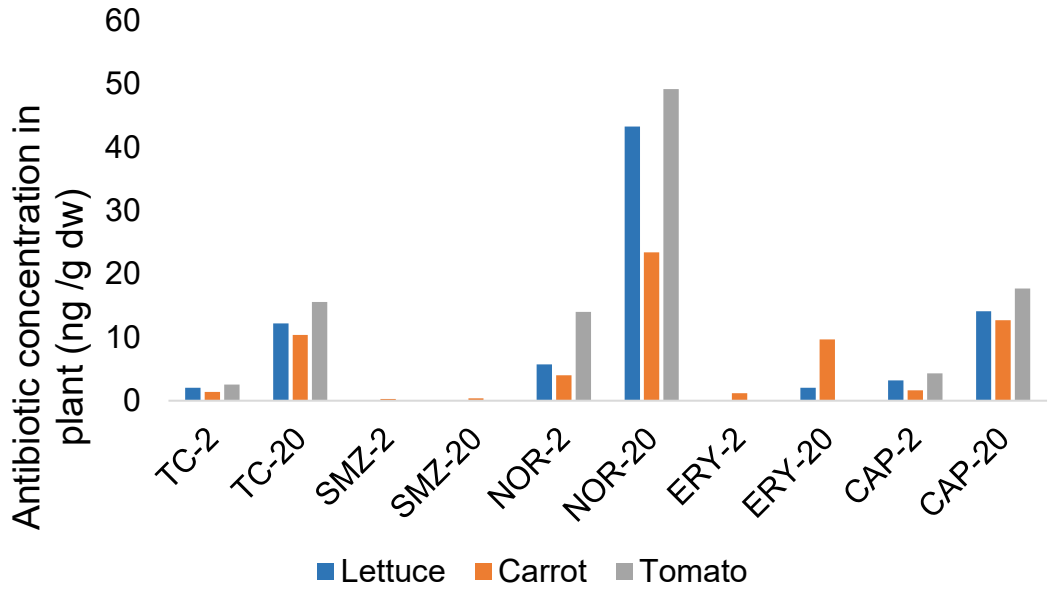


Figure 4. Mean antibiotic concentrations in the common edible part of 3 crop types receiving different initial concentrations of: norfloxacin (NO); erythromycin (ERY); chloramphenicol (CAP), in irrigation water. Irrigation concentration of 2 and 20 µg/L indicated by the suffixes 2 and 20 respectively. n=4. < LOQ was given a value of 0. Data from Pan & Chu 2017.

Pan & Chu (2017) applied either 2 or 20 µg/L of antibiotic as a mixture into the irrigation water that was applied daily. The variability in uptake between crop types and antibiotic is evident with norfloxacin being readily assimilated by the plants (Figure 4). The quantities of antibiotics taken up in Pan & Chu's study were in the order of 10 times lower than Kodesova's study (Figure 3). This may be due to the lower total amounts applied (~ 1.4 mg and < 0.5 mg for Kodesova et al. (2019) and Pan & Chu (2017) respectively), but soil type, duration of exposure/harvest, crop type and antibiotic type may also have contributed to the differences. Further evidence of the variability in the extent of plant uptake depending on plant type and antibiotic, is provided by Hussain et al. (2016) who investigated a number of crop types growing in fields that were treated with pharmaceutical wastewater in Pakistan.

In all of the above work, none of the compounds or variables were the same between studies meaning that it was not possible to compare the results of the studies directly, but it does highlight a number of variables that are relevant to the presence of antibiotics in crops.

Hydroponics

Hydroponics refers to crops grown in an aqueous solution, or with an inert substrate (e.g. sand) to support the roots. It could be expected that antibiotic uptake would be greater in plants grown hydroponically compared to those grown in soil, as the

evidence indicates that antibiotics will adsorb within the soil, reducing the quantity available for uptake in plants. This is one parameter that contributes to the variability in the finding.

The findings from hydroponic studies provide further evidence of the variability in uptake depending on antibiotic and plant type, and this is illustrated in Figure 5.

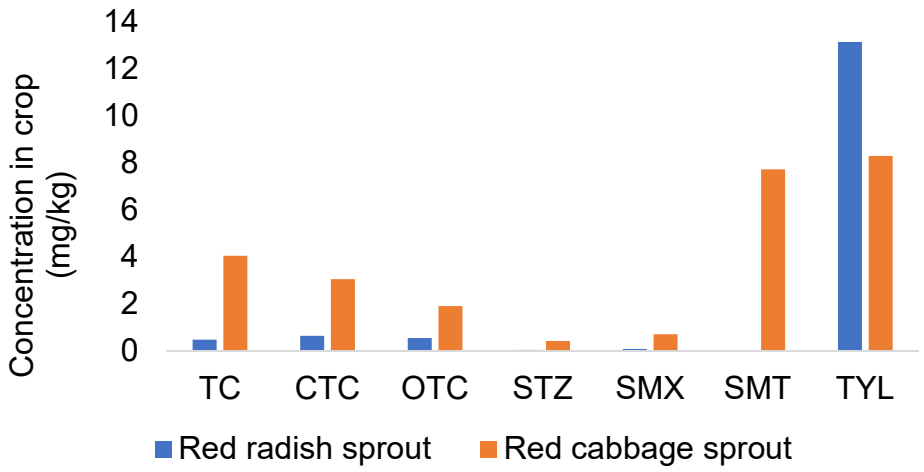


Figure 5. Concentration of antibiotics (tetracycline (TC); chlortetracycline (CTC); oxytetracycline (OTC); sulfathiazole (STZ); sulfamethoxazole (SMX); sulfamethazine (SMT); tylosin (TYL)) in hydroponic seedling sprouts harvested after 8d. Data from (Park et al., 2016).

The data from Christou et al. (2019) illustrate the massive variability that can occur within a single plant type, even within the same experiment, which makes it difficult to make comparisons between compounds and studies (Figure 6). Concentrations in the tomatoes were highest for the fruits that had been on the plant the longest. However, there was a dip in concentrations in the 2nd fruit set for sulfamethoxazole (SMX) applied as a mixture and trimethoprim (TMP) applied as an individual compound illustrating that the conclusions can be affected by the duration of the experiment and whether application is individual or as a mixture; inconsistency was again a key finding. Even within each test, there was variability between the replicates.

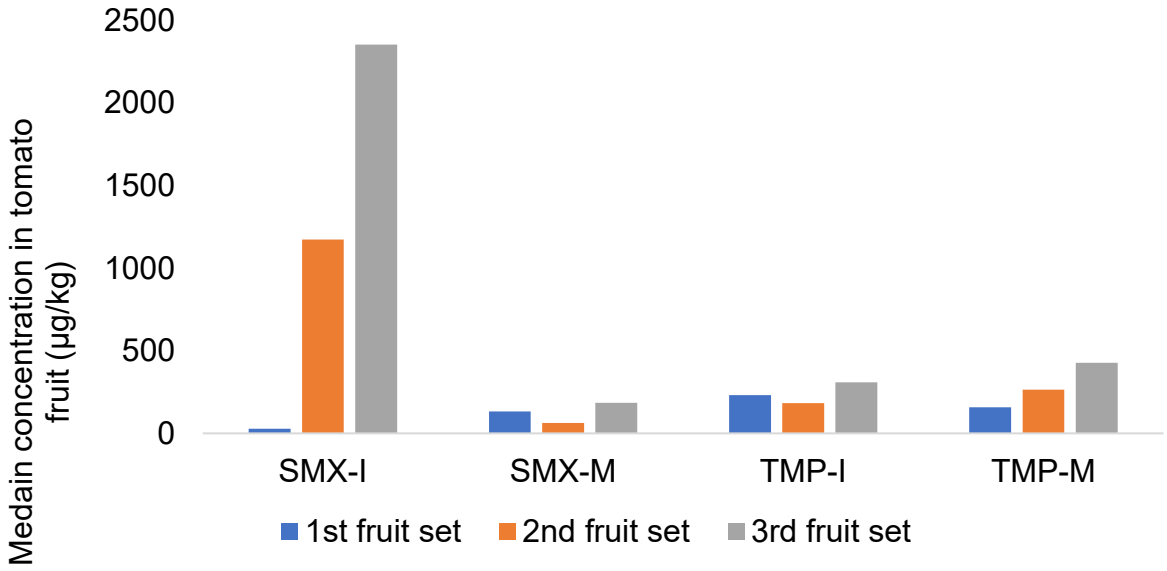


Figure 6. Sulfamethoxazole (SMX) and trimethoprim (TMP) concentrations in tomatoes applied individually (I) or as a mixture (M). Fruit 'sets' were harvested at different times as: 1st: 72-78 days; 2nd: 85-92 days; 3rd 96-102 days. Data from Christou et al., 2019.

In the hydroponic studies there was a clear correlation between initial concentration and concentration in the plant, an observation that was also noted for soil-grown plants.

Table 5. Increase in antibiotic concentration in treated plants as observed by Zhang et al., 2017a and Santiago et al., 2016.

Compound	Increase factor of initial conc.	% increase in plant	Reference
Tetracycline	2	71	Zhang et al. (2017a)
Cephalexin	2	82	
Sulfamethoxazole	2	95	
Ofloxacin	10	94	Santiago et al. (2016)
Ofloxacin	50	288	

What is evident from the hydroponic studies is that the absolute amount of antibiotic detected in the plant is greater than when grown in the soil due to the lack of antibiotic sorption and hence a higher bioavailable amount. Plant concentrations were in the order of 10-1000 µg/kg compared to not detected-10 µg/kg in plants grown in soil.

Antimicrobial Resistance Genes in Crops

Whilst the Web of Science search returned over 40 hits for ARG/ARB in crops/vegetables/foodstuffs, over half these papers were for post-harvest foods. There is clear evidence that ARGs are translocated to crops, but, as with antibiotics, the number of biotic and abiotic factors involved precludes any prediction of the fate of ARGs in the environment/plant with any confidence. The bacteria and/or genes were considered concurrently, and a summary of the findings is presented below.

Several studies have been performed examining the prevalence of ARGs or ARBs on a wide variety of crops grown in manure-amended soils. However, the majority of these have been on experimental plots, rather than surveys of real-world samples. For example, a study of the phyllosphere and root endosphere of lettuce plants grown in soil amended with composted poultry and cattle manure found a number of ARGs (including ARGs for multidrug, beta lactam, aminoglycoside, Macrolide-Lincosamide-Streptogramin B, tetracycline and vancomycin resistance), and evidence of transfer between soil and plant tissues (Zhang et al., 2019c). Growth interval and crop type have been shown to impact ARG transfer from manure amended soil to the phyllosphere and root endosphere in lettuce and endive (Wang et al., 2015). Another study on a wide variety of vegetable types in control, swine- and dairy-manured soil found a number of ARBs (coliforms resistant to amoxicillin-clavulanic acid, ampicillin, cefoxitin, chloramphenicol, nitrofurantoin, co-trimoxazole and chlortetracycline) in soil that had never been manured (Marti et al., 2013). It was also found that manuring increased ARB abundance in soil, but not on the produce itself, although some genes were detected on vegetables only when harvested from manured soil (Table 6).

Table 6. Gene targets that were detected on at least one vegetable sample grown in soil fertilized without or with manure (dairy or swine manure). Primers for blaCTX-M and blaTEM were used only in 2012, whereas all others were used in both years. Data from Marti et al., 2013.

Vegetable	Detected gene(s)[a]					
	No manure		Dairy manure		Swine manure	
	2011	2012	2011	2012	2011	2012
Tomato	*tet*(T), *str*(A)	IncP *oriT*, *incY*, *int2*, *int3*, *tet*(A), *tet*(S), *aad*(A), *str*(A), *str*(B), *erm*(B), *erm*(E), *bla*$_{CTX-M}$, *bla*$_{VIM}$, *bla*$_{TEM}$	IncP *oriV*	*tet*(T), *erm*(F), *bla*$_{PSE}$, *bla*$_{OXA-20}$		*tet*(T), *erm*(F)
Pepper	*int3*, *tet*(T), *str*(B), *sul1*, *vat*(B), *bla*$_{OXAII}$	NA				

Vegetable	Detected gene(s)[a]					
	No manure		Dairy manure		Swine manure	
	2011	2012	2011	2012	2011	2012
Cucumber	IncP *oriT*, IncP *trfA1*, *str*(B), *sul1*, *erm*(B), *bla*$_{OXAII}$	NA	sul2		sul2	
Radish	IncP *oriT*, IncQ *oriV*, *int3*, *aad*(A), *str*(A), *str*(B), *sul1*, *erm*(B), *bla*$_{OXAII}$	IncP *oriT*, IncQ *oriV*, *int2*, *int3*, *tet*(A), *aad*(A), *str*(A), *str*(B), *sul1*, *erm*(B), *erm*(E), *bla*$_{CTX-M}$, *bla*$_{VIM}$, *bla*$_{TEM}$		*erm*(F)	*erm*(A)	*erm*(A), *bla*$_{OXA-20}$
Carrot	IncP *oriT*, IncQ *oriV*, *aad*(A), *str*(A), *str*(B), *sul1*, *erm*(C)	IncP *oriT*, IncQ *oriV*, *int1*, *tet*(A), *tet*(S), *sul1*, *erm*(B), *erm*(E), *bla*$_{VIM}$, *bla*$_{TEM}$		*qnr*(B)		*tet*(B), *tet*(T), *bla*$_{OXA-20}$
Lettuce	NA	IncP *oriT*, IncQ *repB*, *incW*, *int3*, *tet*(A), *tet*(Q), *tet*(S), *aad*(A), *str*(A), *sul1*, *erm*(B), *bla*$_{OXA1}$, *bla*$_{VIM}$, *bla*$_{TEM}$				

The effect of irrigation water on ARG presence has also been investigated. A large study of three crops (lettuce, broad bean and tomato) in Spain found that crop type was the major driver of ARG distribution, and that ARG loads and bacterial diversity decreased from soil to fruit (Cerqueira et al., 2019) (Figure 7). This study did attempt to examine the effect of treated wastewater on crops, but this was confounded by the fact that fields watered with groundwater had been manured with pigeon manure. Marano et al., (2019) found that after several months of irrigation with treated wastewater, crops had lower ARG abundance than in early season samples. The resistance gene *bla*$_{TEM}$ was not linked to irrigation with treated wastewater as abundance was often higher in samples irrigated with tap water (either surface water, ground water or desalinated water). Effluent-derived ARB did not persist in soil or crops. A study of pak choi grown hydroponically in water directly treated with antibiotics found significant increases in both ARBs and ARGs between plants grown in control and treated water (Zhang et al., 2017a).

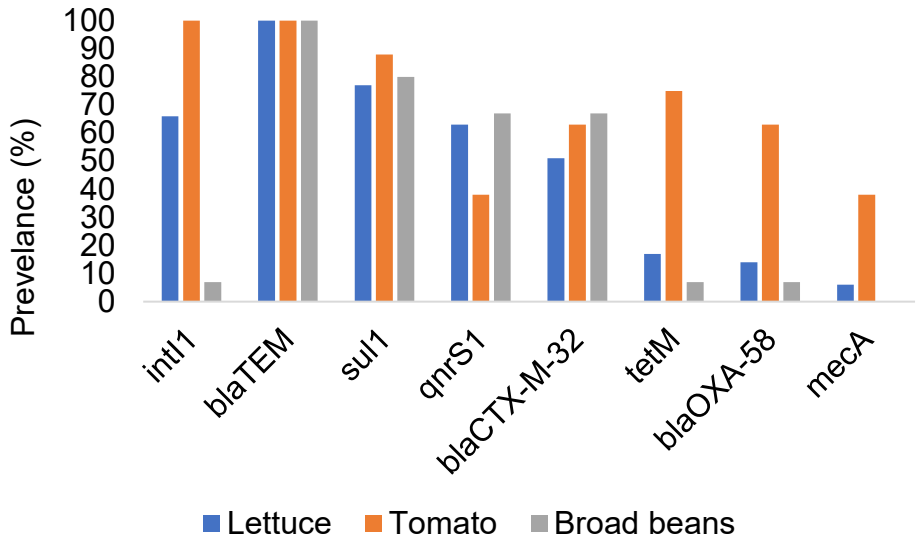


Figure 7. Prevalence of ARGs and insertion elements in lettuce, tomato and broad beans. Data from Cerqueira et al., 2019.

Negreanu et al. (2012) concluded that the overall impact of treated wastewater-associated bacteria on the soil microbiome was negligible, as the resistant bacteria were unable to compete or survive in the soils environment and their control soil contained ARBs that was indicative of native antimicrobial resistance. However this conclusion may be an over-simplification of reality and it conflicts with the findings of Fahrenfeld et al. (2013) who detected elevated levels of *sul1* and *sul2* in soil after repeated irrigation with secondary wastewater effluent. The large number of variables that impact on the uptake of ARG in crops would necessitate a meta-analysis approach before conclusions could be made with any degree of confidence about the risk associated with ARG in crops.

Summary

Antibiotics are readily assimilated into plants, but the results are highly variable. Concentrations in soil-grown crops are generally in the order of <10µg/kg. Plant uptake in hydroponic systems is greater.

Influential variables include:

- Compound;
- Plant type;
- Soil type and physical condition e.g. soil moisture;
- Initial concentration- which is related to manure type and pre-treatment/application method/irrigation water;
- Exposure time.

ARGs are readily translocated into crops but the extent is dependent on the variables above, plus factors that influence plant growth.

Selection Pressure for Antimicrobial Resistance from Non-Antibiotic Sources

There is significant evidence that compounds other than antibiotics are able to favour the emergence or persistence of AMR, via a variety of mechanisms. These can include; cross-resistance, whereby the same mechanisms supply resistance to more than one antimicrobial compound; co-selection, when genes conferring antibiotic resistance are located on the same mobile genetic element (MGE) as genes encoding resistance to other agents; and adaptive resistance, where resistance is due to differences in gene expression caused by environmental variation. Some of the more common agents that enhance antimicrobial resistance, and those most relevant to crop agriculture, are described below.

Metals

The co-occurrence of AMR and resistance to heavy metals is one the most studied co-selective phenomena. Some of the work undertaken in an agricultural context is correlative. For example, in a large study of diverse bacterial isolates from Spanish olive groves treated with copper (Cu) salts found that there was a strong association between Cu-resistant strains and those resistant to ampicillin, vancomycin, erythromycin and trimethoprim/sulfamethoxazole, though no evidence of a correlation was identified for ceftazidime, gentamicin or ciprofloxacin (Glibota et al., 2019). This work itself does not demonstrate the causal link between Cu tolerance and AMR, nor does it test soils that have not been treated with Cu or investigate the genetic basis for antibiotic or Cu tolerance in any strains. However, the correlative evidence is strong, and the existence of Cu-associated antibiotic resistance in soils with environmentally relevant (i.e. low) levels of Cu contamination is disturbing. The authors suggest a number of reasons for this, including; persistence of resistance after short-term, high intensity Cu contamination; existence of microenvironments (e.g. soil particles) with locally high Cu concentrations driving resistance; and the existence of a microflora with naturally high metal resistance (perhaps indicated by the presence of resistance to other metals, including lead (Pb), zinc (Zn) and cadmium (Cd)). In soil microcosm experiments, silver (Ag) ions were found to increase ARG abundance and shift the ARG profile. Ag nanoparticles did not have the same effect, but did significantly increase the relative abundance of efflux pump genes (Chen et al., 2019). Efflux pumps can be associated with resistance to both antibiotics and a range of other antimicrobial compounds (see below).

Several studies have attempted to unravel the mechanisms behind metal-induced resistance to antibiotics. Exposure below minimum inhibitory concentration (MIC) levels of Ag, Cu and Zn increased the mutation rate and enriched mutants of *Escherichia coli* which were resistant to ciprofloxacin, and Ag also increased mutation rate and resistance to chloramphenicol (Li et al., 2019b). Some of this resistance was temporary, but some persisted after multiple passages through metal-free media. Mutational changes were observed in genes responsible for transcription and translation, cell wall structure, and membrane transport, all of which could be associated with antibiotic resistance. Environmentally relevant concentrations of Cu^{2+}

ions and copper oxide nanoparticles have also been found to increase the frequency of plasmid transfer between *E. coli* and *Pseudomonas putida* (Zhang et al., 2019b). This is likely due to overproduction of reactive oxygen species in the donor leading to stress response, and increased cell membrane permeability, contributing to increased transfer rates. Increased transfer of MGEs like plasmids has a high risk of transferring AMR genes between organisms. In other systems such as wastewater Cu^{2+} ions have been shown to drive changes in abundance of ARGs primarily by shifting the bacterial community structure (Ma et al., 2019).

In terms of agricultural importance, Cu is probably the most relevant of the metals shown to affect antimicrobial resistance. Cu-based antimicrobial (especially fungicidal) compounds have been used since the late nineteenth century, and are the most effective active ingredients available in organic agriculture (Lamichhane et al., 2018). The use of Cu-based plant protection products is restricted, though not prohibited, in the EU under regulation 473/2002, and may be widely used outside the EU. According to the most recent figures from the Pesticide Usage Survey, copper oxychloride was the fifth most widely used fungicide on sugar beet in the UK, and was also applied to wheat, winter barley, spring barley, oats, rye and beans (Garthwaite et al., 2018). However, the total amount of Cu applied was very small, less than 0.2% by mass of all fungicides used. Other potentially important sources of Cu (and other metal) exposure in fields include atmospheric deposition, livestock manure and sewage sludge (Nicholson et al., 2003). Industrial waste is a relatively minor source in the UK but could be important in other countries.

A series of pan-European soil surveys found high Cu concentrations primarily in southern European areas (Greece, Italy, Andalusia), but also parts of the UK (Northern Ireland, Wales and the Midlands) (Lado et al., 2008). Comparisons between surveys over time revealed higher average Cu concentrations in later samples (Saaltink et al., 2013, Albanese et al., 2015), possibly indicating an increase in Cu contamination. An analysis of the factors influencing high soil Cu levels revealed anthropogenic factors, primarily the use of Cu as a fungicide in vineyards, olive groves and orchards, were important drivers of soil Cu levels, as well as soil and climatic factors such as high soil pH, organic carbon and clay, and humid and wet conditions (Ballabio et al., 2018). The use of Cu is permitted in organic farming, and a study of organic and conventional vineyards found higher soil Cu in organic vineyards (Steinmetz et al., 2016). A further potential source of Cu is pig manure, as Cu (primarily in the form of copper sulphate) is fed to pigs as a growth enhancer, and much of this is excreted out in manure. The use of pig manure may therefore be an important source of soil Cu contamination, especially in areas with clay soil (Panagos et al., 2018).

Pesticides

In general, pesticides are an integral part of crop production, preventing or reducing spoilage and destruction by insects, fungi, bacteria etc. and/or reduced yields due to competition for water and nutrients from weeds. In the UK, fungicides and herbicides are the most frequently used pesticide type on arable crops accounting for approximately 70% of the total area treated compared to less than 10% for insecticides

(Garthwaite et al., 2018). The widespread and common use of pesticides on crops makes them a highly relevant factor to consider with regards to additional stressors that could influence the development of AMR selection.

Herbicides are a widely-used group of chemicals, with the second highest area treated in the UK after fungicides, and the highest total weight applied of all broad classes of plant protection products (Garthwaite et al., 2018). Both total area treated, and total weight of herbicide applied increased biannually from 2010 to 2018. These compounds therefore have the potential to be very important in terms of AMR selection.

For herbicides, all the evidence found relating to AMR enhancement were examples of adaptive resistance – herbicides inducing temporary resistance to antibiotics when applied in combination. For example, several studies exposed *E. coli* and *Salmonella enterica* serovar Typhimurium to glyphosate-based (Roundup), dicamba-based (Kamba) and the organo-modified polydimethyl siloxans and carbomethyl cellulos co-formulant, Tween80, in combination with a range of antibiotics. When *E. coli* and *Salmonella* Typhimurium were exposed to low levels of the herbicides they were able to tolerate ciprofloxacin above the MIC (Kurenbach et al., 2018). This temporary raising of the MIC enabled ciprofloxacin-resistant mutants to arise and persist. Other combinations showed mixed responses, with some antibiotic-herbicide/co-formulant combinations increasing the effectiveness of the antibiotic, but most leading to larger amounts of antibiotic being required to kill bacteria than when antibiotics are applied singly (Kurenbach et al., 2017). The responses are also species specific, with *E. coli* generally showing weaker responses than *Salmonella* Typhimurium. The use of glyphosate as an example herbicide is appropriate as it is the most widely used herbicide in the world. In the UK, it accounted for 17% of the total herbicide-treated area in 2018 (Garthwaite et al., 2018).

In terms of the mechanisms of cross-resistance, a study of *Enterobacter* spp. showed that under glyphosate stress, a glyphosate-resistant strain upregulated multidrug resistance genes including efflux pumps (*mppA*, *tehA*, *ycgF*) and a pore protein (*ompC*), which could be used to enhance resistance to both glyphosate and a range antibiotics (Fei et al., 2018). Glyphosate was not found to increase mutation rates (Kurenbach et al., 2018, Tincher et al., 2017), or increase the frequency of chromosomal rearrangements (Tincher et al., 2017). Effects were also shown to vary taxonomically (Kurenbach et al., 2017), and by antibiotic class. For example, Extended Spectrum Beta Lactamase (ESBL) *E. coli* were found to be less resistant to glyphosate isopropylamine salt, not more resistant (Bote et al., 2019). This is likely due to the very different resistance mechanisms involved in ESBL response (enzymatic cleavage of antibiotic) and glyphosate tolerance (efflux pump mediated removal of herbicide), and indicates that herbicide-mediated adaptive resistance is most relevant for antibiotics where resistance mechanism is due to antibiotic efflux (e.g. chloramphenicol, ciprofloxacin, tetracycline).

Rather than co-selecting for AMR or inducing mutations, the AMR risk from herbicides lies in raising the MIC for antibiotics enabling bacteria to be exposed to tolerable levels

of antibiotic for sufficient time for AMR to evolve. Interestingly, in one study the maximum effect was seen at glyphosate levels higher than are internationally permitted (Kurenbach et al., 2015). This means that, if herbicide levels in food complied with the law, they shouldn't on their own induce AMR evolution in a consumer's microbiome. However, the effects seen were additive when combined with other chemicals (e.g. dicamba, salicylic acid), and a detectable effect was observed after doses lower than the label-specified application rate (Kurenbach et al., 2015), implying a risk still exists. This could be especially important, given the misuse examples highlighted above around mixing of antibiotics, and combined application of antibiotics and pesticides (see section Use of Antibiotics in Crops). The studies to date have focussed on only two herbicides (glyphosate and dicamba), meaning the risks from other widely used herbicides are unknown.

Compared to herbicides, other types of pesticides are poorly studied as a co-selection pressure for AMR. However, a study of the effects of soils treated with monocrotophos (an organophosphate insecticide) was able to isolate 25 monocrotophos-degrading bacteria, of which four *Bacillus* spp. isolates were highly resistant to ampicillin, streptomycin, cephotaxime, tetracycline and chloramphenicol (Rangasamy et al., 2017). Interestingly the mode of action of insecticide/antibiotic resistance was different to that of herbicide/antibiotic resistance. Monocrotophos and antibiotic resistance appeared to be mediated by the same plasmid, and the organophosphorus hydrolase enzyme (responsible for organophosphate degradation) was predicted *in silico* to have high affinity for chloramphenicol, and moderate affinity for ampicillin and cefotaxime. This is certainly an example of co-resistance and could be a mechanism for maintainable cross-resistance. Monocrotophos use is rapidly declining globally, but this may have implications for other organophosphate pesticides.

Other studies looked at ARG prevalence in on-farm biopurification systems (BPS) – a matrix (e.g. wood chips) containing bacterial communities used to degrade pesticides. One study found that class 1 and 2 integrons and sulfonamide resistance genes were highest shortly after BPS commissioning, likely indicating they entered the BPS via a manure feedstock (Dealtry et al., 2014). The aminoglycoside adenylating cassette (*aadA*) was stably maintained in the BPS over time. Most relevantly, class 2 integrons were found to increase in abundance later in the lifetime of the BPS, perhaps indicating a response of their hosts to pesticides. These integrons can, albeit rarely, carry ARGs (Ramirez et al., 2010). Another study sequenced plasmid DNA from a BPS, and identified diverse ARGs conferring resistance to tetracyclines, macrolides, β-lactams, aminoglycosides, bleomycin, fosmidomycin, bacitracin, phenicols and acriflavine. Of these ARGs, 41% were multidrug efflux systems (Martini et al., 2016). Furthermore, multiple genes putatively associated with pesticide degradation were identified, though the colocalization of ARGs and pesticide degradation genes on the same plasmid was not confirmed. This is an important question in terms of pesticide-ARG co-selection and could potentially be addressed using higher throughput or longer read sequencing technologies.

Pollutants

The effects of pollutants on AMR evolution have not been widely studied. However, polyaromatic hydrocarbon (PAH) contamination can lead to dramatic changes in bacterial community composition. Some taxa known to be PAH-degraders (e.g. *Streptomyces* spp.) are also known to harbour ARGs. Expression levels and abundance of ARGs are also higher in PAH-contaminated soils (Gorovtsov et al., 2018). Polychlorinated biphenyls (PCBs) have also been shown to shift the soil microbiota, and triclosan (see Biocides, below) is structurally similar to PCBs. PCB-tolerant strains can also show great genomic instability and possess many MGEs (Gorovtsov et al., 2018). Therefore, it seems likely that PCB contamination may be a risk for AMR selection, but insufficient work has been done to date. As well as these organic pollutants, many of the heavy metals discussed previously may also be present as industrial pollutants.

Biocides

Biocides are of limited relevance to non-livestock agriculture prior to food processing. They are briefly mentioned here, as concerns have been raised about their ability to select for AMR in exposed bacteria (Donaghy et al., 2019). However, the evidence for biocide-induced AMR evolution is mixed. A study of the effects of benzalconium chloride on bacterial communities from wastewater found no evidence for enhanced AMR prevalence, and in fact showed reduced abundance of ARGs and metal resistance genes (Murray et al., 2019). Chronic triclosan exposure was found to induce maintainable triclosan-resistance, and mild, reversible tolerance to antibiotics (Li et al., 2019a), which may be relevant in terms of raising the antibiotic MIC to permit evolution of persistent resistance, as in glyphosate exposure. Other biocides have an even more mixed picture; one study showed chlorine-tolerant bacteria were more resistant to antibiotics than chlorine-sensitive bacteria, and AMR bacteria were found to have higher resistance to free chlorine (Khan et al., 2016); whilst another study showed a reduction in the abundance and diversity of ARGs and MGEs in bacteria from secondary effluent after chlorination (Lin et al., 2016).

Summary

There is significant evidence that metals of agricultural importance can select for AMR. In a European context, the most important application of metals in crop agriculture is the use of Cu-based fungicides in vineyards, olive groves and orchards. Use of Cu, and presence of Cu in soils, is generally lower in the UK, suggesting that the risk of Cu-induced AMR evolution may be lower in the UK, but may be a consideration for imported products. There is compelling, though limited, evidence that herbicides and insecticides can also lead to increased rates of AMR evolution. The mode of action for herbicide-induced AMR evolution so far discovered is the raising of the MIC for particular antibiotics, allowing the presence of antibiotics themselves to induce AMR evolution. As antibiotics are not used in crop agriculture in the UK, the risk here lies in potential introduction of antibiotics via manure, or in imported products from countries where co-application of antibiotics and herbicides does occur. An evidence gap exists

as to the extent to which other pesticides induce AMR evolution themselves without the presence of antibiotics (as appears to be the case with monocrotophos). If this does occur, then the AMR risk of these pesticides may be higher than appreciated to date. However, given the widespread use of pesticides in crop production, a negligible number pesticidal active ingredients have been tested and no evidence was found of any research into the interactions of fungicides and AMR.

Detection of Antibiotics

Methods of Analysis

The issue of the accumulation of veterinary medicine residues (including antibiotics) in cereals, fruit and vegetables, as a consequence of the agricultural use of animal manure as a fertilizer and/or the irrigation of fields with wastewater during crop production, has been of concern for a number of years. This has necessitated the development of analytical methods to monitor the identity and quantity of residues present in edible plant materials.

Historically however, the main emphasis has been on developing methods for the analysis of veterinary medicine residues in animal tissues and studies monitoring the levels of antibiotics in cereals, fruit and vegetables in the available literature are limited. Chen et al. (2019) for example, recently published a review of methods used to monitor antibiotic residues directly in food products. Of the 71 publications cited, only 3 monitored residues in fruit and vegetables.

Many of the published methods available are part of larger studies concerned with determining the environmental fate of veterinary medicines and the bioaccumulation of such residues in edible crops, either in the environment or under controlled laboratory / field conditions (see section Antibiotics in Crops).

Examples of the types of studies carried out are discussed at length above, with a focus on contamination routes that are likely to have the highest risk in terms of crop contamination (manure fertilization and irrigation of fields). Other studies have concentrated on the analysis of antibiotics applied directly to crops. Aldeek et al. (2015), Amelin and Avdeeva (2018), and Canzani et al. (2017) published methods for the determination of penicillins (and their metabolites), used to control bacterial diseases in fruit and vegetables, including haunglongbing (HLB) in citrus crops. Alechaga et al. (2015), Bohm et al. (2010), Chen et al. (2012) reported methods for the analysis of streptomycin used to control fireblight in apples and pears, fruiting crops, tobacco, rice plants and other crops. Methods for the analysis of tetracyclines were reported by Maia et al. (2008).

The analytical methods noted in the above publications are limited by the range of veterinary medicines sought and the plant materials tested.

More recently, there has been an increasing number of publications specifically noting the development of multi-class, multi-residue methods to monitor for an extended range of veterinary medicine residues in fruit, vegetables and cereals. Methods for the simultaneous determination of multi-class veterinary medicine residues have been reported in cereals (Albero et al., 2019), cabbage, cucumber and tomatoes (He et al., 2018), radish, oil-seed rape, celery and coriander (Hu et al., 2014), baby foods (Jia et al., 2014), lettuce, radish and strawberry (Martínez-Piernas et al., 2018) and leafy vegetables (Yu et al., 2018).

An overview of the parameters used in selected methods is shown in Table 7.

Reference	Matrix	Compounds	Sample Preparation	Detection	LODs (µg/kg)	LOQs (µg/kg)
(Ahmed et al., 2015)	Cucumber, tomato & lettuce	Tetracyclines (chlortetracycline, oxytetracycline, tetracycline) and sulfonamides (sulfamethazine, sulfamethoxazole, sulfadimethoxine)	Plants dried; Acidifed methanol, acetone; Oasis HLB	LC-ESI-Ion trap MS (+)		100 µg/kg
(Albero et al., 2019)	Wheat, barley, rice & oats	Fluoroquinolones (ciprofloxacin, danofloxacin, enrofloxacin, norfloxacin), sulfonamides (sulfamethazine, sulfachloropyridazine, sulfadimethoxine, sulfamethoxazole, sulfamethizole, sulfamerazine, sulfadiazine), tetracyclines (chlortetracycline, doxycycline, tetracycline), macrolides (erythromycin, tylosin, tilmicosin) and lincosamides (lincomycin).	Microwave assisted extraction; acetonitrile / methanol / formic acid 0.5% (7:1:2, by vol); PSA dispersive SPE	LC-ESI-MS/MS (+)	0.3 - 2.0 µg/kg	0.8 - 5.8 µg/kg
(Aldeek et al., 2015, Aldeek et al., 2017)	Oranges, lemons, grapefruit	Penicillin G and its metabolites (penilloic acid and penillic acid)	Phosphate buffer (pH7); Oasis HLB	LC-ESI-MS/MS (+)	0.1 µg/kg 0.25 µg/kg (juice)	0.25 µg/kg 1.0 µg/kg (juice)
(Alechaga et al., 2015)	Tomato, zucchini, chard & lettuce	Kasugamycin & streptomycin	Acetonitrile: aqueous TCA (5%), EDTA (1:1, v/v); Oasis HLB	LC-ESI-MS/MS	5 µg/kg	10 µg/kg

Reference	Matrix	Compounds	Sample Preparation	Detection	LODs (µg/kg)	LOQs (µg/kg)
(Amelin and Avdeeva, 2018)	Oranges, lemons, plums, pears, peaches, cabbage, potatoes, onions, carrots, beets, avocados, cucumbers, tomatoes, eggplants	Penicillins G & V	QuEChERS; acetonitrile /EDTA; C_{18}, GCB dispersive SPE	LC-Time-of-Flight MS	0.05 - 0.3 µg/kg	0.2 - 0.9 µg/kg
(Bohm et al., 2010)	Apples	Streptomycin	Phosphate buffer / EDTA / TCA (pH 4); Oasis HLB	LC-ESI-MS/MS (+)	1 µg/kg	2 µg/kg
(Carter et al., 2014)	Radish & rye grass	Included: diclofenac & sulfamethazine	Acetonitrile / water (70:30,v/v); Oasis HLB	LC-ESI-MS/MS		10 µg/kg
(Chen et al., 2012)	Chinese cabbage & cucumber	Streptomycin	Phosphoric acid (pH 1.8); SCX & C18 SPE	LC-Fluorescence	10 µg/kg	30 µg/kg
(Chung et al., 2017)	Radish	Chlortetracycline, enrofloxacin & sulfathiazole	QuEChERS; Acetonitrile / 1% acetic acid / EDTA; PSA, C18 dispersive SPE	LC-ESI-MS/MS (+)	0.6 - 6.0 µg/kg	2.0 - 20 µg/kg
(Conde-Cid et al., 2018)	Corn, grass, potato & wheat	Tetracyclines (tetracycline, oxytetracycline, chlortetracycline,	Lyophilised plant material;	LC-ESI-MS/MS	20 µg/kg	40 µg/kg

Reference	Matrix	Compounds	Sample Preparation	Detection	LODs (µg/kg)	LOQs (µg/kg)
		doxycycline) & sulfonamides (sulfadiazine, sulfamethazine, sulfachloropyridazine and sulfamethoxypyridazine)	acetonitrile / EDTA / McIlvaine buffer pH 4 or 6); SPE clean-up			
(Duelge et al., 2017)	Distillers grain	Erythromycin, penicillin G, virginiamycin M1 and virginiamycin S1	Acetonitrile / buffer (pH5) (1:4, v/v); Oasis HLB	LC-ESI-MS/MS (+)		2.5 - 5 µg/kg
(Gbylik-Sikorska et al., 2019)	Mushrooms	Cephalosporins (cefquinome, cefalonium, cefazolin, cephalexin, cefoperazone), fluoroquinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, norfloxacin, sarafloxacin), lincosamides (lincomycin), macrolides (erythromycin, tylosin, tilmicosin, josamycin, spiramycin), penicillins (ampicillin, amoxicillin, dicloxacillin, cloxacillin, nafcillin, oxacillin, penicillin G, penicillin V), pleuromutilins (tiamulin, valnemulin), sulfonamides (sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamonomethoxine, sulfaquinoxaline, sulfathiazole), diaminopyridine	Acetonitrile/ TCA (5%, v/v)	LC-ESI-MS/MS (+) [QTRAP]	0.3 - 3.0 µg/kg	1 - 10 µg/kg

Reference	Matrix	Compounds	Sample Preparation	Detection	LODs (µg/kg)	LOQs (µg/kg)
		(trimethoprim) and tetracyclines (chlortetracycline, doxycycline, oxytetracycline, tetracycline).				
(He et al., 2018)	Cabbage, cucumber & tomatoes	Sulfonamides (sulfacetamide, sulfisomidine, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfameter, sulfadimidine, sulfamethizole, sulfadoxine, sulfamethoxazole, sulfamoxole, sulfamonomethoxine, sulfisoxazole, sulfabenzamide, sulfaquinoxaline, sulfadiemthoxine), fluoroquinolones (ofloxacin, danofloxacin, enoxacin, enrofloxacin, difloxacin, sarafloxacin, sparfloxacin, ciprofloxacin, norfloxacin, orbifloxacin, lomefloxacin, cinoxacin, fleroxacin, flumequine), quinolones (nalidixic acid, oxolinic acid), macrolides (spiramycin, erythromycin, roxithromycin, azithromycin, clarithromycin, tilmicosin), penicillins (cloxacillin, penicillin G, amoxicillin, ampicillin, penicillin V) and tetracyclines (chlortetracycline, oxytetracycline, tetracycline, doxycycline, demeclocycline).	QuEChERS; acetonitrile / buffer; PSA, C18, GCB dispersive SPE	LC-ESI-MS/MS		2 - 5 µg/kg

Reference	Matrix	Compounds	Sample Preparation	Detection	LODs (µg/kg)	LOQs (µg/kg)
(Hu et al., 2010)	Radish, oil-seed rape, celery, coriander	Sulfonamides (sulfamethoxazole, sulfadoxine, sulfachloropyridazine), chloramphenicol, tetracyclines (oxytetracycline, chlortetracycline, tetracycline), lincomycin and macrolides (ofloxacin, ciprofloxacin, pefloxacin)	Lyophilised plant material; acidified methanol, acetone; Oasis HLB	LC-ESI-MS/MS	0.5 – 1.5 µg/kg	1.8 - 4.0 µg/kg
(Jia et al., 2014)	Baby foods [including cereal, fruit and vegetable based]	Included: Amphenicols (florfenicol, thiamphenicol), androgens (zeranol), anthelmintics (albendazole, albendazole sulfone, oxfendazole, clorsuluron, closantel, febantel, fenbendazole, levamisole, mebendazole, nitroxynil, oxibendazole, oxyclozanide, thiabendazole, triclabendazole), avermectins (ivermectin, moxidectin), coccidiostats (clopidol, monensin, narasin, robenidine), corticoids (betamethazone, dexamethazone), gestagens (altrenogest), macrolides (erythromycin, lincomycin, novobiocin, oleandomycin, tilmicosin, tylosin), NSAIDs (carprofen, diclofenac, ketoprofen, meloxicam), penicillins (amoxicillin, ampicillin, cefapirin, penicillin G), quinolones (ciprofloxacin, danofloxacin, enoxacin, enrofloxacin,	QuEChERS [acetonitrile / water, 84:16,v/v; 1% oxalic acid , w/v)	LC-ESI-Q Orbitrap	0.01 - 5.26 µg/kg (Decision limit)	0.01 - 9.41 µg/kg (Detection capability)

Reference	Matrix	Compounds	Sample Preparation	Detection	LODs (µg/kg)	LOQs (µg/kg)
		flumequine, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin), sulfonamides (dapsone, sulfachloropyridazine, sulfadimethoxine, sulfadoxine, sulfadiazine, sulfamethazine, sulfamethoxypyridazine, sulfathiazole, sulfaquinoxaline, ormetoprim, trimethoprim), tetracyclines (oxytetracycline), tranquilisers (azaperone, carazolol), pleuromutilin (tiamulin).				
(Kang et al., 2013)	Lettuce, spinach, cabbage, carrot, radish, onion, garlic, tomato, pepper, sweetcorn & potato	Chlortetracycline, monensin, sulfamethazine, tylosin & virginiamycin	Phosphate buffer (pH 7)	Bioassay, ELISA		10 µg/kg
(Maia et al., 2009, Maia et al., 2008)	Tomatoes	Oxytetracycline	McIlvaine buffer (pH 8) / EDTA; C18 SPE	LC-Fluorescence	10 µg/kg	30 µg/kg
(Martínez-Piernas et al., 2018)	Lettuce, radish & strawberry	Included: azithomycin, clarithromycin, flumequine, ketoprofen, lincomycin, mefenamic acid, methylprednisolone, metronidazole, nalidixic acid naproxen, salbutamol, sulfadiazine, sulfamethazine, sulfamethoxazole,	QuEChERS; acetonitrile / acetic acid (1%); PSA, C18 dispersive SPE	LC-ESI-MS/MS [QTRAP]	0.01 - 0.5 µg/kg	0.02 - 2.0 µg/kg

Reference	Matrix	Compounds	Sample Preparation	Detection	LODs (µg/kg)	LOQs (µg/kg)
		sulfapyryidine, sulfathiazole and terbutaline				
(Migliore et al., 2010)	Maize	Tetracyclines (oxytetracycline, 4-epi-oxytetracycline, chlortetracycline, 4-epi-chlortetracycline)	Plants dried; Liquid / liquid extraction; Metal chelate affinity chromatography	LC-ESI-MS/MS (+)	1 µg/kg	1 µg/kg
(Nebot et al., 2014)	Baby foods [containing potato, carrots, tomatoes, peas, onions, leeks, corn starch]	Tetracyclines (chlortetracycline, doxycycline, oxytetracycline, tetracycline)	McIlvaine buffer (pH 4) / EDTA; liquid / liquid extraction	LC-ESI-MS/MS (+)	5 µg/kg	11 - 14 µg/kg (Detection capability)
(Wang et al., 2017)	Spring onion	Nitrofurans (furaltadone, furazolidone, nitrofurantoin, nitrofurazone, AOZ, SEM, AHD, AMOZ)	Ethyl acetate; Solvent exchange; Metabolites derivatised	LC-ESI-MS/MS (+)	0.2 - 0.8 µg/kg	
(Wang et al., 2016)	Radish and Pakchoi	Sulfamethoxazole, norfloxacin & doxycycline	Acetonitrile, acidified acetonitrile; Oasis HLB SPE	LC-ESI-MS/MS (+)	1.2 - 1.8 µg/kg	3.6 - 5.0 µg/kg
(Yu et al., 2018)	Leafy Vegetables	Tetracyclines (tetracycline, oxytetracycline, chlortetracycline), macrolides (norfloxacin, ciprofloxacin, enrofloxacin, ofloxacin, difluoxacin) & sulfonamides (sulfadiazine, sulfamethizole,	QuEChERS; acetonitrile / methanol (85:15, v/v); C18, GCB dispersive SPE	LC-ESI-MS/MS (+)	0.33 - 2.92 µg/kg	1.1 - 9.73 µg/kg

Reference	Matrix	Compounds	Sample Preparation	Detection	LODs (µg/kg)	LOQs (µg/kg)
		sulfamethoxypyridazine, sulfaquinoxaline, sulfapyridine, sulfamethoxazole, sulfathiazole, sulfamerazine, sulfisoxazole, sulfadimidine, sulfachlorpyridazine, sulfadimethoxine).				

Table 7. Selected method performance parameters

C_{18} : Octadecyl EDTA: Ethylenediaminetetraacetic acid GCB : Graphitised carbon black

Oasis HLB: Hydrophilic-lipophilic balance (Waters Oasis Co) PSA : Primary secondary amine SCX : Strong cation exchange

SPE : Solid-phase extraction TCA : Trichloroacetic acid

LC-ESI-MS/MS – High performance liquid chromatography-tandem mass spectrometry (with electrospray ionisation)

Extraction and Sample Clean-up

There is no internationally accepted standard method for the extraction and clean-up of veterinary medicine residues from fruit, vegetables and cereals. Generally, methods have been developed for the analysis of specific compounds, in defined matrices. More recently, a method developed for the analysis of pesticide residues in fruit and vegetables, QuEChERS (Quick, Easy, Cheap, Effective, Robust and Safe) has been applied to the analysis of multiple veterinary medicine residues (see Table 7).

Many of the methods noted for specific studies, use a combination of acetonitrile and an acidic buffer for the extraction of residues (Ahmed et al., 2015, Albero et al., 2019, Aldeek et al., 2015, Bohm et al., 2010, Carter et al., 2014, Chen et al., 2012, Conde-Cid et al., 2018, Duelge et al., 2017, Gbylik-Sikorska et al., 2019, Hu et al., 2010, Kang et al., 2013, Maia et al., 2009, Nebot et al., 2014, Wang et al., 2016). The precise method used depended upon the analyte/analyte group being analysed. Multiple clean-up procedures have been employed, many of which use an Oasis HLB solid-phase extraction cartridge (Waters Corporation, USA).

QuEChERS is a multi-class, multi-residue analytical approach involving liquid-liquid partitioning and dispersive solid-phase extraction clean-up, developed by Anastassiades et al. (2003) to extract pesticides residues from food matrices such as fruit, vegetables and cereals. The method has largely superseded traditional clean-up methods due to its fast and easy operation, simplicity, low cost, low solvent use, high reproducibility and the wide range of residues that can be co-extracted. QuEChERS has evolved into two official methods: AOAC Official method 2007.01 - S. Lehotay (2007) and the European Committee for Standardization European Norm (CEN) 15662 – CEN (European Committee for Standardization) (CEN, 2008).

Modifications of the QuEChERS process (extraction procedure, salting-out agent and dispersive solid-phase extraction adsorbents), has allowed this technique to be used for the multi-residue extraction of antibiotics. A common change is the addition of EDTA to the extraction solvent, which reduces the complexation of beta-lactams, macrolides and tetracyclines with cations, to improve recoveries of these compounds. The acidification of the acetonitrile solvent (e.g. 1% formic acid), increases the recoveries of quinolones and sulphonamides. The application of the QuECHERS methodology for the determination of antibiotics in food was reviewed by Zhang et al. (2019a). Several authors have reported the use of a modified QuEChERS extraction for the analysis of veterinary medicine residues in fruit, vegetable and cereals (Amelin and Avdeeva, 2018, He et al., 2018, Hu et al., 2014, Jia et al., 2014, Martínez-Piernas et al., 2018, Yu et al., 2018).

Fera Science Ltd has extensive experience in the analysis of over 550 pesticides in fruit, vegetables and cereals using the QuEChERS method, coupled to either Gas Chromatography - Tandem Mass Spectrometry (GC-MS/MS) or Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS). A single modified QuEChERS-type extraction is also in routine use for the analysis of 67 antimicrobial

compounds (β-lactams, cephalosporins, macrolides, quinolones, tetracyclines and sulphonamides) in animal tissues. These methods could be applied to the analysis of antimicrobial residues in fruit, vegetable and cereals.

Determination of Veterinary Medicines

Liquid chromatography coupled to mass spectrometry has become the most widely used methodology for the qualitative and quantitative determination of veterinary medicine residues. Most of the published methods use triple quadrupole mass spectrometers with electrospray ionisation (LC-ESI-MS/MS). This procedure is highly selective and sensitive and can be used to determine residues of several hundred different compounds, within one analytical run. The introduction of isotopically labelled internal standards has improved the reproducibility of methods, correcting for small day-to-day variations in method performance.

The main limitation of LC-ESI-MS/MS is that it requires a targeted approach and can only be used to determine a known list of analytes. More recently, High-Resolution Mass Spectrometers (HRMS) are increasingly being used for monitoring residues (Time-of-flight mass spectrometry (ToF-MS), Quadrupole Time-of-flight mass spectrometry (Q-TOF-MS) and Orbitrap (LC-Orbitrap-MS)). These detection systems can be used for non-targeted screening of residues, by comparing the mass spectral data obtained with thousands of spectra held within libraries. Currently, the use of HRMS for surveillance of residues is in the developmental stage and issues such as determining limits of detection, method validation criteria etc. remain topics of discussion. It is foreseen that within 5 years, such technologies will be in routine use and become invaluable in the future monitoring of residues.

Summary

Analytical methods to monitor the levels of antibiotics in cereals, fruit and vegetables published in the available literature are limited, as compared with those available for the analysis of animal tissues.

Multi-class, multi-residue methods to monitor for a range of residues are available but further work is required to both extend the range of compounds monitored and to assess their application to crops routinely grown and imported to the UK.

The role of a non-targeted approach to residue monitoring using high HRMS, will become increasingly important in the identification of residues present in edible crops. This approach has the potential to monitor for the presence of residues of thousands of different compounds, of all chemical classes.

Detection of Antibiotic Resistance

Detection of Phenotypic Antimicrobial Resistance and Susceptibility

The phenotypic detection of AMR, by growing bacterial cultures in a medium containing the antibiotic of interest, is the longest standing method for identifying AMR. It is also the only method that can detect antibiotic susceptibility, rather than resistance. That is because, while molecular approaches (see below) may be capable of detecting resistance determinants, there always exists the possibility of a bacterium carrying a previously unreported resistance gene, or a novel allele of an existing gene. This means that, while we may be able to infer resistance to an antibiotic based on the presence of a previously identified resistance determinant, inferring susceptibility to an antibiotic is more challenging. However, there are limitations to traditional culture-based approaches. For example, only a limited number of antibiotics can be assayed on a single agar plate, and specific media are often required for different bacterial species. Furthermore, the time requirements of traditional bacterial culture can prevent rapid treatment decisions being made – even the fastest growing bacterial species can require several hours growth before interpretation of results, and some require much more. While treatment delays may not be a significant issue when assessing AMR in crop production, it is conceivable that at some point a rapid assay may be required to screen plant products for AMR before they are permitted to enter the food chain. Novel phenotype-based approaches have recently been developed, of which examples are given below.

Several approaches are based on detecting the responses of individual cells to exposure to antibiotics. One technique uses a diffraction-based sensor to measure bacterial growth and mobility, from a single cell to hundreds of cells, on exposure to antibiotics (Volbers et al., 2019). This method is both rapid, generating susceptibility profiles in 30 to 40 minutes, and MIC values in 2 to 3 hours, and potentially high throughput, as the physical separation between the 2D diffraction grating and the biological sample permits the use of routine laboratory plasticware and rapid changing of samples. This approach is also label-free, in that it requires no antibodies or similar for bacterial detection. The technology has been demonstrated for multiple antibiotics and bacteria (ampicillin and kanamycin for *E. coli*, vancomycin for *Bacillus subtilis*). However, it does suffer from some of the same drawbacks as conventional culturing – a pure culture of the bacteria is required, and they must be culturable. Even though very low bacterial volumes are required, the technique does rely on measuring the growth rate of bacteria in an artificial media. Therefore, while the technique itself may be rapid to deploy, it is not yet suitable for use in the field or on field samples and may have limited application with slow growing or fastidious bacteria.

A technique that may be more suitable for slow growing bacteria is the use of a nanomotion technique, where bacterial cells are attached to a chemically functionalised cantilever, similar to an atomic force microscope. If the cells are

metabolically active, they induce oscillations in the cantilever. On exposure to inhibitory antibiotics the oscillations cease. This methodology was tested on the slow growing *Bordetella pertussis*, and was able to detect the effect of clarithromycin and erythromycin in 40 minutes, and ampicillin in less than 20 minutes (Villalba et al., 2018). While rapid and capable of working on slow growing organisms, this is still likely to require a culture of the organism of interest before it can be used. Bacteria that can't be grown can still be investigated phenotypically however, for example by hydrodynamic trapping of individual cells (Pitruzzello et al., 2019). The motility and morphology of the trapped cells can be assessed by changes in pixel luminance, and the effects of antibiotics on individual cells within a population assessed.

Targeted Molecular Detection

While phenotypic assessments of antimicrobial resistance and susceptibility have a number of advantages, they do suffer from drawbacks, such as time to result and a lack of information about the genetic mechanisms underlying observed resistance. This could be important when considering the risk of transfer of ARGs to human pathogens, especially in the context of AMR arising in a crop agricultural environment in either environmental bacteria or plant pathogens. Molecular assays have been designed to fill the gap of rapidity and elucidating the genes responsible for AMR.

Targeted assays for ARGs based on Polymerase Chain Reaction (PCR) amplification have a long history, e.g. (Tokue et al., 1992), therefore an in depth discussion is not proposed here, beyond stating that new quantitative PCR (qPCR) assays continue to be developed for speciating plant pathogenic bacteria, and detecting specific ARGs that they carry (Laforest et al., 2019). Modifications of qPCR, such as the use of Peptide Nucleic Acid (PNA) probes and subsequent melt curve analysis can allow an identification of AMR-relevant mutations, for example clarithromycin-resistance conferring mutations of the *Helicobacter pylori* 23S rRNA gene (Jung et al., 2018). These approaches are relatively rapid to design and implement, though point mutations are likely to be less high risk than MGE-mediated resistance-conferring genes when considering movement from a crop environment to human pathogens. Another modification of the qPCR technique, brought about by technological improvements to PCR platforms, is High Throughput qPCR (HT qPCR). These approaches allow the detection and quantification of hundreds of ARGs per sample, which allows the effective of different treatments of conditions on ARG abundance to be assessed (Wang et al., 2014).

Recently there have been several innovations aimed at bringing molecular detection into the field, enabling the maximum advantage to be gained from its rapid nature. The detection of ARG PCR amplicons on a lateral flow immunoassay allow the detection of a positive PCR reaction in 3 minutes, compared with approximately 45 minutes for conventional gel electrophoresis (Zhang et al., 2017b, Seidel et al., 2017). This represents a time saving when assessing the result of a PCR assay, but still requires a PCR reaction to be performed in the field, which could be cumbersome using commonly available hardware. To address this issue, Rajendran et al. (2019), devised

a convection PCR device, with lateral flow assay and smartphone-based reader, to permit sample-to-result analysis away from a centralised laboratory. Other technologies more amenable to in-field use than PCR have also been developed, such as LAMP (Loop-mediated isothermal AMPlification). Being isothermal, LAMP reactions require less sophisticated equipment than PCR, and LAMP assays to particular ARGs have been developed e.g. (Mu et al., 2016, Nakano et al., 2015, Rodriguez-Manzano et al., 2020). LAMP assays have potential to simultaneously detect multiple ARGs or to detect an ARG and a particular causative pathogen of interest, though the design of such assays can or is likely to be complex (Podushkina et al., 2019). LAMP has even been used to infer phenotypic susceptibility, by comparing the time-to-positive result of *E. coli* cultures that have and have not been exposed to ampicillin, trimethoprim-sulfamethoxazole, and fosfomycin (Ota et al., 2019). Another approach is to target mRNA, which implies that the gene in question has been transcribed and may indicate phenotypic resistance. This has been combined with naked-eye detection based on the formation of DNA hydrogels to allow rapid, low cost detection of transcripts (Choi et al., 2019).

Non-Targeted Molecular Detection

High Throughput Sequencing or Next Generation Sequencing based methods for detecting ARGs have been reviewed extensively elsewhere, e.g. (Oniciuc et al., 2018, Su et al., 2019), and again it is not proposed to cover this again here. In the general case these methods could be thought of as falling into two categories; Whole Genome Sequencing (WGS) and metagenomics. WGS involves genomic analysis of a bacterial isolate, and inference of AMR profile from comparing the genes identified with one or more reference databases. The benefits of such an approach involve streamlining of methods – WGS is becoming widely employed for typing and epidemiological investigations, and detection of ARGs uses the same data without the need for additional culture-based tests. The limitations include the currently incomplete understanding of the genetic basis of resistance. Metagenomic approaches are similar, in that ARG presence is detected from DNA sequence data. In this case, sequence data is generated for a whole DNA extract from the matrix or bacterial community of interest, rather than from an isolated culture. The advantages of this are that ARGs can be detected regardless of the organism or MGE (e.g. plasmid) on which they are carried, and with sufficient sequencing depth or length MGEs and multidrug resistant organisms can be identified. These could all be very important and relevant considerations in terms of AMR evolution in crop context, as ARGs are most likely to arise in environmental or plant pathogenic/commensal organisms. Those that are the highest risk to human health are likely to be those that are transferrable to human pathogens. However, this approach is still relatively new, and therefore expensive and computationally intensive. Metagenomics generally also permits only relative quantification of targets within a sample, not absolute quantification.

Other approaches have been used to combine non-targeted and targeted molecular detection to identify phenotypic resistance. One such approach uses transcriptomics

to identify genes which are upregulated when bacteria are exposed to antibiotics (Bhattacharyya et al., 2019). These transcripts then serve as the targets for a targeted assay, allowing rapid phenotyping from molecular results. Furthermore, the potential exists to combine the advantages of both targeted approaches and high throughput sequencing. For example, primer walking approaches have been combined with long-read sequencing technologies to characterise the boundaries between genetically modified elements and the host chromosome (Fraiture et al., 2019). This approach could be used to add information about host species or plasmid to the results gained from PCR-based assays for AMR genes.

Summary

Many targeted PCR-based assays already exist and could be deployed. Field deployability is possible but is usually used for informing treatment decisions. This may be a consideration if plant diseases continue to be treated with antibiotics, and indeed could improve antibiotic stewardship. Alternately, there may be a role for in field testing if products are to be screened for ARGs before export/import. Metagenomic approaches allow the detection of ARGs regardless of host, which may improve assessment of risk of transfer to human microbiome/pathogens.

Conclusions

The objective of this review was to describe the current state of knowledge of the risk of AMR in crop-based agriculture and point towards research gaps and opportunities.

Antibiotic application in crop agriculture is often poorly documented, although where information is available use appears to be significantly lower than in livestock agriculture. However, antibiotics are used for the treatment of several plant diseases in a number of countries. Furthermore, there is evidence that in some countries undocumented use is widespread. The risk of AMR transmission to the consumer is therefore likely to be low in produce from the majority of countries, especially those with strong regulations in place, where antibiotic use is very low or non-existent. The risk is likely to be higher in produce from countries where antibiotic use is unknown or suspected, however without further information about use and misuse, or testing of produce from higher risk countries, the risk is difficult to assess more accurately.

The evolution of resistance to the applied antibiotics has been observed in plant pathogenic and epiphytic bacteria. Beyond the direct application of antibiotics, contamination of crop plants with antibiotic residues and AMR genes and bacteria from soil amendments may be an important AMR risk. Antibiotics have been shown to be taken up by the crop plants from soil, and to a greater extent from hydroponic systems. However, the picture here is complex, with compound, plant species or variety, soil type and physical condition e.g. soil moisture, initial concentration and exposure time all having an impact. Different antibiotics are likely to have different breakdown rates, so it is possible that any antibiotic compounds applied will have degraded by the time the produce reaches consumers. The persistence of AMR genes or bacteria is unknown, but likely to be much higher, and possibly differing among different bacterial taxa. Therefore, the risk of contamination with AMR organisms is likely higher than contamination with the antibiotics themselves. Again, the risk here is difficult to assess without knowledge of the rates of AMR evolution and in producer countries and persistence in transit, or at least without data on contamination of plant products when they reach the UK.

An additional complication arises as a number of agriculturally important chemicals, including metals, pesticides and pollutants, can lead to enhanced evolution of antibiotic resistance in bacteria. Some of these, such as copper compounds, are widely used in both crop and livestock agriculture, so could represent an AMR risk even in produce from countries where antibiotics are not used in crop agriculture. Many agrichemical compounds have not been assessed for their AMR-inducing effects, and as such no conclusions can be drawn about their contribution to consumers' AMR exposure risk.

Analytical methods to monitor the levels of antibiotics in cereals, fruit and vegetables published in the available literature are limited. Multi-class, multi-residue methods to monitor for a range of residues are available but further work is required to both extend

the range of compounds monitored and to assess their application to crops routinely grown and imported to the UK.

Antimicrobial resistance can be tested for either phenotypically (by monitoring bacterial response to antibiotics) or genotypically (by detecting the AMR genes and mutations present). Novel methods are allowing rapid phenotypic assessment of low numbers of bacteria (i.e. without prior culturing) but these are currently far from deployable. Molecular methods of detection of AMR genes are either targeted or non-targeted. Targeted methods are often relatively cheap and rapid, and some technologies (e.g. LAMP) have the potential to be field deployable. Non-targeted methods are based on high throughput sequencing, which has a relatively high per-sample cost, but theoretically enables the detection of all known AMR genes, regardless of the bacterial species in which they are found.

Based purely on the known amounts of antibiotics applied to crops, the risk of AMR from crop agriculture appears lower than for livestock agriculture. However, with unsanctioned use in some less economically developed countries, contamination of antibiotics and AMR genes from soil amendments, and the effects of other agrichemicals, further research to elucidate risk seems warranted.

Research Gaps

Research gaps can be divided into technical improvements to methods, improved understanding of antibiotics or AMR in the crop environment, and improved understanding of antibiotic practises in crop agriculture.

Technical Improvements

Multi-class, multi-residue methods currently used (at Fera Science Ltd) for the analysis of antimicrobial residues in animal tissues need to be assessed for their applicability to the analysis of such residues in fruit, vegetables and cereals. The finalised method(s) should be validated in accordance with the ISO 17025 standard.

In the longer term (within 5 years), the use of High-Resolution Mass Spectrometers should be assessed for the monitoring of unknown compounds in fruit, vegetables and cereals. Such technologies would allow the identification of residues of many classes of chemicals e.g. pesticides, mycotoxins, veterinary medicines, to be monitored within a single analytical run.

Antibiotics and AMR in the Crop Environment

A greater understanding of antibiotic metabolism in plants is required. High variation is seen depending on compound, concentration, and variety (some cultivars of the same plant had wide variation). This could potentially help with future risk assessments, e.g. compound X applied to cultivar Y is rapidly metabolised, and therefore represents a lower risk of antibiotic exposure to the consumer. However, conversely low amounts of antibiotic can lead to higher rates of evolution of AMR, as bacteria are exposed to the antibiotic without being killed or inhibited. The great variety in metabolism seen means relatively large amounts of data would be required to build predictive correlations. Somewhat related to the lack of information around metabolism, is the need to investigate whether there is evidence for bioaccumulation of antibiotic in different parts of the plant. This could mean that some parts of the plant may be low risk, or not amenable to testing, and others would be higher risk.

Hydroponic systems show greater levels of antibiotic uptake by plants than soil-based systems. There is uncertainty around what implications this has for new ‘vertical farming’ techniques. Vertical farms represent a ‘closed’ system, and as such is there any risk of increased antibiotic use to prevent disease occurrence? Or does the enhanced biosecurity of a vertical system mean introduction of bacterial pathogens is a lower risk and therefore antibiotic applications will be lower? Certain foodstuffs known to be treated with antibiotics (tomatoes, peppers etc) are relevant to vertical farming conditions. These systems and the AMR risk they may present would likely benefit from further evaluation.

A truly One Health approach is required to elucidate transmission pathways between livestock, crops, the environment, and humans. These transmissions pathways are likely to be complex and multi-directional; for example, transmission of AMR genes

from crops to livestock could occur via feed, and from livestock to crops via manure. It is possible that high throughput sequencing approaches may be useful for understanding such transmission.

Evidence for metal co-selection of AMR was seen even after low level exposure. The mechanisms around co-selection after low-level exposure may differ from those elucidated under higher levels of exposure, and the persistence of AMR genes in the environment after exposure has been suggested. Experiments to determine the persistence of AMR genes and bacteria would be straightforward and beneficial. Resistance evolution or enhancement from other agrichemicals is largely unknown, but potentially highly relevant in the global context of small but increasing antibiotic use, combined with widespread pesticide use. At the very least, any evidence for co-selection from some of the most commonly applied classes of pesticide, such as herbicides and fungicides, should be explored.

Fungi themselves may constitute additional AMR risks that were outside the scope of this review. The evolution of fungicide resistance is well recognised in plant pathogenic fungi (Lucas et al., 2015), and this may cause a human health risk if it arises in or is transferred to a human-pathogenic fungus. Indeed, any fungicide resistance genes that conferred cross-resistance to antibiotics could be available for bacteria to acquire via horizontal gene transfer.

Antibiotic Use

The returns to the survey were disappointing, and little is known about direct application of antibiotics in many countries. Methods to encourage participation in such information sharing activities could be considered. Other sources of information around antibiotic use could also be interrogated, for example trade data on import and export flows of antibiotics primarily used in plants (e.g. kasugamycin).

In parallel, an intelligence-led surveillance approach could be taken. Knowledge has been gained on the sorts of crops that are treated with antibiotics. These crops could be sampled after import from countries which are either unknown risk but large-scale suppliers, or countries where misuse is suspected, or both. They could then be tested for antibiotics, antibiotic breakdown products (as a marker of prior use of antibiotics), and AMR bacteria genes. Methods are available at Fera Science Ltd, which could be modified and validated to carry out such a survey of selected antimicrobials.

We know that antibiotics are most commonly applied to high-value crops such as pome and stone fruit, citrus, and vegetables. It would therefore seem sensible to target any monitoring of imports for antibiotics or AMR genes to commodities such as apples, pears, citrus fruit and *Prunus* fruit, along with fresh vegetables such as tomatoes, peppers, cauliflowers, beans etc, and other fruit such as kiwi, watermelon, grapes and mango.

A monitoring regime for AMR on produce should include checking consignments from all countries known to use antibiotics, but it should also focus on LMICs for which we

have no information regarding antibiotic use, or where we believe antibiotic controls to be weak. Consignments of high-risk commodities from these countries should be looked at in proportion to the size of the trade.

While we must be concerned about the risks of AMR development through application of antibiotics to food plants as a plant protection product, particularly in environments where there is little control or regulation, where does direct application of antibiotics, in a well-controlled and regulated fashion, sit in terms of risk compared with potential contamination through contaminated water or manure? And what are the risks from non-food crops, including horticultural plants, for which the literature base is even lower.

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Supplementary Materials 1: Questionnaire on Use of Antibiotics in Crop Production

Country	
Name of Organisation	
Name of responder	

1. Does your country currently have any national regulations controlling the use of antibiotics in crop production intended for human consumption or animal feed?	YES / NO
If YES, please answer questions 2 - 7, If NO, please answer questions 8 - 10	

2. Do these regulations PROHIBIT the use of all antibiotics on crops?	YES / NO
If YES, please go to Question 3, If NO, please go to Question 4.	
3. Are there any derogations which allow use of antibiotics in crop production under specific circumstances, e.g. emergency measures to control of outbreaks of quarantine bacteria (Please provide detail below)	YES / NO
4. What antibiotics do your country's regulations allow for, if any, including any derogations of the regulation that otherwise does not allow use?	

5. If possible, where antibiotics are permitted (either for general use or under a derogation) please provide data on the extent-of-use over the last 3 years, the antibiotics used, the crop, the disease being controlled, productions system (field/ glasshouse), and the frequency of use/volume etc?					
Antibiotic	Crop	Disease being controlled	Production system (field or glasshouse)	Frequency of use	Volume
6. Have there been any documented cases of malpractice where antibiotics have been applied to crops contrary to the regulations.					YES / NO
7. If yes, which antibiotics have been used on what crops?					

8. Are you aware of any use of antibiotics within your country on crops?	YES / NO
If YES,	

9. What crops and for what markets are these intended for?

10. If possible, please provide data in the table below on the extent-of-use over the last 3 years, the antibiotics used, the crop, the disease being controlled, productions system (field/ glasshouse), and the frequency of use/volume etc?

Antibiotic	Crop	Disease being controlled	Production system (field or glasshouse)	Frequency of use	Volume

Overview questions (all responders)	
11. On the basis of your above answers (e.g. the extent antibiotics are regulated/ not regulated, in use/not in use, any malpractice), as the Government NPPO how would you rate the current risks of antibiotic use on crops being a causal factor of Antimicrobial Resistance (AMR)	LOW / MEDIUM / HIGH
12. To your knowledge is the use of antibiotics as a control measure for any bacterial diseases of crops under discussion in your country?	YES / NO

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