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Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 1: *Methodology, general data gaps and uncertainties*

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Abstract

The European Commission requested EFSA to assess, in collaboration with EMA, the specific concentrations of antimicrobials resulting from cross-contamination in non-target feed for food-producing animals below which there would not be an effect on the emergence of, and/or selection for, resistance in microbial agents relevant for human and animal health, as well as the levels of the antimicrobials which could have a growth promotion/increase yield effect. The assessment was performed for 24 antimicrobial active substances, as specified in the mandate. This scientific opinion describes the methodology used, and the main associated data gaps and uncertainties. To estimate the antimicrobial levels in the non-target feed that would not result in emergence of, and/or selection for, resistance, a model was developed. This 'Feed Antimicrobial Resistance Selection Concentration' (FARSC) model is based on the minimal selective concentration (MSC), or the predicted MSC (PMSC) if MSC for the most susceptible bacterial species is unavailable, the fraction of antimicrobial dose available for exposure to microorganisms in the large intestine or rumen (considering pharmacokinetic parameters), the daily faecal output or rumen volume and the daily feed intake. Currently, lack of data prevents the establishment of PMSC and/or FARSC for several antimicrobials and animal species. To address growth promotion, data from an extensive literature search were used. Specific assessments of the different substances grouped by antimicrobial classes are addressed in separate scientific opinions. General conclusions and recommendations were made.

Keywords: antimicrobial resistance, antimicrobial resistance selection concentration (FARSC), minimal selective concentration (MSC), sub-inhibitory concentration, growth promotion, yield increase, food-producing animals

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Combatting antimicrobial resistance (AMR) is a priority in the European Union (EU). Several measures have already been put in place to limit its development. In 2006, the EU banned the use of antibiotics as feed additives for growth promotion. The provisions established in the new Medicated Feed Regulation[1] are further concrete actions to deal with the issue. The cross-contamination of non-target feed (feed, whether medicated or not, which is not intended to contain a specific active substance) with antimicrobials has been identified as one of the core issues addressed in this context. Cross-contamination may occur during manufacture, processing, storage or transport of feed where the same production and processing equipment, including for mobile mixing, storage facilities or means of transport are used for feed with different components. For the purposes of the Medicated Feed Regulation, the concept of cross-contamination is used specifically to designate the transfer of traces of an active substance contained in a medicated feed to a non-target feed. Contamination of non-target feed with active substances contained in medicated feed should be avoided or kept as low as possible. The respective Article 7 on cross-contamination stipulates in paragraph 3: The Commission shall, by 28 January 2023, adopt delegated acts in accordance with Article 20 in order to supplement this Regulation by establishing, as regards the antimicrobial active substances listed in Annex II, specific maximum levels of cross-contamination for active substances in non-target feed and methods of analysis for active substances in feed. Regarding maximum levels of cross-contamination, those delegated acts shall be based on a scientific risk assessment carried out by EFSA. Moreover, recital 17 of that Regulation highlights the protection of animal health, human health and the environment and suggests EFSA to do the risk assessment in cooperation with the European Medicines Agency (EMA). Finally, Article 107 of the new Regulation on veterinary medicinal products[2] stipulates in its paragraph 2: Antimicrobial medicinal products shall not be used in animals for the purpose of promoting growth nor to increase yield. The development of AMR and the resulting impact on human health, animal health and the environment, represents an important consequence of low-level concentration of antimicrobials in feed. Only very limited data are available on the chain between a low concentration of an antimicrobial in feed, the development of resistance to microbial agents relevant for human and animal health (zoonotic bacteria, commensals, animal pathogens) and the possible transfer of resistance determinants and/or resistant agents to humans. Therefore, it would be appropriate to investigate the effect of the exposure to the antimicrobials at low concentrations via feed on the microbiota of animals, and in particular the selection for resistance in microbial agents relevant for human and animal health. Subject to availability of supporting scientific evidence, the impact of the low-level presence in feed on the environment should be considered, too. Further, in order to avoid any misuse of the antimicrobials, use levels of the antimicrobials for promoting growth or increasing yield should, where applicable, be identified and assessed. With respect to the scope of the non-target feed to be addressed it has to be noted that medicated feed for food-producing animals and medicated pet food are produced and distributed in separate ways. Moreover, the antimicrobials at stake are not authorised for incorporation into medicated pet food. Therefore, the scope of this assessment should be focused on the low-level concentration of the antimicrobials in feed for food-producing animals. The European Commission will mandate the European Union Reference Laboratory (EURL) for Feed Additives (Commission Directorate General JRC, Geel) to recommend methods of analysis for the antimicrobials in feed. For this task, some data might be relevant which is to be compiled for the assessment of the cross-contamination levels. Therefore, an exchange of information between the EURL, EFSA and EMA, including national authorisation authorities, would be useful. In order to protect animal health, human health and the environment, maximum levels of cross-contamination for active substances in non-target feed should be established, based on a scientific risk assessment performed by the European Food Safety Authority (EFSA) and in cooperation with EMA. The maximum levels should be compared with the use levels of the antimicrobials for promoting growth or increasing yield, where applicable, to determine a final appropriate level.

[1] Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed, amending Regulation (EC) No 183/2005 of the European Parliament and of the Council and repealing Council Directive 90/167/EEC. OJ L 4, 7.1.2019, p. 1–23.

[2] Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC. OJ L 4, 7.1.2019, p. 43–167.

TERMS OF REFERENCE AS PROVIDED TO EFSA:

The European Commission requests the EFSA to assess the impact of the presence of low-level concentration in feed of the antimicrobial active substances listed in the Annex on animal health, human health and, where possible, on the environment. In particular, EFSA, in close collaboration with EMA, is asked, by 30 September 2021:

1) To assess the specific concentrations of antimicrobials resulting from cross-contamination in non-target feed for food-producing animals, below which there would not be an effect on the emergence of, and/or selection for, resistance in microbial agents relevant for human and animal health, i.e. the end point for this assessment should be the excretion of resistant bacteria from the animals. However, the assessment should also consider the impact on the environment of the low-level concentrations in feed, where possible.
2) To assess which levels of the antimicrobials have a growth promotion/increase yield effect.

Annex: List of antimicrobial active substances to be assessed

Active substance

1. Amoxicillin
2. Amprolium
3. Apramycin
4. Chlortetracycline
5. Colistin
6. Doxycycline
7. Florfenicol
8. Flumequine
9. Lincomycin
10. Neomycin
11. Spectinomycin
12. Sulfonamides
13. Tetracycline
14. Oxytetracycline
15. Oxolinic Acid
16. Paromomycin
17. Penicillin V
18. Tiamulin
19. Thiamphenicol
20. Tilmicosin
21. Trimethoprim
22. Tylosin
23. Valnemulin
24. Tylvalosin

1.2. Interpretation of the Terms of Reference

The terms of reference of the current opinion require an assessment of the antimicrobial concentrations in non-target feed below which there would not be an effect on the emergence of, and/or selection for, resistant bacteria as well as levels that have a growth promotion effect and increase yield. Therefore, depending on the availability of data, the outcome of the assessments will be the calculation or description of these levels, considering only scientific aspects relevant to derive these concentrations.

All other aspects relating to:

- use of antimicrobial medications or general aspects relating to the occurrence of antimicrobial resistance in bacteria from animals, feed and/or the environment;
- use of good manufacturing practices when producing feed, other possible sources of traces of antimicrobials in non-medicated feed (e.g. co-products of the ethanol industry) or any potential synergy between feed medication carry-over residues and occurrence of antimicrobials in the farm environment;
- feasibility aspects such as compatibility of the antimicrobial concentration values that would be obtained in the current assessment with practices in feed manufacturing, storage, transport, analysis or regulatory surveillance (e.g. detection limits);
- proposing recommendations to set maximum antimicrobial levels derived from cross-contamination in feed,

are outside the scope of the current opinion and of EFSA, whose remit is limited to risk assessment and scientific advice and does not include risk management. As indicated in the background above, such aspects will be evaluated at a later stage by risk managers, who will aim to establish, according to the Medicated Feed Regulation 2019/4, specific maximum levels of cross-contamination for active substances in non-target feed and methods of analysis for active substances in feed.

Depending on the availability of data, extrapolations may be needed, uncertainties will be explored, data gaps will be listed and recommendations to fill those data gaps will be made.

To answer ToR1, the ***Assessment question 1*** **(AQ1**) that will be answered is: which are the specific antimicrobial concentrations in non-target feed below which there would not be emergence of, and/or selection for, resistance in the large intestines/rumen?

Low levels of antimicrobials contaminating a non-target feed could lead to the development of resistance in the microbiota of animals consuming the feed. To avoid the emergence and/or selection of resistant bacteria, concentrations at or above the lowest concentration of antimicrobials that promote the emergence and/or give the resistant strains a competitive advantage should not be present.

The levels of antimicrobials below which selection for AMR is not expected to occur that will be provided in this opinion are those that would be present in the feed immediately before consumption. The selective activity of feed antimicrobial residues reaching the rumen and large intestine will be assessed in order to calculate these values.

The end point will be the bacteria present in the rumen and large intestine, where selection of resistant bacteria by the intake of the antimicrobial should not occur at the specified concentrations of antimicrobials in feed. The reasons for selecting these bacteria as the end point are: (i) rumen and large intestinal bacteria are exposed to antimicrobials ingested through feed intake; (ii) there is a high density and diversity of bacteria at rumen and large intestine level which will increase the possibilities of emerging resistance; (iii) the release of a great number of rumen and large intestinal bacteria exposed to antimicrobials that can further disperse systemically within the animal, on foods derived from the animal and in the environment via shedding in faeces, *post-* or *ante-mortem*.

Assessment of specific antimicrobials will only be performed in relation to the animal species for which minimal selective concentrations (MSC) and pharmacokinetics parameters associated with oral administration (at least the oral bioavailability) are available. If pharmacokinetics data are limited, different scenarios will be considered.

Among food-producing animals, based on their importance in the EU production, it was agreed to select swine, ruminants (cattle, goats, sheep), poultry (broilers, laying hens), rabbits, finfish (salmon) and horses as targets for the assessment.

The bacterial species to be included in the assessment will not be restricted to the target bacterial species considered in the therapeutic indications for each antimicrobial, but will include all bacterial species for which susceptibility data are available, to capture the width of the sensitivity distribution. This wide bacterial diversity functions as a proxy for the diversity of intestinal and rumen bacteria (either commensals or pathogens) that could potentially be exposed to antimicrobial residues within the animal after the ingestion of feed, as explained below.

The possible impact on the environment of the low-level concentrations in feed was not assessed. Resistant bacteria or antimicrobial residues released from the animals due to these low concentrations may impact on the environment, but the magnitude and duration of the impact cannot currently be evaluated due to lack of data. Thus, the possible impact on the environment of the low-level concentrations in feed was not assessed as agreed with the requestor.

To answer ToR2, '*To assess which levels of the antimicrobials have a growth promotion/increase yield effect*', the (FEEDAP) Panel will collect and examine the available data. Only food-producing animals will be considered in the assessment.

The ***Assessment question (AQ2)*** is: which are the specific antimicrobial concentrations in feed of food-producing animals that have an effect in terms of growth promotion/increased yield?

'Growth promotion' covers growth, lactating, laying and reproductive, quantitative and/or qualitative, performance of animals. The end points considered as a sign of growth promotion/increase yield effect on animals include: (i) Intake-related parameters: feed intake, feed/gain ratio (feed conversion ratio), feed efficiency, feed intake/milk yield, feed intake/egg mass; (ii) Weight-related parameters: body weight, body weight gain; (iii) Carcass-related parameters: carcass weight, carcass yield, carcass chemical composition, relative weight of the (different sections of) intestine; (iv) Milk or egg production/quality: milk yield, fat/protein yield, egg production/laying rate, egg weight, egg mass; (v) Digestibility/utilisation of nutrients: utilisation of some nutrients (e.g. dry matter (DM), Ca, P), digestibility; (vi) Health-related parameters: reduction of morbidity and/or mortality; (vii) Herd/flock-related parameters; (viii) Other end points: e.g. intestinal morphological characteristics (*villi* height/width), changes in microbiota.

Any level of antimicrobial showing an effect (positive or negative) on these parameters will be reported.

1.3. Additional information

1.3.1. Definitions and information relevant for the present opinion according to the Regulation (EU) 2019/4

'(6) **Medicated feed** is one of the routes for the oral administration of veterinary medicinal products. Medicated feed is a homogeneous mixture of feed and veterinary medicinal products. Other routes for oral administration, such as mixing of water for drinking with a veterinary medicinal product or manual mixing of a veterinary medicinal product into feed should not fall within the scope of this Regulation. The authorisation for use in feed, the manufacture, distribution, advertising and supervision of those veterinary medicinal products are governed by Regulation (EU) 2019/6 of the European Parliament and of the Council.'

'(16) **Cross-contamination** may occur during manufacture, processing, storage or transport of feed where the same production and processing equipment, including for mobile mixing, storage facilities or means of transport are used for feed with different components. For the purposes of this Regulation, the concept of cross-contamination is used specifically to designate the transfer of traces of an active substance contained in a medicated feed to a non-target feed. Contamination of non-target feed with active substances contained in medicated feed should be avoided or kept as low as possible.'

'(17) In order to protect animal health, human health and the environment, **maximum levels of cross-contamination for active substances in non-target feed** should be established, based on a scientific risk assessment performed by the European Food Safety Authority (EFSA) and in cooperation with the European Medicines Agency, as well as taking into account the application of good manufacturing practice and the 'as low as reasonably achievable' ('ALARA') principle. Until the completion of that scientific risk assessment, national maximum levels of cross-contamination for active substances in non-target feed, regardless of its origin, should apply, taking into account the unavoidable cross-contamination and the risk caused by the active substances concerned'.

According to Art. 3 of the Regulation (EU) 2019/4, the following definitions also apply:

(a) "**medicated feed**" means a feed, which is ready to be directly fed to animals without further processing, consisting of a homogenous mixture of one or more veterinary medicinal products or intermediate products with feed materials or compound feed;

(b) "**intermediate product"** means a feed, which is not ready to be directly fed to animals without further processing, consisting of a homogenous mixture of one or more veterinary medicinal products with feed materials or compound feed, exclusively intended to be used for the manufacture of medicated feed;

(c) "**non-target feed"** means feed, whether medicated or not, which is not intended to contain a specific active substance;

(d) "**cross-contamination"** means contamination of a non-target feed with an active substance originating from the previous use of the facilities or equipment.'

The importance of antimicrobial resistance from the One-health perspective is included also in the preambles of the Regulation (EU) 2019/4:

'(22) It is important to take into consideration the international dimension of the development of antimicrobial resistance. **Antimicrobial resistant organisms** can spread to humans and animals in the Union and third countries through consumption of products of animal origin, from direct contact with animals or humans or by other means. This has been recognised in Article 118 of Regulation 2019/6 which provides that operators in third countries are to respect certain conditions relating to antimicrobial resistance for animals and products of animal origin exported from such third countries to the Union. This is to be taken into consideration also in respect of the use of antimicrobial medicinal products concerned if they are administered via medicated feed. Furthermore, in the context of international cooperation and in line with the activities and policies of international organisations such as the World Health Organization (WHO) Global Action Plan and the Strategy on Antimicrobial Resistance and the Prudent use of Antimicrobials of the World Organization- for Animal Health, steps restricting the use of medicated feed containing antimicrobials in order to prevent a disease should be considered worldwide for animals and products of animal origin exported from third countries to the Union'.

'(30) The "**One Health"** concept, endorsed by the WHO and the World Organization for Animal Health (OIE), recognises that human health, animal health and ecosystems are interconnected and it is therefore essential for both animal and human health to ensure prudent use of antimicrobial medicinal products in food-producing animals'.

'(31) On 17 June 2016, the Council adopted conclusions on the next steps under a **One Health approach to combat antimicrobial resistance**. On 13 September 2018, the European Parliament adopted a resolution on a European One Health Action Plan against Antimicrobial Resistance'.

1.3.2. Antimicrobial resistance

Food-producing animal production is an important reservoir, in EU and non-EU countries, of antimicrobial-resistant bacteria with relevance for human and animal health (EMA CVMP and EFSA BIOHAZ Panel, 2017; EFSA and ECDC, 2020).

Antimicrobials are currently used in food-producing animal production for treatment, prevention and/or metaphylaxis of a large number of infections, and also for growth promotion in non-EU countries. In the EU, in future, use of antimicrobials for prophylaxis or for metaphylaxis is to be restricted as addressed by Regulation (EU) 2019/6 and use in medicated feed for prophylaxis is to be prohibited under Regulation (EU) 2019/4. Antimicrobial resistance emergence and spread is promoted by exposure of bacteria to antimicrobials, and also favoured in settings where numerous and diverse bacteria coexist, such as in animal production and storage or processing of faecal waste or run-off and wastewater from farms and abattoirs. The evidence to date demonstrates that exposure to sub-inhibitory concentrations of some antimicrobials does not only select for resistant bacteria but also contributes to an increase in the rate of DNA mutation and emergence of multidrug-resistant mutants, as well as promoting dissemination of antimicrobial resistance (Andersson and Hughes, 2014; Nové et al., 2020).

Antimicrobial use has always been associated with the development of resistance via induction or selection of mutant strains, including spontaneous mutants that always occur within a bacterial population but do not normally persist, and selection of naturally resistant organisms or stimulation of transfer of mobile genetic elements (MGEs) (Baylay et al., 2020). Such phenomena can occur in bacteria from humans, animals and the environment, either saprophytic, commensal or pathogenic. When antimicrobials are present, susceptible bacterial cells are placed at a selective disadvantage, leaving or selecting resistant strains that continue to multiply; the so-called antimicrobial selective pressure effect. Bacteria can acquire resistance as a consequence of mutations, or due to the acquisition of foreign DNA by horizontal gene transfer (HGT), usually involving MGEs such as plasmids and integrative conjugative elements (frequently carrying transposons, insertion sequences, integrons), or bacteriophages, and/or membrane vesicles (Partridge et al., 2018; Abe et al., 2020). Once acquired, antimicrobial resistance genes can be transferred to the cell progeny by vertical transmission, or to other bacteria of the same or different species by horizontal transmission, when associated with MGEs. These resistance genes encode different mechanisms that may be classified within four large groups, as follows: (1) Enzymatic inactivation/modification where bacteria produce enzymes that are capable of modifying or destroying the antimicrobial before it reaches its target, such as ß-lactamases which degrade penicillins and cephalosporins or aminoglycoside modifying genes. (2) Alternative pathways, such as in the resistance to antimicrobials that inhibit dihydrofolate synthesis, e.g. sulfonamides and trimethoprim. (3) Decreased accumulation of the antimicrobial, via a reduction in drug uptake (e.g. altered porins) or enhanced export of antimicrobials from the bacterial cell (efflux) e.g. imipenem resistance mediated by OprD function impairment and tetracycline extrusion from the cell by acquired/ activated efflux pumps. (4) Modifications of the antimicrobial target, thereby decreasing the affinity for the drug, such as in resistance to macrolides and (fluoro)quinolone antimicrobials.

Resistance to an antimicrobial class can occur as a result of different biochemical pathways that can be simultaneously present in the same bacterial cell, leading to a cumulative effect, usually resulting in increasing levels of resistance. As an example, the coexistence in the same bacterial cell of several fluoroquinolone resistance mechanisms e.g.: mutations in gyrase and topoisomerase IV, the target proteins of fluoroquinolones and increased efflux due to QepA or production of a modifying enzyme, the AAC(6′)-Ib-cr (Jeong et al., 2008; Tao et al., 2020), provides an additive combination of mechanisms resulting in the bacteria being resistant to higher concentrations of the antimicrobial. Similarly, higher minimum inhibitory concentrations (MICs) may be associated with the accumulation of multiple copies of the same AMR gene (Decano et al., 2019; Vinué Santolalla et al., 2019). Of note, some mechanisms of resistance for a given antimicrobial are more common for some bacterial species/

groups, such as, for the β-lactams, the predominance of β-lactamases in Gram-negative bacteria vs. the modifications in β-lactams target site: the penicillin-binding proteins (PBPs) in Gram-positive bacteria.

Selection and enrichment of resistant bacteria due to sub-minimal inhibitory concentration (Sub-MIC) of antimicrobials

Numerous studies have demonstrated that levels above the minimal inhibitory concentration (MIC) of an antimicrobial will result in the selection of antimicrobial resistant bacteria. However, the role of sub-MIC concentrations in enrichment of antimicrobial resistance is less well studied and defined. Since antimicrobial concentrations due to cross-contamination of non-medicated feed are likely to be well below the MIC of the various antimicrobials included in the present assessment for the relevant target bacteria, it is of utmost importance to understand how sub-MIC levels of antimicrobials impact on the selection of resistance and to find sensitive and accurate methods to determine which are the lowest selective concentrations for different antimicrobials. Relevant in this context is the demonstration in publications during the last decade that antimicrobial levels that are several 100-fold below the MIC of susceptible bacteria (e.g. *E. coli, S. enterica*) can not only enrich for pre-existing resistant bacteria (Gullberg et al., 2011, 2014; Liu et al., 2011; Andersson and Hughes, 2014) but also select *de novo* (Wistrand-Yuen et al., 2018) for high-level resistant bacteria (see Section 2.2.1.3.1.2). For example, a ciprofloxacin concentration of 100 pg/mL (230-fold below MIC) could enrich for a pre-existing resistant *gyrA* mutant (Gullberg et al., 2011). Recently published data (Wistrand-Yuen et al., 2018) also show that antimicrobial concentrations well below the MIC can select from a susceptible starting strain for highly resistant (above clinical breakpoints) *E. coli* bacteria. These findings unambiguously demonstrate that sub-MIC concentrations can be selective and underline the potential for very low levels of antimicrobials to act as drivers of resistance evolution in natural settings. A central concept that was introduced based on these studies is the **Minimal Selective Concentration (MSC),** which designates the lowest drug concentration that can result in enrichment of resistant bacteria in a mixture of resistant and susceptible strains that are isogenic except for the resistance mutation. Apart from the above studies, this experimental measure has been utilised in several other studies, including resistance selection in complex communities (Lundström et al., 2016; Khan et al., 2017; Kraupner et al., 2018; Klümper et al., 2019; Murray et al., 2020; Stanton et al., 2020), indicating its usefulness as a key parameter to use for assessing risk of resistance selection. In the scientific opinion text below, the use of MSC for risk assessments will be further described.

1.3.3. Establishment of limits for consumer safety of antimicrobial residues in foodstuffs derived from animals treated with veterinary medicinal products – EMA/VICH approach (VICH GL36)

Veterinary medicinal products used in the treatment or prevention of disease in food-producing animals may give rise to residues of such products or their metabolites in foodstuffs obtained from treated animals. In order to ensure consumer safety, an assessment of the safety of residues of all pharmacologically active substances contained in veterinary products for food-producing animals should be conducted in accordance with Regulation (EC) No 470/2009[3].

The approach used by the Committee for Medicinal Products for Veterinary Use (CVMP) for the evaluation of the consumer safety of active substances used in veterinary medicinal products is based on the determination of the acceptable daily intake (ADI) and of maximum residues limits (MRLs). MRLs are the maximum allowed concentration of residues in a food product obtained from an animal that has been treated with a veterinary medicinal product and are established based on the calculated ADIs from preclinical data and residue depletion studies in target animal species. Commission Regulation (EU) 2018/782[4] describes the methodology to be used in the scientific risk assessment and establishment of risk management recommendations relevant to MRL applications. Accordingly, the ADI is the total amount of residue (expressed per unit body weight) that can be ingested daily over a lifetime without a health risk to the consumer. The ADI is set on the basis of the lowest value obtained

[3] Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council. OJ L 152, 16.6.2009, p. 11–22.

[4] Commission Regulation (EU) 2018/782 of 29 May 2018 establishing the methodological principles for the risk assessment and risk management recommendations referred to in Regulation (EC) No 470/2009. OJ L 132, 30.5.2018, p. 5–30.

from toxicological, pharmacological or microbiological studies. The microbiological studies are addressed in VICH GL36(R2) (EMA/CVMP, 2003). They focus on determining if the substance and/or its residues will have an effect on the human intestinal flora, by assessing the 'disruption of the colonisation barrier' and the 'increase of the population(s) of resistant bacteria'. For most antimicrobial substances a microbiological ADI (mADI) is calculated on the basis of antimicrobial susceptibility testing (MIC determination) of representative microorganisms of the human gut flora, as well as on parameters influencing the amount of active drug at the gut level.

As described in the VICH GL 36(R2) (EMA/CVMP, 2003), the mADI could be determined from two microbiological end points, and each of them can be addressed by *in vitro* or *in vivo* studies:

i) 'Disruption of the colonization barrier. Changes in bacterial populations are indirect indicators of potential disruption of the colonization barrier'. When the ADI is derived from MICs, the susceptibility of 'a minimum of 10 isolates from each of the genera' of the most representative of the healthy human flora should be obtained. 'Depending on the drug and the data available at the time of assessment, the concentration used to derive mADI could be the lowest obtained MIC, the lowest MIC_{50} or MIC_{calc}', as illustrated in the guideline.

ii) 'Increase in the population(s) of resistant bacteria in the human colon.' If the end point of concern is an increase in the population(s) of resistant bacteria, MICs are not used to address the resistance end point, but no-observed-adverse-effect concentrations (NOAECs) need to be derived from different *in vitro* studies or no-observed-adverse-effect level (NOAEL) from *in vivo* studies as described in the guideline. As mentioned in the guideline, 'studies to evaluate the emergence of resistance should take into account the organisms of concern in the intestinal tract and the documented resistance mechanisms to the drug class. [...] Changes in the proportions of resistant organisms during pre-treatment, treatment and post-treatment periods can be evaluated by enumeration techniques on media with and without the antimicrobial drug, applying phenotypic and molecular methodologies.'

Accordingly, an increase of the population(s) of resistant bacteria is assessed. For the purposes of VICH GL36(R2) guideline (EMA/CVMP, 2003), 'resistance is defined as the increase of the population(s) of bacteria in the intestinal tract that is (are) insensitive [or show reduced susceptibility] to the test drug or other antimicrobial drugs. This effect may be due either to the acquisition of resistance by organisms which were previously sensitive or to a relative increase in the proportion of organisms that are already less sensitive to the drug'.

Semi-continuous, continuous and fed-batch culture test systems are recommended by the guideline. 'The NOAEC derived from the lower 90% confidence limit for the mean NOAEC from *in vitro* systems should be used to account for the variability of the data. Therefore, in this formula uncertainty factors are not generally needed to determine the microbiological ADI. However, where there are concerns arising from inadequacies in the quality or quantity of *in vitro* data used in determining the NOAEC, the incorporation of an uncertainty factor may be warranted.'

$$\text{mADI} = \frac{\text{NOAEC} \times \text{volume of colon content}\left(500\,\frac{\text{mL}}{\text{day}}\right)}{\text{Fraction of oral dose available to microorganisms} \times 60\ \text{kg person}}$$

Changes in resistant populations can also be assessed *in vivo* (HFA rodents).

Two end points must be considered to derive the mADI. The most sensitive end point of the two will be retained as the basis for the mADI.

For a number of antimicrobials, the disruption of the colonisation barrier was considered the most sensitive end point, and therefore, a microbiological ADI was calculated for these substances on the basis of *in vitro* susceptibility testing obtained by standard test methods (MIC), using the following relevant intestinal bacteria (*E. coli*, and species of *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Eubacterium* (*Collinsella*), *Fusobacterium*, *Lactobacillus*, *Peptostreptococcus*/*Peptococcus*).

The main limitations associated with the estimation of NOAEC (increase of resistance) are:

- Limited bacterial groups diversity, as only a small number of culturable bacterial genera are considered representative of the human microbiota and assessed;
- Bacterial species representativeness of the genus indicated are not specified in guidelines, resulting in uncertainty on whether the more sensitive species are tested;
- Animal species representativeness of the *in vivo* studies to investigate resistance development.

1.3.4. Animal production and feeding

The different animal species are kept in a variety of production systems, some of which are relatively standard (e.g. poultry and pigs), and intensive, particularly for poultry and pigs, whereas others, particularly for beef herds, may be highly variable. Also, the type of feed and its preparation (e.g. purchased compound feed, home mixing, forages, liquid feed) used varies depending on the animal species (see detailed information in Annex A, Table A.1).

Most compound feed is formulated at 15% moisture, or less, limiting bacterial growth, so mutations and transfer of AMR are unlikely in properly stored dry feed (Hinton, 1993). Moist conditions during cooling of heat-treated feed, or storage that is subject to condensation may permit bacterial growth, but the influence of this on the occurrence or transfer of AMR is not known (Vukmirović et al., 2017). In this opinion, only the selective activity of feed antimicrobial residues that occurs after consumption of feed that contains residues derived from a previous batch of medicated feed is assessed.

1.3.5. Antimicrobials in animal production

Antimicrobials are used in animal production via medicated feed to treat or control disease in large groups of animals. Other routes of administration of antimicrobials are used in animal production and are not addressed in this opinion. The use of antibiotics for growth promotion has been forbidden in the European Union since 2006.[5] However, outside of the EU antimicrobials are still used as growth promoters at levels much lower than the MIC or recommended doses for administration via feed (Brown et al., 2017). In addition, sick animals may not consume the expected allowance of feed, so are likely to be under-dosed. Antimicrobials, other than coccidiostats or histomonostats, cannot be authorised in the EU to be used as 'feed additives' (Regulation (EC) No 1831/2003[6]) and can only be used subject to a veterinary diagnosis and prescription.

For information on antimicrobials allowed for use in the EU for food-producing species, see Section 2.1.1.1.

1.3.6. Pharmacokinetics, degradation and modification of antimicrobials

After ingestion of cross-contaminated non-target feed, the main location for resistance selection is considered to be within the digestive tract, and particularly the region of the intestinal tract where there is the greatest density and diversity of bacteria, even if the selection of resistance in commensal bacteria from other body sites (e.g. nasopharynx, skin and urogenital area) cannot be excluded.

To predict the influence of the cross-contamination of animal feed by antimicrobials on the selection of resistance in intestinal bacteria, the antimicrobial concentrations in the large intestines obtained after ingestion of the feed should be known or predicted. Following the ingestion of the feed, the active intestinal concentrations will mainly depend on:

- the ingested dose (percentage of contamination=mg of the antimicrobial per kg feed),
- the bioavailability after oral administration (e.g. if the antimicrobial is extensively absorbed in the proximal part of intestines, low concentrations will reach the large intestines).
- the elimination process of the antimicrobials (e.g. if eliminated in urine or metabolised to produce an inactive metabolite, the concentrations in intestines will be lower).
- the degradation or antimicrobial binding to metals or other components of the intestinal content that reduce the fraction of active antimicrobial in the intestines or provide protection against degradation.

2. Data and methodologies

The data used and the methodologies applied or developed to address ToR1 and ToR2 followed different approaches as described below.

[5] https://ec.europa.eu/commission/presscorner/detail/en/IP_05_1687

[6] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29–43.

2.1. Data

2.1.1. Data for ToR1

2.1.1.1. Data from the European Medicines Agency (EMA)

End point data from relevant studies used for the derivation of the mADI is published in European Public MRL Assessment Reports (EPMARs) (also called Summary Reports for older MRL applications[7]) and was assessed for this scientific opinion. Moreover, access to detailed study summaries aimed to determine the potential impact of the substance to the intestinal flora when ingested by consumers, has been made available to the experts when necessary by EMA, respecting the confidentiality agreement as established by the Memorandum of Understanding on working arrangements that is in place between EMA and EFSA.[8]

Anti-infectious agents/antibiotics allowed for use in the EU for food-producing species according to MRL Regulation (EU) 37/2010 are included in Annex B (Table B.1). A selection of the information on the antimicrobial substances under assessment for the considered animal species/categories is included in Table A.1, Annex A.

2.1.1.2. Public data on minimal inhibitory concentrations for antimicrobials under assessment

The selective pressure that an antimicrobial agent exerts on a bacterial strain is correlated to the MIC of that specific antimicrobial agent (Oz et al., 2014). Therefore, different sources were examined to retrieve MIC data regarding the antimicrobials to be assessed in this scientific opinion (see Appendix A). MIC data collections that are useful in this context must have been obtained using standard methods (i.e. CLSI or EUCAST) and, where possible, derive from diverse sources of isolates, both in terms of species and geographical origin, to ensure the widest possible representation of bacterial populations.

EUCAST has MIC distributions for a wide range of bacteria and antimicrobial agents (http://www.eucast.org/mic_distributions_and_ecoffs/). These distributions are based on a large amount of collated MIC data from clinical, commensal and environmental bacteria from worldwide sources, including veterinary programmes (e.g. VetCAST). As EUCAST distributions adhere to specific quality standards (http://www.eucast.org/documents/sops/), and are not restricted to any specific time period, host and geographical origin, they are currently the best available resource to represent the variability of MIC values within and between bacterial populations. The EUCAST MIC distributions in this context are considered to be representative of the diversity of bacterial populations. MIC distributions for each antimicrobial agent included in the mandate were downloaded from the EUCAST website (https://mic.eucast.org/Eucast2/, last accessed 15.5.2021). For every antimicrobial, there are different numbers of bacterial species that have been tested in this way, ranging from approximately 100 bacterial species with MIC distributions for tetracycline down to no bacterial species having MIC distributions for amprolium, apramycin, chlortetracycline, oxolinic acid, paromomycin, thiamphenicol, valnemulin and tylvalosin.

Based on the fact that EUCAST appears to be the most comprehensive source of MIC data, it was decided to search for additional MIC data only for the antimicrobial agents for which EUCAST MIC distributions are available for less than 10 bacterial species (see Appendix A for additional data sources). These include MIC distributions for (i) flumequine, lincomycin, tiamulin, thiamphenicol, tilmicosin and tylosin, which have limited representation in EUCAST; and (ii) amprolium, apramycin, oxolinic acid, paromomycin, valnemulin and tylvalosin, which are not represented in EUCAST at all. The data sources further checked are shown in Appendix A.

2.1.1.3. Other data

- For the purpose of this report, only some relatively standardised animal production systems are included. Further to this, the production systems included are stratified by age-class where deemed relevant (Annex A, Table A.1).

[7] https://www.ema.europa.eu/en/medicines/field_ema_web_categories%253Aname_field/Veterinary/ema_group_types/ema_document-maximum_residue_limits_report?sort=field_ema_public_date

[8] https://www.ema.europa.eu/en/documents/other/memorandum-understanding-working-arrangements-between-european-medicines-agency-european-food-safety_en.pdf

- A normalised body weight (EFSA FEEDAP Panel, 2017), and estimated daily faecal output mass/dry matter and volume of rumen content were used as indicated below (see Section 2.1.1.3.1, Table 1). These do not cover all breeds or hybrid lines within a species but are considered to be representative of the presumed most likely breeds and hybrid lines.
- Published data on PK of antimicrobials after oral administration and on binding and activity of antimicrobials in intestinal contents were used, whenever available.

2.1.1.3.1. Methodology to calculate daily output of faeces

Daily output of fresh faeces (kg fresh matter (FM)/animal per day) was calculated for animal species and categories, as they are indicated in the EFSA FEEDAP Panel Guidance on the assessment of safety of feed additives for the target species (EFSA FEEDAP Panel, 2017) and the respective EU Regulations.[6,9]

For this purpose, default values (EFSA FEEDAP Panel, 2017) for body weight (BW, kg) and feed intake (kg dry matter(DM)/animal per day) were used (Table 1).

For most animal categories, data for daily output of dried faeces (kg DM/1,000 kg bw per day) were obtained by the American Society of Agricultural Engineers database (ASAE, 2003, 2005), and specifically the total solid faecal values (kg DM) produced per 1,000 kg animal bw per day, which were then transformed into dried faeces per animal unit per day (kg DM/animal per day), using the default values for body weight for each animal category.

Exceptionally, due to the lack of suitable data, to calculate daily output of fresh faeces (kg FM/animal per day) in horse, rabbit and salmon, the fundamental concept of nutrient digestibility of feed was used (McDonald et al., 2011). Digestibility is a measure of the proportion of a feed that is digestible. The digestibility of a nutrient is measured as the difference between the amount of nutrient consumed minus the amount of nutrient excreted in the faeces, according to Equation 1:

Equation 1:

$$\text{Coefficient of Nutrient digestibility} = \frac{\text{nutrient consumed} - \text{nutrient in faeces}}{\text{nutrient consumed}}$$

Then, animal nutrient excretion is calculated, according to Equation 2:

Equation 2:

$$\text{Coefficient of Nutrient excretion} = 1 - \text{Coefficient of Nutrient digestibility}$$

Literature data were used to obtain a coefficient of DM digestibility of 0.75 for horse and rabbit (McDonald et al., 2011), and a coefficient of DM digestibility of 0.83 for salmon (Reid et al., 2009), and then, coefficients of DM excretion were calculated to 0.25 for horse and rabbit and 0.17 for salmon.

Data for daily output of dried faeces (kg DM/animal per day) were calculated by multiplying the default values for feed intake for horse, rabbit and salmon, and the respective coefficient of DM excretion.

Finally, DM content of faeces for all animal categories was obtained from various sources (Flachowsky, 1997, for most animal categories; Kraugerud et al., 2007, for salmon; Carabaño et al., 2010, for rabbit; Table 1), and then used to transform daily output of dried faeces (kg DM/animal per day) into daily output of fresh faeces (kg FM/animal per day).

[9] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1–65.

Table 1: Animal-related data (body weight, feed intake, daily output fresh faeces and volume of rumen content) used for the Feed Antimicrobial Resistance Selection Concentration (FARSC) calculations

Animal category[(a)]	Body weight (kg)[(a)]	Feed intake (kg dry matter (DM)/ animal per day)[(a)]	Daily output of dried faeces (kg DM/ animal per day)[(b)]	DM content of faeces (g DM/kg fresh matter (FM))[(c)]	Daily output of fresh faeces (kg FM/animal per day)	Volume of rumen content (solid + Liquid phase, L)[(d)]
Chicken for fattening	2	0.158	0.044	330	0.133	
Laying hen	2	0.106	0.032	200	0.160	
Turkey for fattening	3	0.176	0.036	330	0.109	
Piglet	20	0.88	0.22	250	0.88	
Pig for fattening	60	2.20	0.66	250	2.64	
Sow lactating	175	5.28	1.925	250	7.70	
Veal calf (milk replacer)	100	1.89	0.52	220	2.36	
Cattle for fattening	400	8.0	3.4	180	18.89	60–120
Dairy cow	650	20.0	7.8	140	55.71	90–180
Sheep	60	1.2	0.66	450	1.47	9–18 (1/10 of dairy cows)
Goat	60	1.2	0.78	450	1.73	9–18 (1/10 of dairy cows)
Horse	400	8.0	2.0	240	8.33	
Rabbit	2	0.1	0.025	470	0.053	
Salmon	0.12	0.0021	0.000357	150	0.00238	

(a): EFSA FEEDAP Panel (2017).
(b): ASAE Standards (2003), for most animal categories; Reid et al. (2009), for salmon; McDonald et al. (2011), for horse and rabbit.
(c): Flachowsky (1997), for most animal categories; Kraugerud et al. (2007), for salmon; Carabaño et al. (2010), for rabbit.
(d): Data collected from the literature and expert knowledge (Kararli (1995), for cattle for fattening, sheep and goat; Gasa et al. (1991), for dairy cows).

2.1.2. Data for ToR2

To answer the ToR2, the data available in the public literature were collected through an extensive literature search (see Section 2.2.2). In addition to the literature search, EFSA consulted other institutions for relevant existing evaluations/authorisations on antimicrobials as growth promoters and the respective information.

2.2. Methodologies

2.2.1. Methodology for ToR1

2.2.1.1. Literature search

The information used on mechanisms of antimicrobial resistance was based on expert knowledge supported by extensive literature searches, as described in Bortolaia et al. (2020). Also, expert knowledge and literature searches were used to retrieve information on development/selection/spread of resistance to antimicrobials, including at low level exposure, and methods for determining the lowest concentration of antimicrobials not selecting for antimicrobial resistance. In all these cases, the searches were broadened out using 'footnote chasing' (White et al., 1992), reference citation and supplemented by citation input by Working Group (WG) members and information about relevant publications provided by members of the EFSA BIOHAZ, AHAW and FEEDAP Panels. This activity continued throughout the term of the mandate until the WG members were satisfied that a thorough coverage of the subject had been achieved. The relevance of each record in terms of providing robust

information on the aforementioned topics was assessed by screening the title, keywords and the abstract and based on the knowledge and expertise of the WG members.

AMR surveillance reports were retrieved using the knowledge available within the EU Reference Laboratory for Antimicrobial Resistance Network (https://www.eurl-ar.eu/). Only reports available online and written in English were selected.

For pharmacokinetics and predicted intestinal concentrations, a literature search was carried out in PubMed using a broad range of terms covering, for each drug and each animal species (cattle, calf, sheep, lamb, goat, pig, horse, poultry, broiler, rabbit) "urinary excretion AND oral", "bioavailability AND oral", "fecal/faecal AND concentrations", "intestinal AND concentrations", "digestive AND concentrations", "activity AND intestinal", "binding AND intestinal". For each drug, the research was conducted with the name of the drug, the name of the class and the names of the related drugs. As an example, for tetracyclines, the data on tetracycline, chlortetracycline and oxytetracycline were collected. Many data being very old, no limit was applied for the date of publication. Two books of reference in veterinary pharmacology (Veterinary Pharmacology and Therapeutics, Riviere and Papich, 10th edition) and veterinary antimicrobial therapy (Antimicrobial therapy in Veterinary Medicine, Giguère, Prescott and Dowling, 5th edition) were also consulted to collect the cited primary references.

For data relating to intestinal and faecal volume, mass and faecal dry matter output parameters of food-producing animals, searches were carried out in Google, Google Scholar and Scopus using exploratory keywords relating to the production sector, intestine/gut, faecal output/dry matter, etc., while snowballing (Sayers, 2007) was further used to identify additional literature. Considering the large differences in existing physiological data within animal species and categories, data obtained are representative of values that apply to European farming conditions of food-producing animals.

To search for effects of sub-inhibitory concentrations of antimicrobials, searches using keywords related to low or sub-inhibitory concentrations (e.g. "sub-MIC", "sub-inhibitory", "low level(s)", "low concentration(s)"), combined with antimicrobial resistance (e.g. "AMR", selection), horizontal gene transfer, virulence and/or mutagenesis and with the different antimicrobial classes or single substances under assessment were performed. Examples of the hits retrieved were included in the different assessments (see the complementing Scientific Opinions Parts 2–13, EFSA BIOHAZ Panel, 2021a–l, and also the Virtual Issue).

2.2.1.2. Public consultation

In line with EFSA's policy on openness and transparency, and in order for EFSA to receive input from interested parties, relevant sections of the draft scientific opinion on maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed were released for written public consultation (from 7 October to 18 November 2020). Those sections open for consultation included the methodology proposed to answer the European Commission Mandate's ToR 1, which is related to antimicrobial resistance development, the uncertainties associated with this methodology and the data gaps and other considerations identified. Comments received were discussed by the experts of the WG and BIOHAZ Panel, and the opinion was updated when required. The outcome containing all information in relation to the public consultation, including the comments received and the explanations on the way these comments were considered for the finalisation of the opinion is included in Annex C.[10]

2.2.1.3. Model development to determine the levels in non-target feed below which there is no emergence of, and/or selection for, resistance in the animal microbiota

To indicate the specific antimicrobial concentrations in non-target feed that do not promote emergence of, and/or select for resistance, a two-step approach was followed. First, to identify the available scientific methodological approaches to determine the lowest concentrations of different antimicrobials that does not select for bacterial resistance (Section 2.2.1.3.1). Subsequently (Section 2.2.1.3.2), and to estimate the active fraction of the consumed antimicrobial reaching the animal compartment where contact with microbiota occurs, an approach considering different factors (e.g. pharmacokinetic parameters, degradation) was developed.

[10] The outcome of the public consultation was endorsed by the BIOHAZ Panel in the form of a Technical Report at its 148th Plenary meeting held on 15–16 September 2021. Due to the implementation of the new OpenEFSA portal, there has been a change and the outcome of the public consultation is now reported as an Annex to the Opinion, and the Question number initially assigned to the Technical Report is no longer of use.

2.2.1.3.1. First step: Determination of the lowest concentration of antimicrobials not selecting for antimicrobial resistance

The key objective in this opinion is to determine the concentration of different antimicrobials, for which, if bacteria were exposed to them at this concentration or lower, no selection and/or enrichment of resistant bacteria (that are either pre-existent or emerge *de novo* via mutational changes or lateral gene transfer) would occur after antimicrobial exposure.

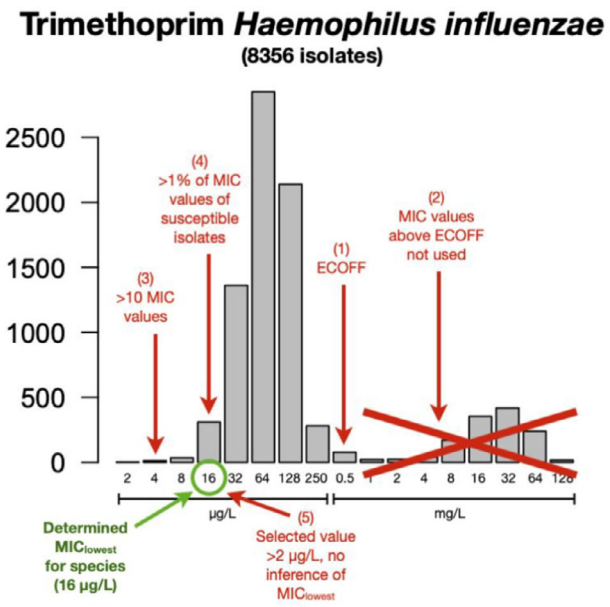
A number of different approaches are available to determine the antimicrobial levels below which selection and enrichment of resistant bacteria (which may either be pre-existing or emerge *de novo* via mutational changes or lateral gene transfer) is not detected. All these methods have different underlying assumptions, advantages and disadvantages; while some methodologies are based on simplified *in vitro* experimental systems, others try to mimic a more natural situation. Importantly, they also have very different sensitivities to detect selection for resistance by low antimicrobial levels. In this context, it has to be remembered that a majority of the selective processes in biology, including antimicrobial resistance evolution, occur over long time periods and act on small selective differences that might be very difficult to detect if the assay is not sensitive enough (Andersson and Hughes, 2014). To exemplify, the problems we face today in human and veterinary medicine regarding the very high frequency of resistant pathogenic bacteria is the result of about 80 years of antimicrobial use that has gradually resulted in enrichment of resistant bacteria by natural selection. Thus, the selective advantage (i.e. the growth difference between a susceptible and a resistant bacterium at a given antimicrobial concentration) can be very small but still, as a result of many generations of growth (i.e. over a long time), generate a strong enrichment of resistant bacteria.

This creates a dilemma since most natural study set-ups (e.g. where researchers look for an increase in the fraction of resistant bacteria in the intestinal microbiome of an animal that is treated with antimicrobials for a relatively short time, i.e. typically days to weeks, as compared to a non-treated animal) are also the least sensitive, especially when performed as short-term experiments. In contrast, methods performed under highly controlled laboratory conditions are less natural, but they have a much higher sensitivity and allow for the detection of very small selective effects at low antimicrobial levels (please see further explanation in Sections 2.2.1.3.1.2 and 3.1).

Below, three different methods, including predicted no effect concentration (PNEC), minimal selective concentration (MSC) and predictive minimal selective concentration (PMSC), for determining the lowest concentration of antimicrobials not selecting for antimicrobial resistance are presented and their strengths and limitations discussed.

2.2.1.3.1.1. Predicted No Effect Concentration (PNEC) for resistance selection

Bengtsson-Palme and Larsson (2016) used MIC distributions from the EUCAST database to calculate PNEC for resistance selection. The fundamental idea underpinning this modelling is that a concentration of an antimicrobial that inhibits the growth of (or kills) any one of the strains in a community will inevitably, in the long run, select for a more resistant microbial community, either through strain replacement or via acquisition of resistance mutations or genes. In short, the Bengtsson-Palme and Larsson approach is based on MIC data obtained from the public EUCAST database. A six-step procedure was followed: (1) For each species and tested antimicrobial the ECOFF value is extracted from EUCAST. (2) To avoid resistant isolates influencing the analysis, all MIC data above the EUCAST ECOFF value were discarded (if ECOFF is defined; if not defined, all MIC data available are used). (3) The lowest MIC with at least 10 observations was identified for each species. (4) The 1% lowest observed MIC was identified (i.e. the concentration where 99% of the tested isolates for a given bacterial species had a higher MIC) to avoid including outliers that may be present in the database due to an individual experimental mistake or a strain mix-up. If this value was below the concentration identified in step 3, the concentration identified in step 3 was used instead as the MIC_{lowest}; (5) if the observed MIC_{lowest} is the lowest concentration tested, the actual MIC_{lowest} was inferred by calculating the mean log_2-distance from the peak MIC of the EUCAST distribution across all other species tested against the antibiotic and then applying the same log_2-distance from the peak of the currently considered species (see Bengtsson-Palme and Larsson, 2016 for specific details). (6) The smallest MIC_{lowest} value across all species for a given antimicrobial was chosen as the overall MIC_{lowest} for that antimicrobial. As an example, in Figure 1, the calculation of $MIC_{lowest.}$ *Haemophilus influenzae* (ECOFF = 0.5 mg/L) was the species presenting the smallest MIC (4 μg/L; in at least 10 observations) and when calculating 1% lowest observed MIC, 16 μg/L was obtained for the $MIC_{lowest.}$ As this concentration does not correspond to the lowest concentration tested, no further correction was required (step 5 not applied).



The y-axis shows number of isolates in EUCAST with a specific MIC (x-axis). After applying the five steps shown in the figure, the MIC_{lowest} values were compared for all species tested against trimethoprim in EUCAST (step 6). *Haemophilus influenza* was the species with the smallest MIC_{lowest} value for trimethoprim, and thus, 16 µg/L was selected as the overall MIC_{lowest} for trimethoprim.

Figure 1: Example of calculation of the lowest MIC of trimethoprim for *Haemophilus influenzae*

Importantly, selecting the lowest MIC following the steps described ensures that the procedure is protective of all strains within every species. Selecting, e.g. the median MIC value in the distribution would result in selecting for the most resistant part of the population, which is to be avoided. The Perl code to perform these steps is available from Annex D.

For many species, the numbers of tested strains/isolates are low and to compensate for this, the lowest MICs for antimicrobials with less than 40 species tested were adjusted for the number of tested species. This adjustment was based on modelling of the MIC distributions for antimicrobials with more than 40 tested species. The best-fitting model for this correction turned out to be one where the observed lowest MIC for an antimicrobial is multiplied by the number of tested species for the same antimicrobial and then divided by 41. After this correction, PNECs for resistance selection were calculated by applying an assessment factor of 10 to account for differences between MICs and minimal selective concentrations as elaborated on in Bengtsson-Palme and Larsson (2016) and shown in Figure 2.

Example calculation of PNEC									
Escherichia coli	***Mannheimia haemolytica***	***Neisseria gonorrhoeae***	***Pasteurella multocida***	***Salmonella spp.***	***Serratia spp.***	***Staphylococcus aureus***	***Staphylococcus haemolyticus***	***Staphylococcus hominis***	***Staphylococcus hyicus***
Lowest MIC in EUCAST (µg/mL)									
8	**8**	**2**	**8**	**16**	**8**	**64**	**8**	**2**	**32**
								Lowest MIC across species (µg/mL)	**2**
								Adjusted for low species coverage (2 µg/mL x 10 / 41)	**0.488**
								Application of assessment factor (10)	**0.0488**
								Rounding down to the closest step of the EUCAST testing scale (µg/mL)	**0.032**
								Final PNEC (µg/L)	**32**

Figure 2: Example of the Predicted No Effect Concentration (PNEC) calculation

The AMR Industry Alliance has adopted this method to calculate PNECs as the best available targets to help limit the selection pressure on the aquatic microbiome from discharges of antimicrobials from manufacturing sites (Tell et al., 2019).

The main uncertainty specific to this calculation is whether the lowest observed MIC corresponds to the actual lowest MIC across all bacteria, i.e. whether the distribution of tested species in EUCAST is representative for all bacteria in nature. For antimicrobials with more than 40 tested species, this effect was estimated to be negligible in an analysis made in the Bengtsson-Palme and Larsson paper. This means that this uncertainty can basically be ignored for antimicrobials with more than 40 tested species in EUCAST, but the fewer the number of tested species, the larger this uncertainty will be. The model used by Bengtsson-Palme and Larsson accounts for that effect, but it is unknown how reliable the estimates are for antimicrobials with just a small number of bacterial species tested. Importantly, though, a central part of the approach is to include all tested bacteria in this MIC calculation, rather than limiting the analysis to what may seem like relevant bacteria in a particular environment (such as the animal intestine). This is because this wide diversity of bacteria functions as a proxy, capturing the width of the sensitivity distribution. The use of the sensitivity of all bacterial species included in EUCAST as a proxy for the general sensitivity of bacterial species is reasonable, based on the assumption that antimicrobial sensitivity is not significantly different for human-associated bacteria compared to other bacterial species, such as those present in the animal intestine. At present, the existing data suggest that this assumption is valid. The distribution of antimicrobial sensitivities for human-associated bacteria is wide and does not seem to be significantly different from the sensitivity distribution for non-human associated bacteria. For these reasons, cherry-picking which bacteria to include from the distribution results in a less accurate number for the PNEC, as fewer representatives would be included, increasing the uncertainty around the lowest MIC. These considerations, which are also relevant for the methodology described in Section 2.2.1.3.1.2, have been included in Sections 3.1 (Data gaps and other important considerations) and 3.3 (Uncertainty analysis).

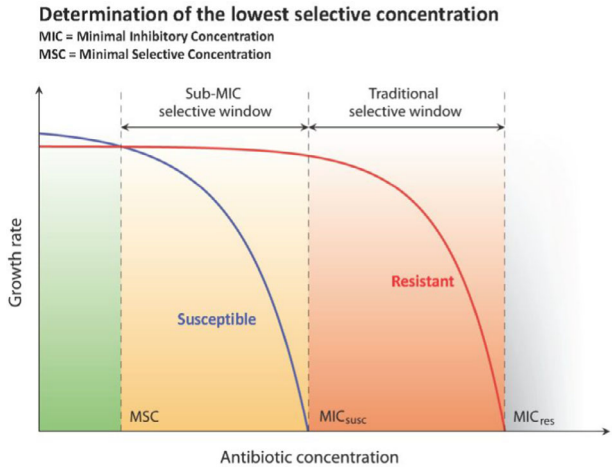
Furthermore, as described in detail by Bengtsson-Palme and Larsson (2016), the relationship between the MICs derived from EUCAST and the concentrations that potentially would be selective below the MIC (see the discussion on minimal selective concentrations below) is also a factor of uncertainty in the PNEC estimation process. Therefore, depending on the context, Bengtsson-Palme and Larsson emphasise that additional safety margins should be employed if there are reasons to believe that certain environments need particular protection, or if there is experimental data suggesting high selective potency for certain antimicrobials substantially below the MIC. As the assessment factor used is intentionally small, exposure limits for antimicrobials should never exceed the suggested PNECs unless there is strong and relevant experimental evidence for lack of effects at higher concentrations.

As the PNECs explicitly also take other factors related to the risk assessment process into account (including a trade-off between feasibility and small risks for selective capacity of antimicrobials), they may not be fully protective against resistance development in all situations. We have therefore opted for a more stringent approach to predict no-effect levels for resistance selection (see below).

2.2.1.3.1.2. Minimal selective concentration (MSC) and predicted minimal selective concentration (PMSC)

Another approach to assess the risk of resistance selection is based on using experimentally determined minimal selective concentration (MSC). With this approach, one determines which is the lowest concentration that can either enrich for a pre-existing resistant bacterium when in competition with a congenic susceptible strain (method A) or, beginning with a susceptible strain, select for *de novo* resistance (method B). The advantages of these methods over inferred data from MIC determinations are that it is an extremely sensitive and reproducible approach to assess risk of resistance selection and that method B can be applied to any bacterial species or community and antibacterial compound. Method A is technically more challenging and requires that the bacterial species examined is genetically amenable (e.g. through the possibility to introduce genes encoding fluorescent markers such as green fluorescent protein (GFP) that are largely neutral in their effect on bacterial growth). Since at present no/very limited published experimental data is available that is generated using method B (*de novo* selection), this scientific opinion will only describe and use results obtained from method A (competition approach). It can be noted that results from two studies (Gullberg et al., 2011; Wistrand-Yuen et al., 2018) indicate that if one performs a *de novo* selection (method B) at an MSC concentration determined from method A, resistant mutants will be selected and enriched. Thus, this finding validates that the MSC extrapolated from method A can result in *de*

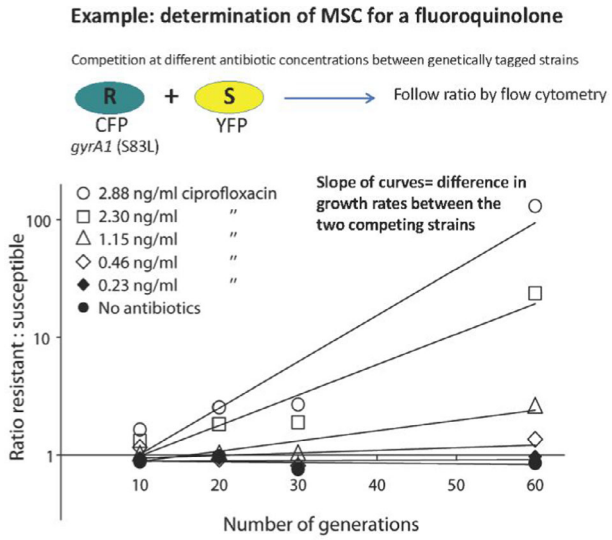
novo selection. Below we outline in Figures 3–5 in detail how method A is used to determine the MSC for an antimicrobial drug. Figure 3 shows the underlying principle of the meaning of an MSC value. Thus, in a competition between isogenic susceptible and resistant bacteria, it designates the concentration at which the selective effect of the antimicrobial is balanced by the fitness cost of the resistance (i.e. the intercept of the two curves in Figure 3). If the antimicrobial concentration is above the MSC, the resistant bacteria will be enriched, and if it is below, no enrichment of the resistant bacteria will occur.



(@copyright Gullberg et al., 2011, permission to reproduce the figure provided by Dan I Anderson)
Green indicates a concentration interval where the susceptible strain (blue line) will outcompete the resistant strain (red line). Orange (sub-MIC selective window) and red (traditional mutant selective window) indicate concentration intervals where the resistant strain will outcompete the susceptible strain.
MIC_{susc}: minimum inhibitory concentration of the antimicrobial for the susceptible strain; MIC_{res}: minimum inhibitory concentration for the resistant strain; MSC: minimal selective concentration.

Figure 3: Schematic representation of growth rates as a function of antimicrobial concentration for susceptible and resistant bacteria

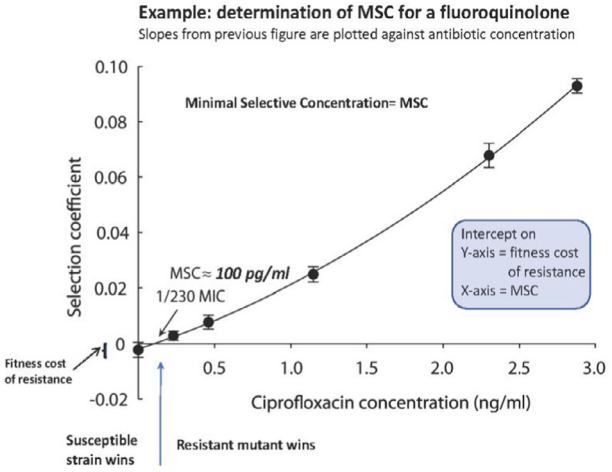
Figures 4 and 5 below show how the MSC value is determined from a competition experiment at different antimicrobial concentrations (i.e. method A). In the example shown in Figure 4, a susceptible and resistant strain (fluoroquinolone resistant due to a *gyrA* mutation) are mixed 1:1, and then, the ratio of resistant/susceptible bacteria is determined (by flow cytometry since the strains are genetically tagged with different fluorescent markers) as a function of number of generations of growth (typically 20–40 generations) at different fluoroquinolone concentrations. In the absence of antimicrobial, the slope is slightly negative, indicating that the susceptible strain outcompetes the resistant strain. As the antimicrobial concentration is increased, the slope becomes positive (i.e. the resistant strain increasingly outcompetes the susceptible strain). At the drug concentration where the slope is zero, the fitness cost of the resistance is balanced by the antimicrobial selective effect = MSC.



On the Y-axis are the ratios of resistant:susceptible bacteria and on X-axis is the number of generations of growth. The strains are genetically tagged with different fluorescence markers allowing flow cytometric detection of large numbers of cells and therefore very precise determinations.

Figure 4: Competition experiments between ciprofloxacin-susceptible and -resistant strains of *E. coli* at different concentrations of antimicrobials as a function of number of generations of growth

To be able to more easily determine the MSC, the slopes (i.e. selection coefficients) obtained in Figure 4 are plotted as a function of antimicrobial concentration, resulting in Figure 5. The intercept on the X-axis is the MSC value and the intercept on the Y-axis is the fitness cost of the resistance. The fitness cost of the resistance is the negative effect the resistance mechanism has on bacterial growth in the absence of antimicrobial. For example, if the fitness cost is 2%, it means that a resistant bacterium grows 2% slower than the corresponding isogenic susceptible strain.



On the Y-axis are the selection coefficients (slope of curves from Figure 4) and on the X-axis are the antimicrobial concentrations.

Figure 5: Calculated selection coefficients as a function of antimicrobial concentrations (data from Figure 4)

Using the above methodology, a few papers (Gullberg et al., 2011, 2014; Andersson and Hughes, 2014; Wistrand-Yuen et al., 2018) have determined MSCs for *E. coli* and *S. enterica* and the antimicrobials streptomycin, tetracycline, ciprofloxacin, trimethoprim and erythromycin, based on method A (competitions). The MSCs are always at least fourfold below the MIC-value and in some

cases, several hundred times lower (e.g. ciprofloxacin 230-fold, illustrated in Figures 2–4 above, and tetracycline 100-fold). It is important to point out that the MSC value will be influenced by the specific resistance mechanism and in particular its fitness cost. Thus, as can be seen in Figure 5, if the fitness cost of the resistance mechanism is increased, the plotted curve will move downwards and the intercept with the X-axis will move towards the right resulting in a higher MSC. In other words, MSC values should be determined for the clinically most prevalent resistance mechanisms to provide the most relevant data.

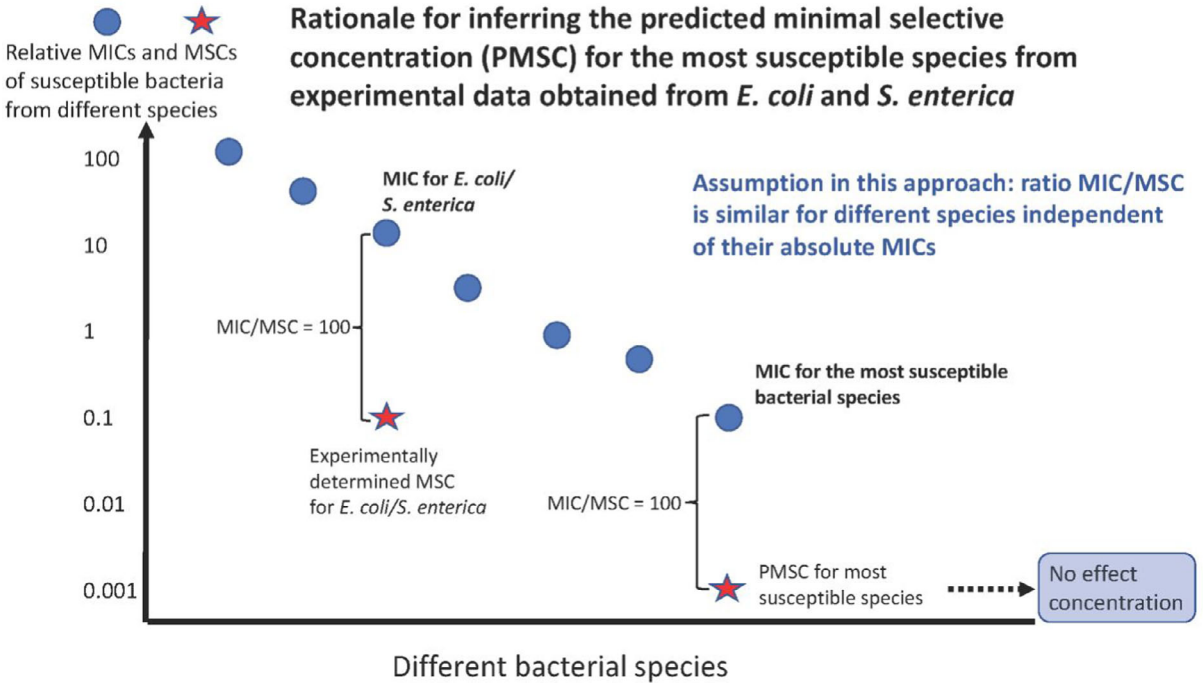
The use of method A and determination of MSC values to guide the establishment of non-selective antimicrobial concentrations is rational and scientifically sound but it also has, at present, associated limitations that have to be considered. These limitations are associated with a lack of experimental data and MSC determinations for different species and strains, antimicrobials and environmental conditions. Thus, the data available at present are only for specific strains of *E. coli* and *S. enterica* and the antimicrobials tetracycline, ciprofloxacin, trimethoprim, erythromycin and streptomycin. Because experimental data are limited, certain extrapolations would have to be made. Below we outline three major limitations and the approaches we have chosen to address them.

(i) Impact of complexity on determined MSCs. Since MSC values are determined under very defined conditions with one specific strain of a species present, a second limitation is to what extent increased complexity due to presence of, e.g. other bacterial species, predators and altered growth conditions can influence the MSC. *A priori* it is difficult to make any general predictions about how a community setting would affect the MSC. However, it has been argued that a community could act to reduce the free concentration of a drug and/or increase the cost of the resistance, thereby increasing the apparent MSC (Klümper et al., 2019). A limited number of studies have allowed single species vs. community comparisons to be made. For one case (tetracycline), the MSC is 15-fold lower in the community compared to the defined single species set-up (1 μg/L vs. 15 μg/L) (Gullberg et al., 2011; Lundström et al., 2016). For the two other cases (ciprofloxacin and gentamicin/kanamycin), the pattern is the opposite and the MSC is 10-fold higher for the community compared to the defined single species and strain set-up (1 μg/L vs. 0.1 μg/L for ciprofloxacin, Gullberg et al., 2011; Kraupner et al., 2018) and approximately 40-fold higher for gentamicin and kanamycin (1 μg/L vs. 0.025 μg/L) (Klümper et al., 2019). The potential reasons as to why the MSC values differ between single-species and community measurements are unknown but could, e.g. be associated with differences in the availability of free antimicrobials (assumed to be lower in a complex community, e.g. because of possible uptake of the antimicrobial by bacteria or binding to extracellular material) in the experimental system or the specific species/strains examined or the effect of nutrient limitation or chemical and environmental stress (which may also increase the rate of MGE transfer in complex bacterial communities – see Zhang et al., 2020). Another concern is that a defined single-species experiment and a community set-up cannot be directly compared because in the community set-ups, the number of generations of growth is generally unknown, meaning that the selective strength of an antimicrobial is harder to assess. Thus, the sensitivity of the MSC determination is dependent on the number of generations of growth (i.e. more generations of growth mean higher sensitivity) and an objective comparison of two different experimental systems would require that the number of generations of growth is known in both of them. In addition, growth conditions might be different in the assay systems. As a consequence of the above considerations and available data, we cannot make any generalisations regarding MSC-values obtained for single-species/strains vs. communities and whether it is expected they would differ in a systematic and predictable way. These considerations have been included in Sections 3.1 (Data gaps and other important considerations) and 3.3 (Uncertainty analysis).

(ii) Extrapolation between different bacterial species. A second limitation is that, at present, MSCs have only been experimentally determined for *E. coli* and *S. enterica*. A key question is how one can extrapolate from these data to obtain the MSC for an untested bacterial species. In this assessment, this problem was circumvented by making the assumption that the MIC/MSC ratio is the same for the tested and untested bacterial species and that the resistance mechanisms are similar. Thus, the MSC for the untested bacterial species is inferred from the MIC of the most susceptible untested species/strain (MIC_{lowest}) and the MIC/MSC ratio of the tested species/strain (MIC_{test}/MSC_{test})**,** resulting in a **predicted MSC value, PMSC** (see Figure 6). Here, the data obtained from experimental measurements of MSCs in *E. coli* and *S. enterica* are used to calculate **the MSCs for the *most susceptible species* using the MIC_{lowest} values based on the PNEC approach** (see Section 2.2.1.3.1.1). Thus, based on the experimentally determined MIC and MSC for species A and MIC value for species B (which is the species with the lowest MIC value), one infers the PMSC for

species B based on the assumption that MIC/MSC ratios for a given antimicrobial are similar for different species/strains. The advantage with this method is that it is based on experimentally derived MIC and MSC values and the reasonable assumption that the MIC/MSC ratio for a given antimicrobial remains similar for species that have different MIC values. This approach can be considered a worst-case scenario for a PMSC calculation since it is based on the most susceptible relevant animal/human bacteria. It is very unlikely, therefore, that an antimicrobial concentration that is under this derived value will confer enrichment of, and/or selection for, resistant bacteria. These and related considerations have been included in Sections 3.1 (Data gaps and other important considerations) and 3.3 (Uncertainty analysis).

(iii) Extrapolation from one antimicrobial to another within an antimicrobial class. One limitation is to what extent one can extrapolate data for one specific antimicrobial within a class to other antimicrobials within the same class. For example, if the MIC/MSC ratio has been determined for the fluoroquinolone ciprofloxacin, is it then reasonable to assume that the MIC/MSC ratios are similar for all fluoroquinolones? The assumption made in this assessment is that they are similar only if the different antimicrobials within a class have a similar MIC (considering the accepted MIC variation described for the methodology, ISO 20776-1:2019, 2019, CLSI, 2018), and share a mechanism of action and resistance mechanisms. This would mean, ciprofloxacin values would not be extrapolated to other quinolones like oxolinic acid or flumequine, whereas data for tetracycline could be used for oxy-, chlor- and doxycycline. These considerations have been included in Sections 3.1 (Data gaps and other important considerations) and 3.3 (Uncertainty analysis).



On the Y-axis are the MIC/MSC ratios and along the X-axis are different bacterial species with different MIC values for a given antimicrobial. MIC and MSC are experimentally determined (MIC_{test} and MSC_{test}) for one species (represented by *E. coli/S. enterica*) and it is assumed that the MIC/MSC ratio is similar for the species that is most susceptible (MIC_{lowest}). For this species, the predicted MSC can then be derived as: PMSC = $MIC_{lowest}/MIC_{test}/MSC_{test}$.

Figure 6: Predicted Minimal Selective Concentration (PMSC) values and no-effect concentration for species where only minimum inhibitory concentration (MIC) is known

Based on the above reasoning, the PMSCs for different antimicrobials and their relation to the PNECs were calculated (Table 2). As indicated in the data section, the MIC_{lowest} used were those presented by the most susceptible bacteria included in the EUCAST MIC distributions (https://mic.eucast.org/Eucast2/ last accessed 15.5.2021).

Table 2: Calculation of the predicted minimal selective concentration (PMSC) for different antimicrobials from MSC determination based on competition experiments

Antimicrobial (all values in mg/L	MIC_{test}	MSC_{test}	MIC_{test}/ MSC_{test} ratios	MIC_{Lowest} of all species included in EUCAST data (MIC_{lowest})	Predicted MSC (PMSC) for most susceptible species (MIC_{lowest}/ MIC_{test}/MSC_{test})
Tetracycline	1.5 (*S. enterica*)	0.015 (*S. enterica*)	100	0.016	0.00016
Ciprofloxacin	0.023 (*E. coli*)	0.0001 (*E. coli*)	230	0.002	0.0000087
Trimethoprim	0.2 (*E. coli*)	0.002 (*E. coli*)	100	0.016	0.00016
Erythromycin	12 (*E. coli*)	0.2 (*E. coli*)	60	0.008	0.00013
Streptomycin	4 (*S. enterica*)	1 (*S. enterica*)	4	0.25	0.0625
Kanamycin	0.5 (*E. coli*)	0.3 (*E. coli*)	1.7	0.125	0.0735

MIC: minimum inhibitory concentration. MSC: minimal selective concentration. MSC_{test}: MSC experimentally determined. MIC_{lowest}: lowest MIC data calculated based on EUCAST database (EUCAST database last accessed 15 May 2021) as described in Bengtsson-Palme and Larsson (2016), see Methodology Section 2.2.1.3.1.1.

A general concern with using MIC values to assess the risk of resistance selection

It is important to note that the two methods described above (PNEC and PMSC) that are used for determining the non-selective drug concentration are in some manner linked to measurements of the minimal inhibitory concentration (MIC) of an antimicrobial. Thus, any caveats associated with using MIC values will also be part of the uncertainty of these methods. MIC values can be determined by a number of different methods and they are defined as the lowest concentration of drug that prevents bacterial growth. Typically, an MIC assay will involve exposing bacterial cells to a gradient of antimicrobial concentrations and then determining at which drug concentration there is no bacterial growth. Since MIC assays are associated with several caveats, these need to be understood so MIC values are applied and interpreted in a correct and rational manner.

First, MIC values are contextual and will depend on the method used (e.g. broth micro-dilution or agar plate dilution-based MICs, agar plate strips diffusion (E-tests), growth rate assays) and the specific growth conditions (temperature, growth media, time of incubation, recent culture history, etc.) under which they are tested. Thus, it is important that comparative studies take these potential differences in methodology into account (e.g. MIC values determined with strips diffusion on agar plates are not necessarily the same as those determined in liquid culture by broth micro-dilution). Second, MIC values are only semi-quantitative because they generally use a two-step concentration serial dilution range (i.e. 1, ½, ¼ and so on), and generally, no information is obtained for the intermediate concentrations. Third, for the majority of assays used, the read-out is based on a visual inspection of a culture or plate, which is subjective. This is a particular problem for broth micro-dilution where the apparent absence of growth does not necessarily mean no growth at all but could rather indicate a certain reduction in growth. Thus, the difference between no growth and some limited growth is difficult to assess and a two-dilution tolerance is usually applied to reflect this variability in determining the MIC value. Fourth, and most importantly, MIC values do not *per se* give any information regarding the risk of resistance selection (even though they are sometimes used as if they would provide such information). It is clear that if the level of the drug is above the MIC, there will be selection for antimicrobial resistance, but the key question is how far below the MIC value a drug concentration can still result in selection for resistance. The PNEC and PMSC methods all attempt to address this question using different approaches and it was therefore attempted to sort out their respective problems and benefits as described in the text below.

In spite of the above general concerns regarding MIC determinations, for some data sets (e.g. those from EUCAST), there is a strict procedure and definition for determination of the MIC values which reduces variability in growth conditions and therefore generates better inter-study reliability.

Sensitivity of MSC vs. *in vivo* experiments

In a typical animal experiment exposure to an antimicrobial occurs over a certain time period with subsequent collection of faeces and enumeration (e.g. by selective plating and CFU determination of live bacteria and/or determination of abundance of specific resistance genes by various metagenomics

approaches) to assess whether the frequency of resistant bacteria/resistance genes is increasing in the intestine. Because of the relative lack of sensitivity of these enumeration methods, combined with the generally observed extensive variability between individual animals, these assays can only detect strong selective effects. In contrast, the *in vitro* MSC assay has a much higher sensitivity allowing for detection of much smaller selective differences. For example, for the MSC assay, an experiment running for 2 days with 10 replicates will allow the detection of selective differences as small as 0.1%. If an *in vivo* animal exposure experiment is run for a few weeks (which is often the case), the sensitivity of detection of selection and enrichment of resistant bacteria is several 100-fold lower than for the MSC competition experiment. In other words, to obtain a similar detection sensitivity in an animal set-up to that of the *in vitro* competition, the exposure time would have to be largely extended and the assay would need to include many animals to detect weak selection due to low antimicrobial concentrations and obtain statistically significant results.

Further considerations have been discussed in Sections 3.1 (Data gaps and other important considerations) and 3.3 (Uncertainty analysis).

In Table 3, a comparison of the different methods (PNEC, MSC and PMSC) that have been considered in this opinion is outlined, along with their strengths and weaknesses for the determination of the lowest concentration of antimicrobials not selecting for antimicrobial resistance.

Antimicrobial concentrations and exposure times

It is important to note that the estimated PMSC is the antimicrobial concentration below which there is no expectation of enrichment of resistant bacteria, irrespective of time of exposure. In contrast, if the antimicrobial concentration is above the PMSC, resistance selection could occur, and the rate by which the resistant bacteria are enriched will depend on the concentration (how far above PMSC) and the length of time of exposure above the PMSC. For these assessments, we consider long-term chronic exposure over the whole lifetime of an animal (which depending on animal species may vary between a few weeks to several years), but we acknowledge that within that time period, exposure will be intermittent and variable (exactly as it is in humans when antimicrobials are used to treat disease) resulting in exposure both below and above PMSC. However, the key point is that even short/intermittent exposure where the concentration is above PMSC can result in enrichment of resistant bacteria.

Table 3: Overview of methodologies to determine the lowest concentration of antimicrobials not selecting for antimicrobial resistance

	PNEC	MSC	PMSC
Brief description	A theoretical method to predict antimicrobial no-effect concentrations that do not select for antimicrobial resistance based on MIC data	An experimental approach to find the lowest antimicrobial concentrations that can select for antimicrobial resistance	A method that merges elements of the PNEC and MSC methods to produce a hybrid method utilising the benefits of both
Goal of methodology[(a)]	Avoid selection for antimicrobial resistance by protecting the most sensitive species of bacteria	Avoid selection for antimicrobial resistance by finding the lowest concentration that would select for resistance under a worst-case scenario	Avoid selection for antimicrobial resistance in the most sensitive species of bacteria by estimating its MSC
Process	• Obtain the MIC distribution for all species for a given antimicrobial • Identify the lowest MIC in the distribution • Compensate for small number of tested species (if < 40)[(b)] • Apply an assessment factor to arrive at the PNEC	• Compare the fitness of two isogenic strains that only differ in a specific resistance factor in competition experiments with different concentrations of antimicrobials • Use the experimental data to determine the concentration where the resistance factor does not provide the resistant strain with a growth advantage	• Obtain the MIC distribution for all species for a given antimicrobial • Identify the lowest MIC in the distribution (MIC_{lowest}) • Compensate for small number of tested species(if < 40)[(b)] • Calculate MIC_{test}/MSC_{test} ratio • Calculate PMSC = $MIC_{lowest}/MIC_{test}/MSC_{test}$
Required data	MIC data for the relevant antimicrobial for more than one species	Experimental data for every combination of antimicrobial, species and resistance factor of interest	MIC data for the relevant antimicrobial for more than one species as well as MSC data for at least one species for the relevant antimicrobial
Strengths	• Much data already exists • Relatively easy to generate new data	• Extremely sensitive compared to the other two methods • Directly measures the desired end point • Can clearly show if resistance selection below the MIC takes place	• Extremely sensitive • Relatively easy to generate new data • Stronger link to the desired end point comparing to PNECs and MICs-
Weaknesses	• For some antimicrobials, MIC data are available for only a limited number of bacterial species and might not be representative for the relevant bacteria in animals • Uncertain as to what extent the PNEC protects against resistance selection below the MIC	• Very little MSC data exists • Time-consuming and somewhat costly to generate new data, at least at a large scale • Tested strains should ideally be genetically tractable and have known resistance mechanisms	• Very little MSC data exist. • For some antimicrobials, MIC data are available for only a limited number of bacterial species and might not be representative for the relevant bacteria in animals. • Tested strains should ideally be genetically tractable and have known resistance mechanisms

	PNEC	MSC	PMSC
Regulatory uses	Suggested by O'Neill (2016) to be used as discharge limits for antimicrobials from pharmaceutical production. Adopted by the Industry AMR Alliance for self-regulation of antimicrobial emissions from production facilities.	None	Proposed for use in this document
Example values Tetracycline Trimethoprim	0.001 mg/L 0.001 mg/L	0.015 mg/L 0.002 mg/L	0.00016 mg/L 0.00016 mg/L

MIC: minimum inhibitory concentration; MIC_{lowest}: minimum inhibitory concentration of the most susceptible species/strain included in the EUCAST database (accessed May 2021) as described in Bengtsson-Palme and Larsson (2016) and Section 2.2.1.3.1.1; MIC_{test}: minimum inhibitory concentration of the susceptible isolate used in the competition experiments to calculate the MSC; MSC: minimal selective concentration; PMSC: predicted MSC; PNEC: Predicted No-Effect Concentration.
(a): MSC values are typically determined for only one species, whereas the derived PNEC and PMSC values are expected to be non-selective for any bacterial species.
(b): As described in Bengtsson-Palme and Larsson (2016).

Concluding remarks

The experimental approach that is conceptually sound and highly sensitive for identifying the lowest antimicrobial concentrations that can select for antimicrobial resistance is MSC determination by competition experiments. However, there are very little MSC data available.

In the absence of an experimental MSC value for the most sensitive bacterial species, a model was developed to predict the MSC of any species (PMSC). The PMSC method merges elements of the PNEC and MSC methods to produce an extremely sensitive hybrid method utilising the benefits of both. Thus, it is expected that an antimicrobial level in natural settings that is below the PMSC will not cause selection for resistant bacteria. Conversely, using a method with low sensitivity and that cannot detect small selective differences (such as e.g. an analysis of enrichment of resistant bacteria in an animal or a fermenter-based gut model after a short antimicrobial exposure) would be associated with the risk that an antimicrobial selective effect goes undetected.

In addition, *in vivo* models that closely mimic real-life conditions require complex experimental settings that are very difficult to standardise (e.g. variation in microbial community composition depending on source of faeces/intestinal contents, individual animals and animal species, feed used, etc.) and compare between laboratories. In contrast, the high sensitivity of methodologies based on *in vitro* competition assays, such as PMSC, can be fully defined and controlled, generating better inter-study comparability. Finally, there are to date no data available from more complex models for the substances under evaluation in this Opinion.

It is therefore the opinion of the WG and the Panel that in the absence of MSC for the most susceptible bacterial species, the PMSC model presented is also conceptually sound, highly sensitive, allowing the determination of the concentrations of antimicrobials below which selection of resistance for any bacteria would not be expected.

2.2.1.3.2. Second step: estimation of the maximum concentrations of antimicrobials in feed that would not select for antimicrobial resistance in the rumen and large intestines

In order to compare antimicrobial concentrations in the digestive tract of the animals to the PMSC, a prediction of the activity of the drug and of the drug concentrations in the rumen and large intestine after the consumption of cross-contaminated feed was needed.

The proposed approach to predict the **Antimicrobial Resistance Selection Quantity (ARSQ)** in the distal part of the intestines was derived from the equation for determination of mADI (VICH GL36 (R2), EMA/CVMP, 2003) whereas a different calculation was made to predict the ARSQ in the rumen.

mADI is an estimate of the total amount of residue (expressed per unit body weight) that can be ingested daily over a lifetime without leading to higher concentrations in the colon than a defined maximal concentration. For the mADI calculation, the selected maximal concentration can either be based on MIC_{calc}/NOAEC obtained from disruption of barrier effect investigations or on an NOAEC obtained from increase of resistance investigations (see Section 1.3.3).

The mADI (VICH GL36(R2), EMA/CVMP, 2003), expressed in mg/kg bw, is calculated from Equation 3:

Equation 3

$$\text{mADI} = \frac{\text{NOAEC} \times \text{volume of colon content}\left(500\,\frac{\text{mL}}{\text{day}}\right)}{\text{Fraction of oral dose available to microorganisms} \times 60\ \text{kg person}},$$

where NOAEC is derived from the lower 90% confidence limit for the mean NOAECs from *in vitro* or *in vivo* systems.

The NOAEC was replaced by the PMSC in mg/L as described above for the calculation of ARSQ.

Since the data on the volume of colon content were not available for the considered animal species and the large intestine volume (relative to the bodyweight) can vary greatly between species, the volume of the colon content was substituted by the **daily output of fresh faeces** in kg/day, or in L/day if a density of faeces around 1 kg/L is assumed.

By using the daily output of fresh faeces in the calculations, it was considered that the antimicrobial can diffuse both in liquid and solid compartments and that the occurrence of the antimicrobial passing through the distal part of the intestines (including caeca for poultry) after a daily intake of feed would be similar in faeces over one day, allowing for changes in dry matter that occur before defaecation.

The uncertainty derived from the estimation of volume of large intestines by substituting the volume of faeces will therefore be described and taken into account. The main role of the colon being water reabsorption, the concentrations of antimicrobial in faeces can be higher than in the proximal part of the colon, especially for species with high water reabsorption such as sheep and goats. The publication of Hecker and Grovum (1975) showed that the water content ranged from 7 g of water/g of dry matter in the proximal colon of sheep to 1.5 g of water/g of dry matter in the distal part of the colon, suggesting that concentrations could be fivefold lower in the proximal region of the colon than in faeces. Therefore, the ARSQ obtained by using the volume of faeces could be fivefold lower than the value that would be obtained by directly using the volume of large intestinal content. For cattle, the water content was only reduced by twofold along the colon. No data were found for pigs and poultry.

From the current VICH GL36(R2) (EMA/CVMP, 2003), the **fraction of an oral dose available for large intestine microorganisms** should be based on *in vivo* measurements for the drug administered orally or, alternatively, if sufficient data are not available, the fraction of the dose available for large intestine microorganisms can be calculated as 1 minus the fraction (of an oral dose) excreted in urine. Such data are very rare for animal species, so for all the combinations of drug and species without these data, some estimations of the fraction of an oral dose available for large intestine microorganisms were made from the bioavailability of the drug after oral intake. The oral bioavailability (F), which is the fraction of the dose absorbed from the digestive tract to the plasma, is very often reported in published articles. Approximate digestive concentrations of the drug in contact of microorganisms can be deduced from the non-absorbed fraction of the drug ($1 - F$) as confirmed by an article by Peeters et al. (2016) who showed an inverse correlation between the bioavailability of four drugs (chlortetracycline, doxycycline, sulfadiazine and trimethoprim) after oral administration and their concentrations in the colonic and caecal contents and faeces. The sources of errors that can occur by only considering the bioavailability to estimate drug concentrations in large intestines was assessed for each drug and each species. As some examples, the drug concentrations in the large intestines can be:

- Underestimated if the absorption continues beyond the small intestines
- Underestimated if the absorbed drug is subject to enterohepatic recirculation and is eliminated through the gut.
- Overestimated if the non-absorbed antimicrobials are rapidly and extensively degraded in the gut (e.g. presence of beta-lactamases or other enzymatic degradation by ruminal flora) (Erickson et al., 2014).

More information on this can be found in Section 3.3 and Appendix B.1.

In addition to the factors included in the calculation of mADI in Equation 3, the activity of the drug in the colon was considered. The binding of the drug to celluloses, ions and other substances can be very high in the digestive tract, thereby limiting the impact on intestinal microorganisms. Thus, the data on the binding or the inactivation of the drug were included in the calculations when available. Moreover, when available, the influence of the pH on the drug activity was checked for pH ranging from 5.5 to 7.5 since extremely acidic (< 5.5) or basic pH (> 8) are unlikely in the rumen or large intestines.

Detailed approach:

The maximum acceptable active daily quantity (AADQ) (in mg) of the drug in the large intestines of animals was estimated by the product of PMSC (mg/L) and daily output of fresh faeces (in kg) (Equation 4). The average density of faeces was considered around 1 kg/L with a probable maximal range from 0.5 to 2 kg/L.

Equation 4

$$\text{AADQ(mg)} = \text{PMSC} \times \text{daily faeces}.$$

Since a fraction of the drug called 'I' can be inactive in the digestive tract as a result of binding or degradation, the maximum AADQ of the drug in the large intestines was converted to the maximum acceptable total (inactive and active) daily quantity (ATDQ) (in mg) of the drug in the large intestines (Equation 5).

Equation 5

$$\text{ATDQ(mg)} = \frac{\text{AADQ}}{1 - I},$$

where I is the fraction of drug that is inactive in the large intestines.

If the entire drug is active in the large intestines ($I = 0$), the maximum AADQ of the drug in the large intestines would be equal to the maximum ATDQ.

If a fraction of the drug is inactive, ATDQ would be higher than AADQ.

To predict the antimicrobial resistance selection quantity (ARSQ) expressed in mg of the drug acceptable in the large intestines from ATDQ, the pharmacokinetics of the drug need to be considered. After oral administration, a fraction of the drug can leave the digestive tract before interacting with bacteria by being absorbed to the plasma (this fraction is called F and corresponds to the bioavailability) and should be subtracted to estimate the drug concentrations in the large intestines. However, a fraction (GE) of this absorbed drug can be secondarily eliminated through the gut and so increase drug concentrations in the digestive tract. GE is equal to the fraction of drug eliminated unchanged from plasma to gut and so, the product of F by GE ($F \times GE$) is equal to the fraction of ingested/consumed drug that is first absorbed and then eliminated in gut. Thus, the ARSQ can be calculated by Equation 6.

Equation 6

$$\text{ARSQ (mg)} = \frac{\text{ATDQ}}{1 - F + F \times GE}$$

where F is the fraction of drug absorbed from the digestive tract and GE is the fraction of drug eliminated unchanged from plasma to gut.

If there is no absorption ($F = 0$), the ARSQ would be equal to the ATDQ.

If there is complete absorption ($F = 1$) followed by an exclusive elimination of the unchanged drug from plasma to large intestines ($GE = 1$), the ARSQ would also be equal to the ATDQ.

If there is a very high absorption ($F = 0.9$) not followed by an elimination in the large intestines ($GE = 0$), the ARSQ would be 10-fold higher than the ATDQ.

Finally, the **Feed Antimicrobial Resistance Selection Concentration (FARSC)** (in mg/kg feed) was calculated considering ARSQ (in mg) and the feed intake (in kg DM feed/day) (equation 7). The drug was assumed to only be contained in the DM component of feed.

Equation 7

$$\text{FARSC}_{\text{intestine}}\ (\text{mg/kg food}) = \frac{\text{ARSQ (mg)}}{\text{daily feed intake (kg feed/day)}}.$$

To conclude, the **FARSC**$_{\text{intestine}}$ was calculated from Equation 8, providing the maximal concentration in non-target feed not selecting for resistance:

Equation 8

$$\text{FARSC}_{\text{intestine}}\ (\text{mg/kg food}) = \frac{\text{PMSC} \times \text{daily faeces}}{(1 - I) \times (1 - F + F \times GE) \times \text{daily feed intake}}$$

Assumptions and uncertainties relating to these calculations

The calculation made in Equation 8 implies that, as for the calculation of mADI from VICH GL36 (R2) (EMA/CVMP, 2003) (Equation 3), we did not consider the possible selection of resistance in the small intestine. At a given time point, the drug contained in the intestines was therefore assumed to be entirely contained in the large intestine and homogenously distributed within its contents. Thus, in case of heterogeneity, higher drug concentrations than those predicted could be found at some locations within the large intestines.

Then, by using the daily output of faeces, it was considered that the drug going through the large intestine, including caeca in the case of poultry, after daily ingestion will also be homogeneously recovered in faeces over 1 day.

Since the data on the volume of the large intestine were difficult to obtain from the different animal species and age classes, we considered that the concentration of drug in faeces was representative of the highest concentrations in the large intestine. Indeed, due to water reabsorption through the large intestine, the concentrations in the large intestine, and especially in the proximal part, can be lower than faecal concentrations. These and related considerations have been included in Sections 3.1 (Data gaps and other important considerations) and 3.3 (Uncertainty analysis).

Finally, when the values of *F*, *GE* or *I* were uncertain due to a high variability of the published values or due to extrapolations between species, different values of parameters representing the range of possible values were used for FARSC calculations. This results in the report of a range of possible values for FARSC.

For ruminants after weaning (adults and young for fattening), the maximum concentrations of antimicrobials in feed that would not select for antimicrobial resistance in the rumen were also estimated ($FARSC_{rumen}$). $FARSC_{rumen}$ was not determined for the ruminants before weaning (veal calves) because their rumen was considered to be not sufficiently developed.

$FARSC_{rumen}$ was estimated by considering that the ingested drug would be homogeneously distributed in the rumen.

The maximum acceptable active daily quantity ($AADQ_{rumen}$) (in mg) of the drug in the rumen of animals was estimated by the product of PMSC (mg/L) and volume of rumen (in L, Table 1) (see Equation 9).

Equation 9

$$AADQ_{rumen}\ (mg) = PMSC \times \text{volume of rumen}.$$

Since a fraction of the drug called '*I*' can be inactive in the rumen by binding or degradation, the maximum AADQ of the drug in the rumen was converted in the maximum acceptable total (inactive and active) daily quantity ($ATDQ_{rumen}$) (in mg) of the drug in the rumen (Equation 10).

Equation 10

$$ATDQ_{rumen}\ (mg) = \frac{AADQ}{1-I} = ARSC_{rumen},$$

where *I* is the fraction of drug inactive in the rumen.

In the rumen, $ATDQ_{rumen}$ was equal to $ARSQ_{rumen}$ since we consider that at this step, the absorption of the drug has not started.

Finally, the FARSC (in mg/kg feed) was deducted from $ARSQ_{rumen}$ (in mg) by taking into account the feed intake (in kg feed/day) (equation 11)

Equation 11

$$FARSC_{rumen}\ (mg/kg\ feed) = \frac{ARSQ_{rumen}(mg)}{\text{daily feed intake}(kg\ feed/day)}.$$

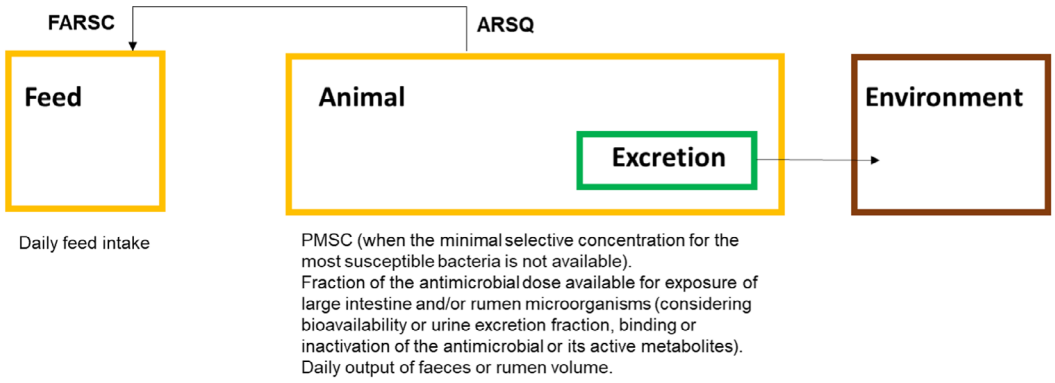
To conclude, the **$FARSC_{rumen}$** corresponding to the maximal concentration in non-target feed not selecting for resistance was calculated from Equation 12:

Equation 12

$$FARSC_{rumen}\ (mg/kg\ feed) = \frac{PMSC \times \text{volume of rumen}}{(1-I) \times \text{daily feed intake}}.$$

2.2.1.3.3. Concluding remarks

Microbiological, pharmacological or physiological concepts affecting feed and animal were discussed and considered for the determination of the maximum antimicrobial residue concentration in non-target feed not selecting for resistance in a certain animal species (FARSC). Selected data were included in the model established (Equations 8 and 12) (Figure 7). This methodological approach will result in a value for antimicrobial concentration in non-target feed below which no emergence of, and/or selection of resistant bacteria would be expected to occur at large intestine or rumen level.



FARSC- Feed Antimicrobial Resistance Selection Concentration. Defines the maximal antimicrobial concentration in non-target feed not selecting for resistance (mg/kg feed)

ARSQ- Antimicrobial Resistance Selection Quantity. Defines the maximal antimicrobial quantity (mg) in the large intestines not selecting for resistant bacteria.

PMSC- Predicted Minimal Selective Concentration. Defines the predicted lowest concentration not selecting for antimicrobial resistance (mg/L).

Figure 7: Compartments and data relevant for the assessments

2.2.2. Methodology for ToR2

2.2.2.1. Source of information

An extensive literature search (ELS) was carried out following the criteria described in the EFSA Application of systematic review methodology to food and feed safety assessments to support decision-making (EFSA, 2010). The literature search was the subject of a procurement granted by EFSA to a contractor. The contract was launched in December 2018.

The literature search strategy and methodology are described in detail in Annex E, and briefly summarised below.

Six databases (Scopus, Web of Science Core Collection, AGRICOLA, AGRIS, CABI - Animal Health & Production Compendium and MEDLINE) were used. No time limit was applied to the search, which was concluded in October 2019.

The search was performed defining the active substance, the animal species and the possible effects on zootechnical parameters, as follows:

The active substances in the search were: amoxicillin, amprolium, apramycin, chlortetracycline, colistin, doxycycline, florfenicol, flumequine, lincomycin, neomycin, spectinomycin, sulfonamides, tetracycline, oxytetracycline, oxolinic acid, paromomycin, penicillin V, tiamulin, thiamphenicol, tilmicosin, trimethoprim, tylosin, valnemulin, tylvalosin.

The food-producing animals included in the search were:

- Ruminant: growing and dairy (cattle, sheep, goats);
- Pigs: weaned, growing and reproductive;
- Equines;
- Rabbits;
- Poultry: chickens and turkeys for fattening, laying hens, turkeys for breeding, minor avian species (ducks, guinea fowl, geese, quails, pheasants, ostriches);
- Fish: salmon; trout; other farmed fish (seabass, seabream, carp, other), crustaceans;
- Other animal species that could be identified as relevant during the literature search.

The end points that were considered as a sign of improved growth or production of the animals, quantitatively and/or qualitatively, were: average daily gain/final weight, feed intake, feed efficiency, feed/milk production ratio, milk yield, fat/protein yield, feed/egg mass ratio, egg production, egg weight, carcass weight, carcass yield, relative weight of the (different sections of) intestine, utilisation of some nutrients (DM, Ca, P), digestibility, homogeneity of the flock/herd, health, i.e. reduction of morbidity and/or mortality and other end points identified as relevant during the literature search.

The relevance of each record in providing information on growth-promoting effects of each of the 24 antimicrobials was assessed by screening the title, keywords and the abstract. The publications were included in the list of selected papers when matching the following eligibility criteria: use of one or more of the active substances listed above, use of one or more of the animal species listed above; oral administration of the active substance(s), presence of control group(s); end points measurement includes one or more of the zootechnical end points listed above.

The papers meeting the criteria described above were selected as possibly relevant and made available to EFSA for further evaluation.

In addition to the literature search, EFSA consulted other Institutions for relevant existing evaluations/authorisations on antimicrobials as growth promoters and the respective information. Since no information which could be considered relevant for the assessment was retrieved, only the results of the ELS search were considered.

2.2.2.2. Selection and evaluation of the relevant studies for the assessment

For each of the 24 antimicrobials, the studies resulting as the outcome of the literature search were evaluated by experts in the field of animal nutrition and animal production to identify the effects of the administration of the selected antimicrobial to food-producing animals. The suitability for the assessment was done by checking the studies against a set of criteria which would not allow a consistent and reliable evaluation of any possible observed growth-promoting/increase yield effects in food-producing animals. Those studies matching one or more of those criteria were excluded from further assessment (see Section 2.2.2.2.1). The remaining studies were assessed to identify the growth-promoting effects of the antimicrobials on food-producing animals (see Section 2.2.2.2.2).

2.2.2.2.1. Studies excluded from the assessment

A series of criteria to identify and exclude the publications not suitable for the assessment were defined. The criteria were: (i) the relevant antimicrobial substance was used in combination with other antimicrobials, but not independently; (ii) the antimicrobial used is not the one under assessment (e.g. a different form of the substance (i.e. tetracycline, oxytetracycline, chlortetracycline); (iii) the antimicrobial was administered to the animals via a route different from oral route[11]; (iv) the antimicrobial was fed to the animals with a therapeutic scope; (v) the animals were subjected to challenges with pathogens to evaluate the effects of the antimicrobial; (vi) the animals included in the study were sick or their general health status was not considered good; (vii) the zootechnical parameters were not reported; (viii) the reporting of the study or the statistical analysis was not sufficient/appropriate to evaluate the effects of the antimicrobial; (ix) any other cause which was considered as relevant to exclude the study from further assessment.

Each study meeting one or more of the aforementioned excluding criteria was removed from the evaluation. The full list of studies excluded and the respective criteria for the exclusion from further evaluation is reported in the Appendices of the complementing Scientific Opinions Parts 2–13, EFSA BIOHAZ Panel, 2021a–l (see also the Virtual Issue).

2.2.2.2.2. Assessment of the effects of the antimicrobials on the performance/yield of food-producing animals

For each of the 24 antimicrobials, all the studies not excluded from the evaluation following the criteria described in Section 2.2.2.2.1 were assessed. To evaluate the effects of the antimicrobials on growth promotion/production yield increase, eight categories of zootechnical end points, on which effects might have been potentially observed, were identified: (i) Intake-related parameters: feed intake, feed/gain ratio, feed efficiency, feed intake/milk yield, feed intake/egg mass; (ii) Weight-related parameters: body weight, body weight gain; (iii) Carcass-related parameters: carcass weight, carcass yield, relative weight of the (different sections of) intestine; (iv) Milk or egg production/quality: milk yield, fat/protein yield, egg production/laying rate, egg weight, egg mass; (v) Digestibility/utilisation of nutrients: utilisation of some nutrients (e.g. DM, Ca, P), digestibility; (vi) Health-related parameters: reduction of morbidity and/or mortality; (vii) Herd/flock-related parameters; (viii) Other end points: e.g. intestinal morphological characteristics (*villi* height/width), changes in microbiota. For each study, any effect reported on one or more of these zootechnical end points was evaluated.

[11] Concerning the oral administration, both feed and water were considered and doses were expressed as mg/kg feed (mg kg DM for ruminants) or mg/L drinking water; in each case, appropriate data on either feed or water intake collected from the relevant statistical unit were necessary. If such data were not available, the study was excluded from the assessment.

Each suitable study was described summarising the main characteristics of the study design and the measured end points; regarding the results, only those end points considered relevant for the assessment of growth promotion/yield, in which a statistically significant effect (positive or negative)[12] was observed, were described and evaluated.[13]

For each antimicrobial assessed, the suitable studies are reported by species.[14] Within each species, the studies were summarised following an alphabetical order of the first author and then by year.

In the description of the studies, where available, the specific form/formulation (e.g. specific salt, commercial product) of the antimicrobial used has been indicated as reported in the original paper. When no detailed information on the specific form/formulation is available, the antimicrobial is reported with the name of the base followed by 'unspecified form'. Additionally, the doses/concentrations reported in the description of each study reflect those indicated in the respective publications.

2.2.3. Uncertainty analysis

The uncertainty in this Scientific Opinion was investigated in a qualitative manner following the procedure detailed in the EFSA guidance on uncertainty analysis in scientific assessments (EFSA Scientific Committee, 2018a,b). The sources of the main uncertainties were identified, and for each of these, the nature or cause of the uncertainties was described by the experts (see Section 3.3 and Appendix B, Tables B.1, B.2, B.3 and B.4). In particular, the overall impact of the identified uncertainties on the final conclusions was expressed in the overall answer to the ToRs using probability terms supported by subjective probability ranges, following the recommendations of the EFSA uncertainty guidance document. The subjective probability ranges expressed were not the result of precise calculation but were agreed by consensus by the members of the working group drafting this scientific output following the discussion of the analyses performed.

Due to the different approaches followed to determine the requested values (model development to address ToR1, extensive literature research to address ToR2), specific methodological considerations apply to each ToR.

To address ToR1:

Expert judgement by WG members was used to estimate the individual impact of each of the uncertainties on the determination, at feed level, of concentrations for specific antimicrobials below which there would not be an effect on the emergence of, and/or selection for, resistance in microbial agents relevant for human and animal health for all substances under assessment. Appendix B.1, Table B.1 presents the general uncertainties associated with the model proposed and data gaps identified. The uncertainties associated with specific substances for which the data available allowed the application of the model are presented in the specific assessments (Scientific Opinions Part 7 – EFSA BIOHAZ Panel, 2021f, Part 12 – EFSA BIOHAZ Panel, 2021k, and Part 13 – EFSA BIOHAZ Panel, 2021l; see also the Virtual Issue).

To address ToR2:

Considering the nature of the data analysed (see Section 2.2.2), the WG members evaluated the uncertainties associated with the methodology and the data used in the analysis, as well as their expected impact on the conclusions.

The sources of uncertainty reported in Appendix B.2, Tables B.2 and B.3 are general and would impact on the assessment of all the antimicrobials. Substance-specific sources of uncertainty associated with the process of evidence synthesis described below are summarised in Appendix B.2, Table B.4.

Evidence synthesis was possible only when more than three studies showing consistent (positive) results in a comparable range of concentrations were available for the antimicrobial in the same species/category. Owing to the heterogeneity of the evidence, meta-analysis could not be applied.

A substance-specific evaluation of the uncertainty was done, considering the total number of studies available and the heterogeneity/homogeneity in the results (i.e. the relative number of studies

[12] The statistical probability for significance was accepted as $p \leq 0.05$ for all animals species except for ruminants with ≤ 0.10.
[13] Differences reported only in feed intake, diarrhoea scoring or in mortality/morbidity were not considered as growth-promoting effects.
[14] Applying the following order: ruminants, pigs, equines, rabbits, poultry, fish, crustacean and other animal species.

giving positive effects, negative effects or without any effect in the same concentration range and for the same end points). The level of uncertainty is reflected in the use of subjective probability ranges when drawing conclusions for the individual antimicrobials, as defined in Appendix B.2, in line with recommendations of the uncertainty guidance.

3. Assessment

The assessments of the specific substances belonging to the different antimicrobial classes are presented in the Scientific opinions as regards maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed (see the complementing Scientific Opinions Parts 2–13, EFSA BIOHAZ Panel, 2021a–l, and also the Virtual Issue).

3.1. Data gaps and other important considerations for ToR1 assessment

3.1.1. Data gaps

There are numerous data gaps, mainly relating to the absence or incompleteness of studies (e.g. MSC, MIC, PK, degradation) on the antimicrobials that are relevant to this assessment. The competition studies that have been carried out to determine MSCs have only included *Salmonella* and *E. coli,* which are relevant target bacteria for such studies of resistance, in that they can be pathogenic and sources or recipients of transferable resistance, but may not be representative for the whole of the intestinal bacterial population, most of which is considered to be non-culturable, or so fastidious that meaningful competition experiments in the presence of antimicrobials could not be reliably carried out. There may also be strain differences within bacterial species, so it is important that the experimental strains provide a sensitive model and are also representative of their species. Furthermore, the MSC has been determined for a limited number of antimicrobials. Questions have also been raised about the representativeness of the competition experiments in relation to complex intestinal microbiota, but it is not possible to carry out such studies in a standardised and repeatable way with complex microbial populations, and the very limited studies that have attempted this provide conflicting results regarding the increased or reduced probability of observing resistance selection.

The lack of data in the literature on the volumes of the digestive compartments and especially the volume of large intestines of different animal species and on the PK of antimicrobials also limited/ compromised the prediction of active antimicrobial concentrations in the rumen and large intestines after consumption of cross-contaminated feed. The bioavailability of most of antimicrobials after oral administration was obtained for many species even though the number of studies per species was low and there can be variability according to age, diet and animal production sector. These data were used to predict the percentage of antimicrobials remaining in the large intestines after ingestion. However, several other factors such as the actual activity of the non-absorbed drug or the possible intestinal excretion of active metabolites after absorption were very poorly described in the literature.

The specific data gaps are considered in more detail below:

MSC/PMSC Data

- MSC data for antimicrobial class/substances:
  - Quinolones: MSC published data are available only for the fluoroquinolone ciprofloxacin (not included in the Mandate list), but not for the quinolones flumequine and oxolinic acid. Due to potential differences in *in vitro* potency (MIC) and activity spectrum between the fluoroquinolones and the quinolones included in the list of antimicrobials to be assessed, oxolinic acid and flumequine, no extrapolation on the MSC data were done.
  - Aminoglycosides: MSC published data are available only for streptomycin and kanamycin (not included in the Mandate list), but not for apramycin, neomycin, spectinomycin, or paromomycin. Due to differences in *in vitro* potency (MIC) and activity spectrum between the aminoglycosides, no extrapolations were done.
  - Macrolides: MSC published data available only for erythromycin (not included in the Mandate list), but not for tilmicosin, tylosin nor tylvalosin. Due to potential differences in *in vitro* potency (MIC) and activity spectrum between the macrolides, no extrapolations were done.

- Amphenicols: MSC published data are available only for florfenicol, but not for thiamphenicol. Due to differences in *in vitro* potency (MIC) and activity spectrum between the amphenicols, no extrapolations were done.
- No MSC data are available for amoxicillin, penicillin V, sulfonamides, amprolium, lincomycin, florfenicol, thiamphenicol, tiamulin, valnemulin or colistin.

— MSC data for the most susceptible species:

- MSC data for the species with the lowest MIC for a certain antimicrobial are lacking. If these data were available, calculation of the PMSC would not be necessary.

— MSC data for Gram-positive species:

- We also tried to gather some MSC data on Gram-positive bacteria from the existing literature. One method used by Greenfield et al. (2018) involves deducing the MSC from a dose-response (growth rate) experiment for two isogenic strains; one resistant and one susceptible to the antimicrobial considered. This methodology was validated by comparison with the experimental competition data obtained from Gullberg et al. (2011) and was shown to provide good predictions. In principle, this method could be used for the species and antimicrobials for which no MSC data presently exists, but it would require experimental determination of the dose-response curve and validation by additional competitions.
- By screening the literature, the work by Ankomah et al. (2013) was identified as a potential source of data for Gram-positive bacteria. However, the data presented there were only for susceptible strains and therefore not deemed useful for the present opinion. Therefore, future studies should include the determination of MSC of Gram-positive bacteria.

Animal-related features

The anatomy of large intestines greatly varies between animal species and even though the topography of the organs has been extensively described among species, their content in terms of volume is rarely available, especially considering the different ages of animals at the time of potential exposure to antimicrobial residues in feed and for fish species, the diversity in size. Therefore, the use of estimated volumes of daily faecal output instead of the volume of the large intestines for each animal category should be considered as a source of uncertainty.

PK Data

The published data for the values of bioavailability (*F*), fraction eliminated in intestines after absorption (*GE*) and fraction of inactive drug in intestinal contents (*I*) were rarely available for each considered species. When the uncertainty on these values was high due to a high variability of the different published values or due to extrapolations between species, different values of parameters representing the range of possible values were used for FARSC calculations. This results in the report of a range of possible values for FARSC.

3.1.2. Other important considerations

Resistant mutants that appear *de novo* or are pre-existing have some probability to increase in frequency, and potentially become fixed in a specific environment (e.g. a human or animal host or population). This probability will depend on several complex evolutionary and ecological factors, including both the bacterial and host population structure, fitness of the resistant mutants in different environments, environmental conditions such as microbiota, levels of nutrients and selectors such as antimicrobial agents as well as other factors (see e.g. Levin et al., 2000; Patwa and Wahl, 2008; Hiltunen et al., 2017; Lakshmaiah Narayana et al., 2020; Leónidas Cardoso et al., 2020). In this opinion, the main focus is on determining the lowest level of an antimicrobial agent that can enrich for a specific resistant mutant under defined experimental conditions in laboratory settings.

Clearly the various factors mentioned above could influence the estimate of the PMSC (it could be either higher or lower). However, they have not been addressed here (with the exception of the reference to the impact of complex communities on selection for which there is a small amount of conflicting data), mainly because of lack of data and estimates of how they potentially could influence the magnitude of the PMSC. This lack of knowledge results in an uncertainty in the estimate of the PMSC.

Ideally, the increase of resistance from residues in animal feed should be directly determined for all 24 antimicrobial substances in the various target animals. Such studies may possibly represent different species-specific real-life conditions such as the pharmacokinetics of the drug and the composition of the commensal bacterial communities, but the potential for variability is huge. Moreover, the prolonged time required for such experiments to reliably demonstrate no selective effect might further hinder the implementation of such studies. Additionally, alternative methods to animal experimentation according to the 3R principles: 'Replacement, Reduction and Refinement' should be considered.[15]

Concerns on the consequences that may result from the conservative approach and the inherent uncertainties (Table B.1, Appendix B.1) were discussed. However, as stated in the interpretation of terms of references (Section 1.2), the possible practical consequences are outside of the scope of this opinion.

3.2. Data gaps for ToR2 assessment

Various data gaps were identified from the results obtained with the extensive literature search, mainly related to (i) the absence of suitable studies in a series of antimicrobials (doxycycline, flumequine, spectinomycin, oxolinic acid, paromomycin, penicillin V, thiamphenicol, trimethoprim, tylvalosin and valnemulin), (ii) the incompleteness of some studies, which caused their exclusion from the assessment, (iii) the scarcity of studies aiming to examine the specific growth-promoting effects of the antimicrobials relevant to this assessment (especially in the EU during the last 15 years and under current farming conditions in Europe, since antibiotics were banned as growth promoters in the EU in 2006), (iv) the lack of specific dose-range finding studies and (v) the lack of relevant data on all food-producing animals.

In addition, despite the information was sought, no data were retrieved from sources other than the public literature (e.g. industry data, other assessments from relevant institutions).

3.3. Uncertainty analysis

With regard to ToR1, the specific uncertainties identified and their impact on the determination of the FARSC are included in Appendix B.1, Table B.1.

With regard to ToR2, a common methodology (extensive literature search) was applied to assess the potential growth-promoting effects of 24 antimicrobial substances. General uncertainties related to the methodology applied and the results of the extensive literature search have been identified and are applicable to all antimicrobials. They are summarised in Appendix B.2, Tables B.2 and B.3. Substance-specific sources of uncertainty associated with the process of evidence synthesis are presented in Appendix B.2, Table B.3.

4. Conclusions

ToR1: **to assess the specific concentrations of antimicrobials resulting from cross-contamination in non-target feed for food-producing animals, below which there would not be an effect on the emergence of, and/or selection for, resistance in microbial agents relevant for human and animal health.**

AQ1. Which are the specific antimicrobial concentrations in non-target feed below which there would not be emergence of, and/or selection for, resistance in the large intestines/rumen?

To address this ToR, a two-step conservative model that is generally applicable for different substances and animal species was developed to also apply to the assessment of antimicrobials.

- Regarding the first step, the determination of an experimental approach to find the lowest antimicrobial concentration that can select for antimicrobial resistance, it was concluded:
    - Experimental evidence shows that antimicrobial concentration values below the MIC can select for or enrich resistant bacteria. Accordingly, the experimental approach for the determination of the minimal selective concentration (MSC, i.e. the lowest concentration that can enrich for a pre-existing resistant bacterium when in competition with a congenic susceptible strain, or, select for *de novo* resistance in a susceptible strain) was considered conceptually sound and highly sensitive. Therefore, MSC was identified as a key parameter to be used for the assessment of resistance selection.

[15] https://ec.europa.eu/environment/chemicals/lab_animals/3r/alternative_en.htm

- As limited MSC data have been generated to date, the concept of predicted MSC (PMSC, based on experimental MSC/MIC strain data and MIC_{lowest}) was developed. When this method is applied, the probability that antimicrobial concentrations below the derived level will confer any enrichment of, and/or selection for, antimicrobial resistant bacteria, irrespective of time of exposure, is estimated to be 1–5% (extremely unlikely).
- If the antimicrobial concentration is above the PMSC, resistance selection could occur, and the rate and extent to which the resistant bacteria are enriched will depend on the concentration (how far above PMSC) and the length of the time of exposure.

- In the second step, to estimate the antimicrobial levels in the non-target feed that would not result in emergence of, and/or selection for, resistance, the concept of Feed Antimicrobial Resistance Selection Concentration (FARSC) was developed. The FARSC model takes into account the MSC or PMSC (when MSC for the most susceptible species is not available), the fraction of antimicrobial dose available for exposure of large intestine and/or rumen microorganisms (taking into account bioavailability and urine excretion fraction, binding or inactivation of the antimicrobial or its active metabolites), the daily faecal output or rumen volume and the daily feed intake, according to the following equations:

$$\text{FARSC}_{\text{intestine}}\ (\text{mg/kg feed}) = \frac{\text{PMSC} \times \text{daily faeces}}{(1-I) \times (1-F+F \times GE) \times \text{daily feed intake}}$$

$$\text{FARSC}_{\text{rumen}}\ (\text{mg/kg feed}) = \frac{\text{PMSC} \times \text{volume of rumen}}{(1-I) \times \text{daily feed intake}}$$

- The probability that an antimicrobial concentration below the FARSC will confer any enrichment of, and/or selection for, resistant bacteria in the intestine and/or rumen is estimated to be 1–5% (extremely unlikely). Several data gaps regarding, e.g. MSC, MIC, PK and/or activity in the digestive tract of particular antimicrobials in relation to animal species that are relevant to this assessment were identified. This lack of knowledge results in uncertainties in the estimation of the PMSCs and FARSCs or prevents the establishment of PMSC and/or FARSC for particular antimicrobials.

A summary of all the assessments done applying the FARSC model (complementing Scientific Opinions Parts 2–13, EFSA BIOHAZ Panel, 2021a–l – see also the Virtual Issue) is presented in Annex F, Table F.1. The FARSC was obtained for florfenicol, tetracycline, chlortetracycline, oxytetracycline, doxycycline and trimethoprim, but it was not possible to estimate it for other substances.

ToR2: **'to assess which levels of the antimicrobials have a growth promotion/increase yield effect.**

AQ2. Which are the specific antimicrobial concentrations in feed of food-producing animals that have an effect in growth promotion/increased yield?

- With the methodology applied, it has been possible to identify studies from the scientific literature suitable for the assessment and, in general, the data retrieved covered only to a limited extent the specificity of the mandate.
- For 10 antimicrobials (doxycycline, flumequine, spectinomycin, oxolinic acid, paromomycin, penicillin V, thiamphenicol, trimethoprim, tylvalosin and valnemulin), no relevant data to assess the growth promotion/increase yield effects in food-producing animals were available.
- Based on the selected suitable studies, for the remaining 14 antimicrobials (amoxicillin, amprolium, apramycin, chlortetracycline, colistin, florfenicol, lincomycin, neomycin, sulfonamides, tetracycline, oxytetracycline, tiamulin, tilmicosin, tylosin), levels causing effects on growth promotion/increased yield were identified and reported; however, this was only possible for a reduced number of animal species/categories. For levels below the lowest value reported, no data showing growth-promoting/increase yield effects were found, and thus, it is not possible to conclude on the effects of those levels.

A summary of the assessments done (complementing Scientific Opinions Parts 2–3, EFSA BIOHAZ Panel, 2021a–l, – see also the Virtual Issue) is presented in Annex F, Table F.2.

5. Recommendations

To determine the FARSC for the substances included in the European Commission mandate, the following studies are required:

- Generation of MIC data using low levels of antimicrobials (below ECOFFs) for a wider range of bacteria and strains.
- Studies to determine the MSCs for all the antimicrobials under assessment for a range of bacteria, including the most susceptible species, using experiments with both pure cultures and complex bacterial communities.
- Studies to determine the lowest antimicrobial levels that promote emergence of resistance (e.g. via the promotion of horizontal resistance gene transfer and/or mutations) using pure cultures and complex bacterial communities.
- Studies to supply missing data and fully evaluate the diversity of parameters for different animal species/breeds/ages/diets, including the total and active concentrations of antimicrobials or their active metabolites in the different segments of the digestive tract after oral administration, taking into account standard pharmacokinetic factors, such as absorption, distribution, metabolism and excretion.
- Studies to determine the antibacterial activity of antimicrobial metabolites occurring in the intestinal tract.
- Development of sensitive, fit for purpose, standardised and robust methods to determine the lowest antimicrobial concentrations that could select for antimicrobial resistance in the target animal species.

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Abbreviations

AADQ	Acceptable Active Daily Quantity
ADI	Acceptable Daily Intake
ALARA	'as low as reasonably achievable'
AMR	antimicrobial resistance
ARG	antimicrobial resistance gene
ARSQ	Antimicrobial Resistance Selection Quantity
ATDQ	Acceptable Total Daily Quantity
BIOHAZ	Biological Hazards
BW	body weight
bw	body weight used in toxicity studies
CFIA	Canadian Food Inspection Agency
CVMP	EMA Committee for Medicinal Products for Veterinary Use
DHFR	dihydrofolate reductase
DM	dry matter
EMA	European Medicines Agency
EPMAR	European Public MRL Assessment Report
EUCAST	European Committee Antimicrobial Susceptibility testing (European Society for Clinical
EURL	European Union Reference Laboratory
F	fraction of the drug that is absorbed from the digestive tract to the blood
FARSC	Feed Antimicrobial Resistance Selection Concentration

FDA	US Food and Drug Administration
FSCJ	Food Safety Commission Japan
FM	Fresh matter
GE	fraction of drug eliminated unchanged from plasma to gut
GFP	green fluorescent protein
GP	growth promotion
HGT	horizontal gene transfer
I	fraction of the drug present in the digestive tracts that would be inactive on the microbiota
mADI	microbiologically acceptable daily intake
MGE	mobile genetic element
MIC	minimal inhibitory concentration
MIC_{lowest}	minimal inhibitory concentration of the most susceptible species/strain included in the EUCAST database for a certain antimicrobial used to calculate the PMSC (see below)
MIC_{res}	minimal inhibitory concentration of the resistant strain
MIC_{susc}	minimal inhibitory concentration of the susceptible strain
MIC_{test}	minimal inhibitory concentration of the susceptible isolate used in the competition experiments to calculate the MSC
MRL	maximum residues limit
MSC	minimal selective concentration
NARMS	US National Antimicrobial Resistance Monitoring System
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
OIE	World Organization for Animal Health
PK	pharmacokinetic(s)
PMSC	Predicted MSC
PNEC	Predicted No-Effect Concentration
TMR	total mixed ration
VETCAST	Veterinary Committee on Antimicrobial susceptibility test (ESCMID)
WG	Working Group

Appendix A – Additional MIC data sources considered

Other data sources were examined to potentially complement the data available in EUCAST. EFSA in EU and NARMS in USA publish the results of programs for surveillance of AMR in pathogenic and commensal bacteria from animals. The available data can be summarised as follows:

EFSA: EFSA collects MIC data from all Member States regarding the European AMR monitoring which targets food-producing animals including cattle under 1 year, pigs, poultry, turkeys and laying hens and foods derived thereof. The bacterial species represented in the EU AMR monitoring are *Salmonella* spp., *Campylobacter* spp. and commensal *E. coli*, and some countries also report MRSA and *Enterococcus* spp. on a voluntary basis. Since 2014, there are standardised sampling scheme and methodology across EU Member States, thus bacterial isolates are representative of production systems with different antimicrobial use. MIC data are available for some bacterial groups and antimicrobials (colistin for *Salmonella enterica* and *E. coli;* sulphonamides and trimethoprim for *Salmonella enterica, E. coli* and MRSA*;* tetracycline for *Salmonella enterica*, *E. coli*, MRSA and *Campylobacter* spp.; tiamulin for MRSA). However, the range of concentrations to be tested for different antimicrobials differs from those required by EUCAST to include data in their MIC database, and thus, in general, the lowest concentrations tested to be reported to EFSA, are higher than the lowest concentrations available in EUCAST MIC distributions. Thus, the data from the EU AMR monitoring were not further examined.

NARMS (US National Antimicrobial Resistance Monitoring System): NARMS collects AMR monitoring data regarding enteric bacteria from humans, food animals and retail meats in USA (https://www.cdc.gov/narms/antibiotics-tested.html and https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/integrated-reportssummaries, https://www.fda.gov/media/79976/download). Furthermore, NARMS collects AMR monitoring data for selected animal pathogens (https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/integrated-reportssummaries). When last accessed (April 2021), MIC data were available for several of the antimicrobials under assessment. Nevertheless, in the ranges of concentrations tested, the lowest concentration for those antimicrobials of interest was higher or equal to the lowest concentration available in EUCAST MIC distributions with the exception of tylosin. Thus, the data for tylosin could be of interest for future assessments.

Public reports from national AMR monitoring programmes encompassing animal pathogens were examined. We focused on European reports (https://www.eurl-ar.eu/monitoring-reports.aspx) and published articles describing AMR monitoring programmes (e.g. VetPath, El Garch et al., 2016; de Jong et al., 2018). However, these programmes differ for the tested bacterial pathogens, antimicrobial agents and concentration ranges, and for time periods. In some of these reports/publications (FINRES-VET,[16] NORM-VET[17] and SVARM-PAT,[18] and VetPath, El Garch et al., 2016; de Jong et al., 2018), it was possible to retrieve MIC distributions for oxolinic acid, valnemulin and tylvalosin, for which no data are available in EUCAST. Data were also available for lincomycin, tilmicosin, tylosin and tiamulin. Furthermore, for, tiamulin and tylosin, the lowest MIC available from the national AMR monitoring programmes in animal pathogens was lower than the one reported in EUCAST and in case of sufficient representativeness (number of isolates, sources, etc.), these lowest values could be also of interest for future assessments.

We also consulted the EARS-Vet Network (Mader et al., 2021) members, and data from the FINRES reports were shared with us.

No MIC data for amprolium, apramycin and paromomycin were found in these MIC data collections at the time of finalising the current assessments (end August 2021).

[16] FINRES-VET reports - Finnish Food Authority (ruokavirasto.fi).
[17] NORM-VET reports (vetinst.no).
[18] Swedres-Svarm reports - SVA.

Appendix B – Uncertainty analysis

B.1. Uncertainty analysis associated with the determination of the Feed Antimicrobial Resistance Concentration (FARSC) applied to estimate the maximum concentrations of antimicrobials in non-target feed that would not select for antimicrobial resistance in the rumen or large intestines

Table B.1: Potential sources of uncertainty identified in the determination of the Feed Antimicrobial Resistance Concentration (FARSC) applied to estimate the maximum concentrations of antimicrobials in non-target feed that would not select for antimicrobial resistance in the rumen or large intestines and assessment of the impact that these uncertainties could have on the conclusions

Source or location of the uncertainty	Nature or cause of uncertainty as described by the experts	Impact of the uncertainty on the determination of the Feed Antimicrobial Resistance Selective Concentration (FARSC)
Determination of the lowest concentration of antimicrobials not selecting for antimicrobial resistance using MSC and PMSC		
	PMSC is linked to measurements of the minimal inhibitory concentration (MIC) of an antimicrobial; MIC determination is a highly sensitive method based on pure cultures. MIC could be slightly higher or lower in the animal intestine compared to *in vitro* tests.	The PMSC and FARSC could be under or overestimated.
	EUCAST data sets contain primarily MIC data from human pathogens, and extrapolation to underrepresented animal pathogens has been made. This was considered a reasonable assumption because: (i) EUCAST has an overrepresentation of pathogenic strains. At the same time, EUCAST has data for a vast diversity of species across the bacterial kingdom, and thus to be biased in a systematic manner, it would be necessary that pathogens would show significantly different antimicrobial sensitivity profiles compared to all other bacteria. As far as we are aware, there is no indication that this would be the case. If anything, the currently available data suggest that the diversity of pathogens in terms of antimicrobial sensitivity would also predict the diversity of non-pathogens. Furthermore, it could be argued that resistance selection in pathogens would be the most important end point from a risk management point of view; (ii) bacteria of animal origin seem to have similar characteristics to those of human origin, and there is no evidence to suggest that animal-associated bacteria would systematically be more or less sensitive than human-associated ones.	If this assumption is not correct, the PMSC, and accordingly the FARSC, could be underestimated, if a genera or species from animal microbiota is identified with higher susceptibility, or underestimated if in general animal pathogens show lower levels of antimicrobial susceptibility as compared to human pathogens.

Source or location of the uncertainty	Nature or cause of uncertainty as described by the experts	Impact of the uncertainty on the determination of the Feed Antimicrobial Resistance Selective Concentration (FARSC)
	Several bacterial members of animal microbiomes are not yet culturable, with no MICs determined.	The PMSC, and accordingly the FARSC, could be underestimated if a genera or species is identified with higher susceptibility.
	The assumption that the MIC/MSC ratio obtained from experimental measurements in *E. coli* and *S. enterica* for a given antimicrobial remains similar for other species that have different MIC values.	If this assumption is not correct the PMSC could be higher or lower, and accordingly, the FARSC could be under- or overestimated.
	The assumption that the MIC/MSC ratio obtained from experimental measurements in different strains of the same species for a given antimicrobial remains similar.	If this assumption is not correct, the PMSC could be lower, and accordingly the FARSC could be under- or overestimated.
	Extrapolation of the MSCs from a defined experimental competition *in vitro* between two different bacterial strains under ideal conditions may not be fully representative of what would be observed in a natural complex community setting with lower nutrient levels, inhibitory agents, competitors and predators. Experimental data suggest that the MSC can, depending on the antimicrobial class, be either higher or lower in a simple *in vitro* competition experiment as compared to a microbial community. A reasonable conservative assumption is that MSC is similar *in vitro* and in communities.	If this assumption is not correct, the PMSC, and accordingly the FARSC, could be either over- or underestimated, depending on the specific species and the targeted community.
Antimicrobial pharmacokinetic and degradation data used for the estimation of the maximum concentrations of antimicrobials in feed that would not select for antimicrobial resistance in the rumen and large intestines		
	The method only considers the concentrations of drug in the rumen and large intestines.	The final estimates of antimicrobial concentrations could be higher or lower in other parts of the digestive tract, and accordingly, the FARSC could be over- or underestimated.
	It is assumed that the antimicrobials contained in the rumen or large intestines are homogeneously distributed within the contents of these organs.	The ARSQ and FARSC could be overestimated as the concentrations are not homogeneous, with possible local higher concentrations than predicted.
	Since the data on the volume of large intestines was difficult to obtain from the different animal species and age classes, the calculation of the ARSQ and consequently FARSC considers that the antimicrobial concentrations in faeces are similar to those in the large intestines.	In most cases the impact would be minimal as the concentrations in the distal part of the digestive tract are similar to the concentrations in faeces. However, the concentrations in the proximal part of the colon can be slightly overestimated since there is water reabsorption between the proximal and the distal part. Since the concentrations could be lower in the colon than those included in the calculation (concentrations in faeces), ARSQ and consequently FARSC could therefore be underestimated in species with drier faeces, or if feeding practices result in unusually dry faeces.

Source or location of the uncertainty	Nature or cause of uncertainty as described by the experts	Impact of the uncertainty on the determination of the Feed Antimicrobial Resistance Selective Concentration (FARSC)
	Large differences exist in physiological data (relating to body weight, feed intake, intestinal and faecal volume, mass and faecal dry matter output parameters) within animal species and categories. Also, high intake of highly refined 'least cost ration' feed often leads to dysbacteriosis and more liquid faeces. Therefore, in order to use the most widely accepted data, standard values for body weight and feed intake provided by the FEEDAP Panel were used and, along with other obtained physiological data (relating to intestinal and faecal volume, mass and faecal dry matter output parameters), the daily outputs of fresh faeces calculated are representative of values that apply to European farming conditions of food-producing animals. However, depending on a more specific food animal production system, species, breed or age, animal physiological data (relating to body weight, feed intake, intestinal and faecal volume, mass and faecal dry matter output parameters) may vary.	As the ratio between feed intake and faecal output remains in general similar within animal species and categories, their impact on the calculation of the ARSQ and FARSC would be expected to be minimal. However, depending on a more specific food animal production system, species, breed or age, animal physiological data (relating to body weight, feed intake, intestinal and faecal volume, mass and faecal dry matter output parameters) could result in an under or overestimation of FARSC.
	The range of individual antimicrobial bioavailability values for each animal species is not available. For some species there are little published data.	The average value for bioavailability extracted from literature for each animal species was used and overestimation or underestimation of ARSQ and FARSC could therefore occur. Simulations were done using a wider range of values to determine the range of values that could be obtained.
	The method is based on the resistance development due to the exposure to one antimicrobial. Synergistic or antagonist effects of different antimicrobial combinations might come into play, but the effects are unknown.	The drug combination effects could - depending on the substances, mechanisms of resistance and bacterial organisms - affect the relevant concentration of individual substances that would have a role in the development and selection of resistance. This could lead to an overestimation or underestimation of FARSC.

ARSQ: Antimicrobial Resistance Selection Quantity; FARSC: Feed Antimicrobial Resistance Selection Concentration; MSC: minimal selective concentration; PMSC: predicted minimal selective concentration.

B.2. Uncertainty analysis associated with the methodology (extensive literature search) applied to assess the potential growth-promoting effects

A common methodology (extensive literature search) was applied to assess the potential growth-promoting effects of 24 antimicrobial substances. The general uncertainties related to the methodology (extensive literature search) are described in Table B.2.

Table B.2: Sources of uncertainty associated with the methodology used for the extensive literature search performed to assess the potential growth-promoting effects of 24 antimicrobial substances and their possible impact on comprehensiveness of the search (number of papers retrieved)

Source of the uncertainty	Nature or cause of the uncertainty	Impact of the uncertainty on the comprehensiveness of the search
General assessment: methodology Extensive literature search (Annex E)		
Search strategy		
Language	The search was performed exclusively in English (at least the abstract for other languages). More hits might be identified including papers in other languages that do not have an English abstract.	Underestimation of the published relevant papers.
Publication type	Papers presenting original data (primary research studies) were considered. Reviews were only included if they presented data that were not available from a primary research study. Records that do not present original/primary data (e.g. reviews, editorials, position papers) were excluded. More hits might have been identified including grey literature (conference proceedings, extended abstracts, book chapters).	Underestimation of the published relevant papers.
Access	Only full text documents were included. Potentially relevant publications, for which it was unsuccessful to obtain a copy, were excluded.	Underestimation of the published relevant papers.
Search terms and search strings used in the ELS (sensitivity)	The search criteria were clearly reported. Some synonyms may have not been used. Less hits might have been identified.	Underestimation of the published relevant papers.
Sources of studies	Bibliographic databases, websites, etc. were clearly reported. Two multi-disciplinary/large databases (Scopus and Web of Science Core Collection and four more specialist/subject-specific databases (AGRICOLA, AGRIS, CABI - Animal Health & Production Compendium and MEDLINE) were searched. The search was complemented by internet searches and manual searches. No data were retrieved from sources other than the public literature (e.g. industry data). More studies (e.g. industry data) could have been retrieved by applying a different methodology (e.g. public call for data).	Underestimation of the existing potentially relevant hits.
Eligibility criteria	The eligibility/inclusion criteria were clearly reported in the protocol. A conservative approach was applied during the screening phase. Some papers might have been not included.	Underestimation of the published relevant papers.
Exclusion criteria	The exclusion criteria applied during the assessment of the evidence are considered appropriate. However, considering that the search is unlimited in time,	Underestimation of the published relevant papers.

Source of the uncertainty	Nature or cause of the uncertainty	Impact of the uncertainty on the comprehensiveness of the search
	some criteria are very strict for old studies (e.g. the lack of statistics for studies published before 1980, as the use of statistics was not common). Some old studies could have been excluded due to the strict criteria applied in the assessment phase, e.g. lack of statistics or unclear reporting. In case of doubts/unclear reporting, the studies were excluded during the assessment phase.	

The extensive literature review conducted was implemented according to the principles of a systematic review. The search strategy applied has high sensitivity, although it could not retrieve data from sources other than the public literature (e.g. industry data). More hits might have been identified including papers in other languages that do not have an English abstract. Some studies could have been excluded based on the application of strict exclusion criteria during the assessment phase. It is expected the number of hits retrieved is an underestimation of the existing potentially relevant hits (see Table B.2). However, it is expected that these factors have limited impact on the final number of studies with adequate quality.

In general, this impact on the conclusions of the assessment is considered minimal. However, the impact is considered not negligible when the number of studies available for the assessment of a certain antimicrobial/animal category is limited, preventing an integration of the evidence.

General uncertainty analysis associated with the results

The sources of uncertainty associated with the results of the extensive literature searches and their possible impact on the level(s) which have growth promotion/increase yield effect are summarised in Table B.3. The sources of uncertainties identified are applicable to all 24 antimicrobials.

Table B.3: Sources of uncertainty associated with the results of the extensive literature search applied to assess the potential growth-promoting effects of 24 antimicrobial substances and their possible impact on the level(s) which have growth promotion/increase yield effect

Source of the uncertainty	Nature or cause of the uncertainty	Impact of the uncertainty on the conclusion on the level(s) which have growth promotion/increase yield effect
General assessment applicable to all 24 antimicrobials		
Methodology, overall insufficient studies	The extensive literature (ELS) review conducted was implemented according to the principles of a systematic review. The search strategy applied has high sensitivity, although it could not retrieve data from sources other than the public literature (e.g. industry data). More hits might have been identified including papers in other languages that do not have an English abstract. Some studies could have been excluded based on the application of strict exclusion criteria during the assessment phase.	Underestimation/Overestimation The impact of the uncertainties identified in ELS (underestimation of the number of papers retrieved) on the conclusions of the assessment(s) would be minimal when the number of papers of adequate quality retrieved for a certain antimicrobial/animal category is high. However, the impact would not be negligible when the number of studies retrieved for a certain antimicrobial/animal category is low. The levels which have a growth promotion/ increase yield effect could be higher or lower.
Scope and study design		
Scope of the experiments and	A number of studies retrieved was not designed to investigate the effects of the substances on growth performance. The	Underestimation/Overestimation As an appropriate data set was not available,

Source of the uncertainty	Nature or cause of the uncertainty	Impact of the uncertainty on the conclusion on the level(s) which have growth promotion/increase yield effect
selection of the concentrations	choice of the concentrations and the completeness of the set of parameters evaluated have been driven by the primary purpose of the study.	the levels which have a growth promotion/ increase yield effect could be higher or lower.
Lack of dose-range finding studies	Specific dose-range findings studies were scarcely identified. Only studies in which a single concentration or few concentrations not consistently tested in different studies were available. The results could therefore reflect point effects and do not allow to identify dose-response relationships.	Underestimation/Overestimation As the complete range of concentrations was not explored, the levels (other than those tested) which have a growth promotion/ increase yield effect could be higher or lower.
Animals		
Animals used	The animals used in the studies retrieved might not be the breeds/strains currently used in the EU for food production, characterised by high performances in terms of food production. Studies performed with animals with lower performance are likely to detect positive effects on grow promotion at lower levels (overestimation of the effects). There is uncertainty in the extrapolation of the results to breeds/strains used in the EU.	Underestimation of the level at which the effect is observed (as the effects could have been detected at lower concentrations in breed/strains characterised by lower performances).
Farming conditions	Papers examined cover studies performed all over the world. Farming conditions might not be the same as those currently allowed in the EU, also regarding animal welfare. Studies performed with animals farmed under conditions not comparable to those allowed in the EU, are likely to detect positive effects on grow promotion at lower levels (overestimation of the effects). There is uncertainty in the extrapolation of the results to EU farming conditions.	Underestimation of the level at which the effect is observed (as the effects could have been detected at lower concentrations in animals farmed under different conditions).
Test item		
Form(s) of antimicrobial used	The specific form of the antimicrobial used in the study (as the '(free) base' substance, its salts or specific products/formulations containing the base substance) has not been clearly described in several publications.	Underestimation/Overestimation
Concentration(s) of the 'base' substance vs. compound/product specific test items	In the majority of the studies it is not specified whether the concentration applied refers to the 'base' substance or the salt/ commercial form, etc. This would lead to an uncertainty on the actual concentration applied, which may have shown growth-promoting effect. In summarising the results, the concentrations have been reported as for 'base' substance when the form of the antimicrobial is not specified.	Underestimation/overestimation of the concentration which may have shown growth-promoting effect.
Calculations of the concentration on	The calculation is based on the total dry matter (DM) intake; when no data of DM of	Underestimation/Overestimation

Source of the uncertainty	Nature or cause of the uncertainty	Impact of the uncertainty on the conclusion on the level(s) which have growth promotion/increase yield effect
Dry Matter (DM) basis in ruminants	the different feed items was given, default values from the literature were used.	
Reporting/Appraisal		
Absence of raw data generated/ analysed in the publication retrieved	The analysis of the results is based on the reporting and the assessment of the data done by the authors of the publications. An independent evaluation of the studies by the assessors was not possible.	Underestimation/Overestimation

The main source of uncertainty in the results of the extensive literature search (see Table B.3) is due to the fact that a number of studies retrieved was not specifically designed to address the assessment question. To properly answer the assessment question, i.e. *to assess which levels of the antimicrobials have a growth promotion/increase yield effect*, studies aiming to examine specific growth-promoting effects of the antimicrobials and dose-range findings studies (designed to identify the concentration which have an effect in growth promotion) would be needed. With few exceptions, these studies were not available. In the lack of studies aiming to examine the specific growth-promoting effects and evaluate dose-response relationships, the exact concentration limits for growth promotion effects could not be established. In the absence of specific studies that would allow to establish the exact concentration limits for growth promotion effects of antimicrobials, the levels in which the effects have been observed were extracted from the studies retrieved and reported in the opinion.

In the available evidence, the study design and the selection of the concentration(s) and end point(s) were rather driven by the primary aim of the study. A single or few concentrations were selected for testing based on expected effects on other parameters. This source of uncertainty has an impact on the conclusions of the assessment, on the reported levels which have a growth promotion/ increase yield effect, which could be higher or lower. The other sources of uncertainty identified are related to the animals and the test item used in the studies retrieved. It is uncertain on whether studies performed with different animal breeds/strains and under different farming conditions compared to the current EU standards reflect the current farming conditions in the EU. The description of the test item (as the base substance or its salt/commercial form) was not always clear, leading to uncertainty on the actual feed concentration, which may have shown growth-promoting effect. When unclear, the concentrations have been reported as for the base substance. Both sources of uncertainty are expected to lead to an overestimation of the effect, but to an underestimation or overestimation of the level at which the effect is observed.

Substance-specific assessment: evidence synthesis

Evidence synthesis was possible for a limited number of antimicrobials (if more than three positive studies were retrieved in the same animal category). The substance-specific sources of uncertainty associated with the process of evidence synthesis are summarised in Table B.4.

Table B.4: Substance-specific sources of uncertainty associated with the process of evidence synthesis

Source of the uncertainty	Nature or cause of the uncertainty	Impact of the uncertainty on the conclusion on the level (s) which have growth promotion/increase yield effect
Substance-specific assessment applicable to a limited number of substances/animal category		
Varying degrees of validity/quality of the individual studies	Although the individual studies have different degrees of internal validity/quality, it was assumed that all studies that were considered eligible have similar validity. In synthesising the evidence, the same weight was given to all the studies considered eligible.	Underestimation/Overestimation

Source of the uncertainty	Nature or cause of the uncertainty	Impact of the uncertainty on the conclusion on the level (s) which have growth promotion/increase yield effect
Heterogeneity of studies	The number of studies retrieved for the different antimicrobials in the different species/categories is highly variable. The studies retrieved are heterogeneous in terms of the study design and the scope of the research, the concentrations were selected over a wide range (up to 3 orders of magnitude), different end points were tested and different results have been reported with the same levels of antimicrobials. Inconsistent results have been reported for the same levels of several antimicrobials, e.g. same level in the same animal species/categories: some studies reporting positive growth promotion effects, some other negative and some other no effects. For other antimicrobials, consistent results have been reported across the studies for the same level of the antimicrobial in the same animal species/category In synthesising the evidence, the uncertainty due to the heterogeneity in the results of the studies was considered.	Underestimation/Overestimation
Publication bias	Studies reporting positive results are more likely to be published compared to studies reporting negative or no effects (overestimation of the effect)	Underestimation/Overestimation The levels which have a growth promotion/increase yield effect could be higher or lower.
Process of evidence synthesis	The evidence retrieved for the different antimicrobials is characterised by heterogeneity (number of studies retrieved, scope of the studies, compound/test item, concentrations and end points tested, results). Although meta-analysis was not applicable, evidence synthesis (including a substance-specific uncertainty analysis) was done when more than 3 studies showing consistent (positive) results in a comparable range of concentrations were available for the antimicrobial in the same species/ category (see Section 2.2.3). However, in the majority of cases, the low number of studies retrieved ($n \leq 3$ or $n = 4–5$ covering a wide range of concentrations, 3 orders of magnitude) prevented evidence synthesis. The uncertainty resulting in the process of evidence synthesis is dependent on the number of publications for the same substance and the consistency of the results (% of studies reporting positive effects vs. % of studies reporting negative or no effects), see Section 2.2.3.	Underestimation/Overestimation The extent of the underestimation or overestimation on the levels which showed growth-promoting effect is modulated by the consistency of the results within the same category.
Evidence integration	In the absence of knowledge on the mode of action leading to growth promotion effects, evidence integration across the different animal categories/species was not done. However, when positive effects observed in piglets were also observed in pigs for fattening, the consistency across categories was considered to strengthen the conclusions for both categories. Consistency of results across categories (i.e. piglets and pigs for fattening) would reduce the uncertainty in the conclusions for both categories.	Underestimation/Overestimation The extent of the underestimation or overestimation on the levels which showed growth-promoting effect is modulated by the consistency of the results across categories.

In general, the evidence retrieved for the different antimicrobials is characterised by heterogeneity (number of studies retrieved, scope of the studies, compound/test item, concentrations and end points tested, results).

For most antimicrobials, data were not retrieved for all food-producing animals. For 10 substances, the literature search did not retrieve any relevant paper preventing to draw conclusions. The number of studies retrieved for the remaining 14 substances was highly variable, ranging from 1 up to 27 (when considering the same target species/category) and had an impact on the conclusions on the level(s) which have growth promotion/increase yield effect (see Section 4 Conclusions and Annex F, Table F.2).

In a limited number of cases, when more than three studies showing consistent (positive) results in a comparable range of concentrations were available for the antimicrobial in the same species/category, evidence synthesis was possible, including a substance-specific assessment of the uncertainty (see Table B.3). In these cases, the level of uncertainty has been specifically assessed considering the consistency in the results observed for the same species/categories in a comparable range of concentrations (see the methodology, Section 2.2.3). This is reflected in the use of subjective probability ranges when drawing conclusions for the individual antimicrobials, as defined in line with recommendations of the uncertainty guidance.

Depending on the total number of studies available for a specific animal category, the consistency of the results (% of studies reporting positive effects vs. % of studies reporting negative or no effects), it is judged

- 66–90% certain ('likely')
- 50–66% certain
- 33–66% certain ('about as likely as not')

that the substance has growth-promoting/increase yield effects in the animal category in a defined concentration range (based on n studies).

When the low number of studies retrieved ($n \leq 3$ or $n = 4–5$ but covering a wide range of concentrations, e.g. 3 orders of magnitude) prevented evidence synthesis, 'it is about as likely as not' (33–66% probability) that the substance has growth-promoting/increased yield effects in the animal category in a defined concentration range (based on n studies).

Appendix C – Supporting information on animal feeding systems

Poultry species reared for meat are normally fed heat-treated crumbs (mechanically crumbled pellets) during the first week of life, followed by a succession of different stages of pellets in which the digestible vegetable protein (mainly soya bean meal) content is reduced and wheat content increased to reduce the cost (Karunajeewa, 1987). Each of these ration changes (typically 3–5 during the life of a broiler chicken) imposes digestive stress on the birds, which may result in dysbacteriosis and the need for antimicrobial treatment. Breeding chickens may be fed heat-treated meals and laying hens are normally fed non heat-treated coarsely-ground meal rations. These are typically produced in a compound feed mill and delivered to farms in bulk each week, to be fed via automated pan or chain feeding systems (Širovnik et al., 2017), or in some smaller flocks, via ad-lib hoppers. Some egg production farms have home-mixing facilities for feed. Free-range flocks have access to pasture and mixed herbal vegetation after the indoor rearing phase, but this does not make a major contribution towards their nutrition.

Pigs are typically fed compound pelleted feed in a similar way to meat poultry species, but the ration is likely to contain a high level of cheaper proteins, such as rapeseed meal, and some of the wheat content may be replaced by barley or maize, in an attempt to reduce the dysbacteriosis caused by high levels of wheat and fine grinding of meal before pelleting. A higher proportion of pig farms than poultry farms use home mixing, apart from for weaning rations, and some farms use liquid feed, which may be just based on water added to meal, but ideally liquid feed should be fermented to enhance its probiotic and acidic properties to benefit gut health. Addition of waste milk-based products to liquid feed is particularly beneficial. In some countries, particular the UK, outdoor pig breeding represents about half of the national breeding herd, of which a very small proportion is organic, requiring certified organic feed (Blair, 2017; Knecht et al., 2017). Pigs rapidly destroy vegetation in their outdoor ranges and are fed large pellets distributed on the ground by a spinner, or, increasingly, in large troughs and ad-lib feed hoppers.

Feeding systems for cattle are very diverse (Humer et al., 2018; McAllister et al., 2020), apart from for veal calves, which are fed on milk substitute via an automated feeder, or grass-fed beef suckler herds, which may be fed almost entirely on grazed grass and forages (silage, hay, straw), supplemented by urea-based mineral licks and minimal rolled cereals or compound feed if required. Dairy cattle are normally fed a higher proportion of compound feed or home-mixed total mixed ration (TMR), which usually comprises a mix or maize silage, rolled grain and vegetable protein meal, plus minerals and vitamins. Some compound feed may be offered in the milking parlour or in robotic milking systems to encourage cows to enter.

Dairy calves are normally removed from the dam within 1–4 days and then fed on waste milk from the herd and a commercial milk replacer via buckets or semi/automated feeding systems. Solid feed in the form of a coarse ration or pellets in increasingly offered from 2 to 3 weeks of age and calves are typically weaned onto concentrates and forage at 6–8 weeks (except for calves destined for veal production). Subsequent feeding programmes depend on whether the calves will be used for dairy herd replacements, intensive beef production or semi-intensive/extensive production. The main difference is the speed of fattening, which for intensive systems requires a high level of cereal and vegetable protein intake (Kertz et al., 2017).

Milk sheep and goats are typically reared and fed in a similar way to dairy cattle, but breeding flocks of sheep are grazed throughout the year in many countries, and only given concentrate ration during the lambing period. Most prime lambs are finished off the ewe during the grazing period but may be given additional concentrate via ad-lib feeders. Weaned lambs that do not make the grade as prime lamb can be finished by grazing on root crops or in systems similar to beef calves. Cull ewes may be brought together by dealers at the end of their breeding life and intensively fattened on cereals and forages to supply the mutton market (Wilson and Brigstocke, 1981).

Horses specifically bred for meat are managed and fed in a similar way to semi-intensive beef cattle on hay and cereals (Raspa et al., 2020).

Rabbits are reared for meat in colonies, usually in cages, and fed a pelleted concentrate ration via manual or automated feeding systems (De Blas and Wisewan, 2020). Pellets often include grass meal as well as vegetable protein and cereal components and are usually fed ad libitum to fattening rabbits, but may be restricted for breeding animals (Maertens, 2020).

Farmed salmon are initially bred and reared in tanks and then fattened in net pens located in offshore, in sea inlets or in lakes (Ellis et al., 2016). They are fed pelleted rations based largely on fishmeal and oils, but may also include mammalian or avian proteins, vegetable proteins and cereals. The pellets are manually or automatically dispensed into the water surrounding the fish (Pelletier and Tyedmers, 2007).

Annex A – Supplementary information on animal production systems

The Annex is provided as a separate excel file containing supplementary information on animal production system and is available under the Supporting information section at https://doi.org/10.2903/j.efsa.2021.6852

Annex B – Antibiotics authorised in the EU for food-producing species according to MRL Regulation (EU) 37/2010

The Annex is provided as a separate Excel file containing the list of antibiotics allowed for use in the EU for food-producing species according to MRL Regulation (EU) 37/2010 (consolidated version of 6 May 2021) and is available under the Supporting information section at https://doi.org/10.2903/j.efsa.2021.6852

Annex C – Outcome of the public consultation on specific sections of the draft scientific opinion on Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 1: Methodology, data gaps and uncertainties

The Annex is provided as a separate PDF file containing the outcome of the Public consultation on specific sections of the 'Draft scientific opinion on Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 1: Methodology, data gaps and uncertainties', including the comments received and how they were taken into account when finalising the scientific Opinion, and is available under the Supporting information section at https://doi.org/10.2903/j.efsa.2021.6852

Annex D – Perl code used to download EUCAST data and extract the MIC_{lowest}

The Annex is provided as text files: three files with Perl code, a brief instructions file and the list of antibiotics used to extract data in this study. The code is available on the EFSA Knowledge Junction community on Zenodo at: https://doi.org/10.5281/zenodo.5567362

Annex E – Extensive literature search on the potential growth-promoting effects of 24 antimicrobial active substances

The Annex is provided as a separate PDF file containing a detailed description of the literature search strategy and methodology applied to address ToR2 and is available under the Supporting information section at https://doi.org/10.2903/j.efsa.2021.6852

Annex F – Summary Tables with the results for the Feed Antimicrobial Resistance Selection Concentration (FARSC) estimation and antimicrobial concentrations with growth promotion/yield increase effects for the 24 substances under assessment

The Annex is provided as a separate PDF file containing a summary table with the results of the Feed Antimicrobial Resistance Concentration (FARSC) estimations (Annex F.1) and a summary table with the levels of antimicrobial substances causing effects on growth promotion/yield increased in food-producing animal species/categories (Annex F.2), available under the Supporting information section at https://doi.org/10.2903/j.efsa.2021.6852