SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 15 September 2021

doi: 10.2903/j.efsa.2021.6855

Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 4: *β-Lactams: amoxicillin and penicillin V*

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Abstract

The specific concentrations of amoxicillin and penicillin V 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 bacteria relevant for human and animal health, as well as the specific antimicrobial concentrations in feed which have an effect in terms of growth promotion/increased yield were assessed by EFSA in collaboration with EMA. Details of the methodology used for this assessment, associated data gaps and uncertainties, are presented in a separate document. To address antimicrobial resistance, the Feed Antimicrobial Resistance Selection Concentration (FARSC) model developed specifically for the assessment was applied. However, due to the lack of data on the parameters required to calculate the FARSC, it was not possible to conclude the assessment until further experimental data become available. To address growth promotion, data from scientific publications obtained from an extensive literature review were used. Levels in feed that showed to have an effect on growth promotion/increased yield were reported for amoxicillin, whilst for penicillin V no suitable data for the assessment were available. It was recommended to carry out studies to generate the data that are required to fill the gaps which prevented the calculation of the FARSC for these two antimicrobials.

Keywords: amoxicillin, penicillin V, antimicrobial resistance, sub-inhibitory concentration, growth promotion, yield increase, food-producing animals

Requestor: European Commission

Question number: EFSA-Q-2021-00504

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Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at https://ess.efsa.europa.eu/doi/doiweb/doisearch.

Acknowledgements: The BIOHAZ Panel, leading Panel in charge of the adoption of the scientific opinion and assessment of Term of Reference 1 (ToR1, antimicrobial resistance) wishes to thank the following for the support provided to this scientific output: EFSA Panel on Animal Health and Welfare (AHAW Panel), who supported ToR1 assessments development and endorsement of those sections under their remit (animal production, main use of antimicrobials); EFSA Panel for Additives and Products or Substances used in Animal Feed (FEEDAP), in charge of the assessment and endorsement of ToR2, and providing advice and data needed for ToR1 assessments; European Medicines Agency (EMA), who was represented by an external expert and EMA secretariat as members of the Working Group (WG); Valeria Bortolaia, who was member of the WG until 17 April 2020; EFSA staff members: Angelica Amaduzzi, Gina Cioacata, Pilar García-Vello, Michaela Hempen, Rita Navarrete, Daniel Plaza and Anita Radovnikovic; EMA staff members: Barbara Freischem, Zoltan Kunsagi, Nicholas Jarrett, Jordi Torren, and Julia Fábrega (currently EFSA staff). The BIOHAZ Panel wishes also to acknowledge the EMA Committee for Medicinal Products for Veterinary Use (CVMP) and their experts.

Suggested citation: EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, Chemaly M, Davies R, De Cesare A, Herman L, Hilbert F, Lindqvist R, Nauta M, Ru G, Simmons M, Skandamis P, Suffredini E, Andersson DI, Bampidis V, Bengtsson-Palme J, Bouchard D, Ferran A, Kouba M, López Puente S, López-Alonso M, Nielsen SS, Pechová A, Petkova M, Girault S, Broglia A, Guerra B, Innocenti ML, Liébana E, López-Gálvez G, Manini P, Stella P and Peixe L, 2021. Scientific Opinion on the maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 4: *β-Lactams: amoxicillin and penicillin V.* EFSA Journal 2021;19(10):6855, 26 pp. https://doi.org/10.2903/j.efsa.2021.6855

ISSN: 1831-4732

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1. Introduction

The European Commission requested the European Food Safety Authority (EFSA) to assess, in collaboration with the European Medicines Agency (EMA), (i) 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 (term of reference 1, ToR1), and (ii) the levels of the antimicrobials which have a growth promotion/increase yield effect (ToR2). The assessment was requested to be conducted for 24 antimicrobial active substances specified in the mandate.[1]

For the different substances (grouped by class if applicable)[1], separate scientific opinions included within the 'Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed' series (Scientific Opinions Part 2 - Part 13, EFSA BIOHAZ Panel, 2021b-l – see also the Virtual Issue; for practical reasons, they will be referred as 'scientific opinion Part X' throughout the current document) were drafted. They present the results of the assessments performed to answer the following questions: *Assessment Question 1 (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, and *AQ2*: which are the specific antimicrobial concentrations in feed of food-producing animals that have an effect in terms of growth promotion/increased yield. The assessments were performed following the methodology described in Section 2 of the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (EFSA BIOHAZ Panel 2021a, see also the Virtual Issue). The present document reports the results of the assessment for the β-lactams: amoxicillin and penicillin V.

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

The background and ToRs provided by the European Commission for the present document are reported in Section 1.1 of the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (see also the Virtual Issue).

1.2. Interpretation of the Terms of Reference

The interpretation of the ToRs, to be followed for the assessment is in Section 1.2 of the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (see also the Virtual Issue).

1.3. Additional information

1.3.1. Short description of the class/substance

The class of β-lactam antimicrobials is a key group of bactericidal drugs used for treating infections caused by both Gram-positive and Gram-negative bacteria in human and veterinary medicine (Bush and Bradford, 2020). The members of the class are classified according to their core ring structures and include penicillins, cephalosporins, carbapenems and monobactams as well as various β-lactamase inhibitors that can be used in combination with the antimicrobial. The two β-lactams of relevance here are penicillin V (phenoxymethylpenicillin, an early discovered penicillin) and amoxicillin (a semisynthetic derivative of penicillin belonging to the aminopenicillin family). The main chemical characteristic common to all β-lactams is the presence of a β-lactam ring in their molecular structure. This ring structure is central to the mechanism of action where the β-lactam binds to penicillin-binding proteins (PBPs), enzymes that are central in synthesis of the peptidoglycan layer, and thereby inhibits formation of the cell wall. β-Lactams show a structural similarity with the terminal amino acid of the NAM/NAG peptide. This similarity allows the antimicrobial binding to the active site of PBPs and a resulting inhibition of the PBPs and cell wall synthesis.

The narrow spectrum of activity of penicillin, mainly restricted to Gram positive bacteria (e.g. staphylococci, streptococci) and Gram-negative cocci (e.g. *Neisseria* spp.) was further constrained by the extensive emergence of resistance in several of those microorganisms (e.g. staphylococci). With the addition of the amino group to penicillin, amoxicillin gained the ability to cross porins of

[1] Aminoglycosides: apramycin, paromomycin, neomycin, spectinomycin; Amprolium; Beta-lactams: amoxicillin, penicillin V; Amphenicols: florfenicol, thiamphenicol; Lincosamides: lincomycin; Macrolides: tilmicosin, tylosin, tylvalosin; Pleuromutilins: tiamulin, valnemulin; Sulfonamides; Polymyxins: colistin; Quinolones: flumequine, oxolinic acid; Tetracyclines: tetracycline, chlortetracycline, oxytetracycline, doxycycline; Diaminopyrimidines: trimethoprim.

Gram-negative bacteria, enlarging the penicillin spectrum of activity namely to *E. coli* and *Salmonella enterica*.

The spectrum of activity, minimum inhibitory concentration (MIC) and pharmacokinetic (PK) values of penicillin and amoxicillin present differences (Bush and Bradford, 2016). Penicillin and amoxicillin will be addressed separately in the framework of this scientific opinion.

1.3.2. Main use[2]

Amoxicillin has a very broad application with a bactericidal effect on many Gram-positive and Gram-negative bacteria. It is therefore also used for a wide range of respiratory, gastrointestinal and urogenital infections along with treatment of secondary infections following viral infections in pigs, and respiratory and gastro-intestinal infections in poultry. These include rhinitis and bronchopneumonia caused by *Pasteurella* spp., rhinitis caused by *Streptococcus* spp., bronchopneumonia caused by *E. coli* or Gram-positive cocci, infections of the urogenital system with, e.g. *E. coli* or *Proteus* spp. In cattle, treatment of mastitis caused by Gram-positive cocci or *E. coli* is an indication. In pigs, amoxicillin can also be used for treatment of *Actinobacillus pleuropneumoniae.*

Penicillin V is administrated orally. It is a first drug of choice against clostridial infections in poultry (Löhren et al., 2008) and it can be used as an in-feed medication for prevention of streptococcal meningitis in pigs (Johnston et al., 1992).

1.3.3. Main pharmacokinetic data

The bioavailability (i.e. the fraction of the antimicrobials absorbed from the digestive tract to the plasma) of amoxicillin and penicillin V after oral administration is generally low in mammals. Higher bioavailability is reported for chickens and turkeys.

Penicillin V

The oral bioavailability of penicillin V is 69% in chicken (EMA/CVMP, 2012).

In fed or fasted pigs, the bioavailability is low with values around 17–19% (Nielsen and Gyrd-Hansen, 1994). In pre-ruminant calves, the mean oral bioavailability is less than 10% of the 10 mg/kg dose and equal to 28.8% for the 20 mg/kg dose and 34.5% for the 40 mg/kg dose (Soback et al., 1987).

In 5- to 12-day-old foals, the bioavailability of penicillin V was described as 16.04 ± 1.23% but in the adult horses, the bioavailability is lower with a value of 1.65 ± 0.55% (Baggot et al., 1990). However, the main limitation of this study is that the intravenous data necessary to calculate the bioavailability in foals were obtained in adult horses. The bioavailability in foals is potentially inaccurate.

For ruminants 6-week-old calves fed exclusively hay, silage and concentrates, it was suggested that penicillin V could be inactivated and degraded in the gastrointestinal tract (Soback et al., 1987). No quantitative data were provided for this phenomenon.

After absorption, penicillins are mainly excreted unchanged in urine and to a lesser extent in bile. Metabolism is considered to be of little importance in the elimination of most penicillins (EMA, 2008).

Amoxicillin

The bioavailability of amoxicillin is 60.2% in turkey (Jerzsele et al., 2011) and around 60-64% in chickens (Anadón et al., 1996; Abo El-Sooud et al., 2004; Jerzsele et al., 2009).

The mean oral bioavailability of amoxicillin in pigs ranges from 22 to 31% in fed and fasted pigs (Agersø and Friis, 1998; Reyns et al., 2007; Sun et al., 2021) and is around 30% in calves (Ziv et al., 1977).

The oral bioavailability of amoxicillin is around 5% to 10% in fasted horses (Wilson et al., 1988; Ensink et al., 1992) and 36% to 43% in neonatal foals (6–7 day-old) (Baggot et al., 1988). However, the foals were not their own control as the oral and iv administrations needed to calculate the bioavailability were performed by two different laboratories in different foals. Thus, the bioavailability in foals is potentially inaccurate.

[2] 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.

No quantitative data on the bioavailability of amoxicillin in ruminant calves were available.

Amoxicillin, like other penicillins, is mainly excreted unchanged in urine and to a lesser extent in bile (EMA, 2008).

Amoxicillin was apparently inactivated by the gastrointestinal contents of early ruminant (6 weeks old) calves (Soback et al., 1987). One study also showed that amoxicillin can be degraded in the small intestine (45% of the initial amount), and that it was mainly due to the action of intestinal tissues (28% of the initial amount) compared to the action of intestinal juices (15% of the initial amount) (Chesa-Jiménez et al., 1994).

1.3.4. Main resistance mechanisms

The effectiveness of all β-lactams relies on their ability to reach the PBP target molecules with the β-lactam ring intact and an ability to bind to the PBP. The two main mechanisms that can generate high-level β-lactam resistance involve the synthesis of enzymes that attack and hydrolyse the β-lactam ring (thereby rendering the antimicrobial unable to bind to the PBPs) and alterations in the PBPs that prevent binding of the antimicrobial (Fisher and Mobashery, 2016; Bush, 2018). Resistance to penicillin V and amoxicillin can be conferred by both of these mechanisms.

For the former mechanism, there exists a myriad of different types of enzymes that have different activities on the different β-lactams. These enzymes are classified into A, B, C and D according to their amino acid sequence (phylogenetic relatedness) and mode of action of hydrolysis (A, C and D uses a serine for hydrolysis and B a zinc ion). In addition, they are classified according to their functional role and to which β-lactam substance they provide resistance (e.g. penicillinases, extended spectrum β-lactamases, ampC β-lactamases, carbapenamases) (Bush and Jacoby, 2010).

The second major mechanism of β-lactam resistance (including to penicillin V and amoxicillin) involves altered PBPs that do not bind to the β-lactam drug. A notable example of this type of resistance includes meticillin resistant *S. aureus* (MRSA) due to the *mec*A gene which encodes an alternative PBP2a that confers resistance to meticillin (Peacock and Paterson, 2015). Similarly, the pioneering work of Tomasz, Spratt and Hakenbeck showed that penicillin resistance in *S. pneumoniae* resulted from horizontal gene transfer (HGT) and recombination of heterologous DNA sequences to generate mosaic PBPs conferring resistance to β-lactams (Spratt, 1994; Tomasz and Munoz, 1995; Hakenbeck et al., 2012). HGT and recombination or mutations of PBP5 in *E. faecium* is also associated with high level resistance to amoxicillin (Novais et al., 2016). In addition, resistance to β-lactams can be generated by point mutations in specific PBPs and (Adler et al., 2016), or by induction of PBPs, that can compensate for the inhibited PBP, observed for mecillinam (Thulin and Andersson, 2019).

Apart from the two major mechanisms described above other mechanisms such as reduced porin activity/level (Martínez-Martínez et al., 1999) or increased drug efflux (Adler et al., 2016) can generate low level resistance.

2. Data and methodologies

The data sources and methodology used for this opinion are described in a dedicated document, the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (see also the Virtual Issue).

3. Assessment

3.1. Introduction

As indicated in the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (see also the Virtual Issue), exposure to low concentrations of antimicrobials (including sub-minimal inhibitory concentrations, sub-MIC) may have different effects on bacterial antimicrobial resistance evolution, properties of bacteria and in animal growth promotion. Some examples including emergence of, and selection for, antimicrobial resistance, mutagenesis, virulence and/or HGT, etc., for the antimicrobials under assessment are shown below.

3.1.1. Resistance development/spread due to sub-MIC concentrations of β-lactams including amoxicillin and penicillin V: examples

In spite of the enormous medical importance of β-lactams, relatively little is known about resistance development at sub-MIC levels of antimicrobials with regard to rates and mechanisms and, in

particular, what are the minimal selective concentrations (MSCs) for the different drugs in this antimicrobial class. Listed below are a number of studies that have examined if and how exposure to sub-inhibitory levels of different β-lactams influence resistance evolution and properties of the bacteria. A majority of these studies have been performed with antimicrobial concentrations that are relatively close MIC values of the strains used. It should be noted that few studies address specifically amoxicillin and penicillin V but there are similarities between the different antimicrobials within the class of β-lactams with regard to structure (i.e. common β-lactam ring), mode of action (i.e. binding to PBPs) and resistance mechanisms (i.e. PBPs modifications, hydrolysis of the β-lactam ring) that justify the inclusion of reports of some cephalosporins and carbapenems to evaluate the effects of sub-MIC levels on selection, de novo evolution and transmission.

3.1.1.1. Effects of sub-MIC concentrations on selection for resistance and mutagenesis

- Subculturing of *S. pneumoniae* in amoxicillin (with/without clavulanate), cefaclor, cefuroxime and cefuroxime at sub-MIC (1/2 to 1/8 of MIC) led to raised MIC after 17-45 serial passages (Pankuch et al., 1998).
- Serial passage of *S. pneumoniae* at sub-MIC levels (exact level not stated in paper) of amoxicillin (with and without clavulanic acid), imipenem, cefixime, cefatrizine, cefadroxil and cefuroxime led to substantial increases in resistance after 11–24 serial passages (Carsenti-Etesse et al., 1995).
- Subculturing of *S. pneumoniae* in amoxicillin-clavulanate at sub-MIC (1/2 to 1/8 of MIC) led to MIC rising from 0.015 to 0.125 mg/mL after 24 passages. Growth for 10 generations in the absence of the antimicrobial reverted the strain to full susceptibility (Davies et al., 1999).

Studies of other β-lactams subclasses also showed sub-MIC resistance development:

- Subculturing of *S. pneumoniae* in ceftriaxone and cefprozil at sub-MIC (1/2 to 1/8 of MIC) led to selection for mutants with substantially raised MICs after 10–50 passages. Growth for 10 generations in the absence of antimicrobial reverted the strain to susceptibility (Nagai et al., 2000).
- Subculturing of *P. aeruginosa* in azlocillin and ceftazidime at 1/2 MIC led to selection for high-level resistant mutants (Wu et al., 1999).
- Exposure of *S. enterica* (var. Typhimurium) to 1/2 MIC of cefotaxime led to an increased ability of the bacteria to colonise mice (Molina-Quiroz et al., 2015).
- Exposure of MRSA to ceftazidime, ceftriaxone and imipenem for 18 serial passages at sub-MIC levels (exact level not stated in paper) can result in selection for heterogeneous vancomycin-intermediate-resistant MRSA (Roch et al., 2014).
- Exposure of *E. coli* expressing bla_{OXA-48} on a clinical plasmid at sub-MIC (1/4 of MIC) of ceftazidime resulted in selection of mutant variants of OXA-48 with only marginally increased resistance but with strong selectable fitness benefits during competition at sub-MIC levels (Fröhlich et al., 2021).
- Exposure of MRSA to sub-MIC concentrations of oxacillin (1/4 of MIC) can cause an SOS response and an associated increase in mutation frequency (Plata et al., 2013).
- Exposure of *E. coli* to sub-MIC levels (1/4 MIC) of ceftazidime can cause induction of the SOS response and a small increase in mutation frequency to other antimicrobials (Thi et al., 2011).
- Exposure of *E. coli* to sub-MIC concentrations of ceftazidime (1/2 to 1/20 of MIC) caused a LexA/RecA-independent induction of the *dinB* gene and an associated increase in mutation frequency (Pérez-Capilla et al., 2005).
- Exposure of *S. marcesens* to sub-MIC (1/2 to 1/4 MIC) of ceftazidime induces transcription of *qnr*B, *qnr*D and *smaqnr* genes via SOS-dependent regulation, potentially providing low-level fluoroquinolone resistance (Briales et al., 2012).

3.1.1.2. Effects of sub-MIC concentrations on horizontal gene transfer and virulence

Several studies have shown that antimicrobials, in particular fluoroquinolones, but also β-lactams, can induce the SOS response and thereby promote emergence of resistance promoting HGT. Listed below are studies showing that sub-MIC antimicrobial concentrations of β-lactams can also have this effect.

- Exposure of *S. aureus* to sub-MIC levels (exact level not stated in paper) of penicillin, ampicillin and cloxacillin and ceftriaxone can induce the SOS response and trigger prophage induction and transfer of a pathogenicity island (Maiques et al., 2006).
- Exposure of *E. coli* O157:H7 to sub-inhibitory concentrations (1/2 to 1/4 of MIC) of imipenem can induce Shiga toxin production in an SOS-independent manner (Nassar et al., 2013).

In summary, even though exposure to sub-MIC concentrations (in the range of 1/2 to 1/20 of the MIC) of various β-lactams can result in the selection for resistant mutants and/or promote resistance spread and emergence, at present no data exist that allow a determination of MSC for any of the target antimicrobials, amoxicillin and penicillin V.

3.2. ToR1. Estimation of the antimicrobial levels in non-target feed that would not result in the selection of resistance: Feed Antimicrobial Resistance Selection Concentration (FARSC)

As explained in the Methodology Section (2.2.1.3) of the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (see also the Virtual Issue), the estimation of this value for these two β-lactams for different animal species, if suitable data were available, would follow a two-step approach as described below:

The first step would be the calculation of the predicted minimal selective concentration (PMSC) for amoxicillin and penicillin V as indicated in Table 1. However, no MSC data required to do the calculations is available for those substances.

Table 1: Calculation of the amoxicillin and penicillin V predicted minimal selective concentration (PMSC)

Antimicrobial (all values in mg/L)	MIC_{test}	MSC_{test}	MIC_{test}/MSC_{test} ratio	MIC_{lowest}	Predicted MSC (PMSC) for most susceptible species ($MIC_{lowest}/MIC_{test}/MSC_{test}$)
Amoxicillin	NA	NA	NA	0.004	NA
Penicillin V	NA	NA	NA	0.004	NA

MIC: minimum inhibitory concentration. MSC: minimal selective concentration. MSC_{test}: MSC experimentally determined. MIC_{lowest}: lowest MIC data amoxicillin and phenoxymethylpenicillin (Penicillin V) calculated based on data from the EUCAST database as described in Bengtsson-Palme and Larsson (2016), see Methodology Section 2.2.1.3.1.1 in the Scientific Opinion Part 1. EUCAST database https://mic.eucast.org/search/ last accessed 15 May 2021. NA: not available.

Due to the lack of PMSC, no FARSC could be calculated. If PMSC was available, the FARSC ($FARSC_{intestine}$ and $FARSC_{rumen}$) corresponding to the maximal concentrations in feed would be calculated for each species from the equations below (for details, see Section 2.2.1.3.2 of the Scientific Opinion Part 1; see also the Virtual Issue) by including specific values for penicillin V and amoxicillin.

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

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

With daily faeces being the daily fresh faecal output in kg, I the inactive fraction, F the fraction available, GE the fraction of the antimicrobial that is secreted back into the intestinal tract for elimination, after initially being absorbed into the bloodstream, and daily feed intake being the daily dry-matter feed intake expressed in kg.

Penicillin V

Penicillin V is poorly absorbed except in chickens. After absorption, the elimination in the intestines is negligible. There is no information on the potential binding of penicillin V to intestinal contents.

The values of *F, GE* and *I* extracted from literature for the calculations of FARSC are summarised in Table 2. The set of values (scenario 1) corresponds to the average of published values.

Table 2: Pharmacokinetic (PK) values used for the calculation of Feed Antimicrobial Resistance Selection Concentration (FARSC) of penicillin V for the different animal species

Penicillin V data	Scenario #1
Inactive fraction (*I*)	NA
Bioavailability (*F*) calves	0.1
Bioavailability (*F*) pigs	0.18
Bioavailability (*F*) horses	0.02
Bioavailability (*F*) chickens	0.7
Gastrointestinal elimination (*GE*)	0

Inactive fraction (*I*) is the fraction of antimicrobial that would not have any activity on bacteria. Bioavailability (*F*) is the fraction of antimicrobial that is absorbed from the digestive tract to the blood. The fraction remaining in the digestive tract and that could be available for the bacteria is equal to (1 – *F*). Gastrointestinal elimination (*GE*) is the fraction of the antimicrobial that is secreted back into the intestinal tract for elimination, after initially being absorbed into the bloodstream. NA = not available.

Amoxicillin

Oral bioavailability of amoxicillin is less than 30% in adult monogastric mammals and around 30% in calves. The absorption is higher in chickens and turkeys with a bioavailability of around 60%. After absorption, the elimination in the intestines is negligible. There is no information on the potential binding of amoxicillin to intestinal contents, but one study demonstrated an inactivation of amoxicillin in the small intestines of rats.

The values of *F*, *I* and *GE* extracted from literature for the calculations of FARSC are summarised in Table 3. The first set of values (scenario 1) corresponds to the average of published values while scenario 2 corresponds to a scenario that would lead to lower FARSC and scenario 3 to a scenario that would lead to higher FARSC.

Table 3: Pharmacokinetic (PK) values used for the calculation of Feed Antimicrobial Resistance Selection Concentration (FARSC) of amoxicillin for the different animal species

Florfenicol data	Scenario #1	Scenario #2	Scenario #3
Inactive fraction (*I*)	NA	–	–
Bioavailability (*F*) pig	0.25	0.15	0.35
Bioavailability (*F*) poultry	0.6	–	–
Bioavailability (*F*) calves	0.3	–	–
Bioavailability (*F*) turkeys	0.6	–	–
Bioavailability (*F*) horses	0.07	0.05	0.1
Gastrointestinal elimination (*GE*)	0	0	0

Inactive fraction (*I*) is the fraction of antimicrobial that would not have any activity on bacteria. Bioavailability (*F*) is the fraction of antimicrobial that is absorbed from the digestive tract to the blood. The fraction remaining in the digestive tract and that could be available for the bacteria is equal to (1 – *F*). Gastrointestinal elimination (*GE*) is the fraction of the antimicrobial that is secreted back into the intestinal tract for elimination, after initially being absorbed into the bloodstream. NA = not available.

Due to the absence of MSC and other PK data the estimation of the FARSC for amoxicillin and penicillin V was not possible.

3.2.1. Associated data gaps and uncertainties

With regard to the uncertainties and data gaps described in the Scientific Opinion Part 1 (Sections 3.1 and 3.3; see also the Virtual Issue) we identified the following for the β-lactams under assessment:

i) MSC data: no data for MSCs are available.
ii) Bioavailability: quantitative data are not available for each species. There are no data for adult ruminants (penicillin V and amoxicillin) and for turkeys (penicillin V). For other species, the value of bioavailability was sometimes extracted from only one or two studies and the selected values can thus be inaccurate.
iii) Inactive fraction: there is no information for penicillin V. One study demonstrated an inactivation of amoxicillin in the small intestines of rats but there are no quantitative data on

intestinal degradation of amoxicillin in large intestines in target animal species. Few data are available on the effect of β-lactamases produced in the intestine.

iv) Ruminants: penicillin V and amoxicillin were described as being inactivated and degraded in the gastrointestinal tract of ruminants but no quantitative data are available

3.2.2. Concluding remarks

Due to the lack of data on the parameters required to calculate the FARSC, it is not possible to conclude the ToR1 assessment until further experimental data are available.

3.3. ToR2. Specific antimicrobials concentrations in feed which have an effect in terms of growth promotion/increased yield

3.3.1. Amoxicillin

3.3.1.1. Literature search results

The literature search, conducted according to the methodology described in Section 2.2.2.1 of the Scientific Opinion Part 1 (see also the Virtual Issue), resulted in 1,161 papers mentioning amoxicillin and any of the food-producing animal species considered[3] and any of the performance parameters identified as relevant for the assessment of the possible growth-promoting effects of amoxicillin.[4] After removing the reports not matching the eligibility criteria, 22 publications were identified.

3.3.1.2. Evaluation of the studies

The 22 publications identified in the literature search were appraised for suitability for the assessment of the effects of amoxicillin on growth or yield of food-producing animals; this appraisal was performed by checking each study against a series of pre-defined exclusion criteria (see Section 2.2.2.2.1 of the Scientific Opinion Part 1; see also the Virtual Issue).[5] A total of 19 publications were not considered suitable for the assessment because of several shortcomings identified in the design of the study or in the reporting of the results. The list of excluded publications and their shortcomings are presented in Appendix A.1 (Table A.1).

The publications considered suitable for the assessment are described and assessed in Section 3.3.1.3.

3.3.1.3. Assessment of the effects of amoxicillin on growth performance and yield

Three publications were considered suitable for the assessment of the effects of amoxicillin on growth and yield performance in food-producing animals. The effects of the administration of the antimicrobial on the endpoints described in Section 2.2.2.2.2 of the Scientific Opinion Part 1 (see also the Virtual Issue) were evaluated. The selected publications and the effects on the relevant endpoints are described below. The summary of the studies includes the description of the source of amoxicillin used either as the base or as any specific form/commercial preparation, and the concentration(s) applied as reported in each study; where a specific compound has been used, the calculation of the concentration applied to the base substance is provided.

3.3.1.3.1. Studies in poultry

In the study of Abaza et al. (2006), a total of 180-laying hens (32 weeks old) and 24 cockerels (local Egyptian strain 'Al-Salam') were divided into individual cages and allocated to six dietary

[3] Ruminants: growing and dairy (cattle, sheep, goats, buffaloes); 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, ostrich); fish: salmon, trout, other farmed fish (seabass, seabream, carp, other); crustaceans; other animal species.

[4] (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, 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. DM, Ca, P), digestibility; (vi) Health-related parameters: reduction of morbidity and/or mortality; (vii) Herd/flock related parameters; (viii) Other endpoints: e.g. intestinal morphological characteristics (*villi* height/width), changes in microbiota.

[5] The following exclusion criteria were applied: 'Combination of substances administered to the animals', 'Antimicrobial used different from the one under assessment', 'Administration via route different from oral', 'Use of the antimicrobial with a therapeutic scope', 'Animals subjected to challenges with pathogens', 'Animals in the study sick or not in good health, Zootechnical parameters not reported', 'Insufficient reporting/statistics', 'Other (indicate)'.

treatments. Two were the relevant treatments: a control and a treatment consisting of amoxicillin 0.02% (20 g amoxicillin trihydrate per 100 g of product, Adwia Co., Egypt) at a concentration of 40 mg amoxicillin trihydrate/kg feed (corresponding to 34.8 mg amoxicillin/kg feed); the basal diet was based on maize-soybean. The study lasted 11 weeks. The number and weight of eggs were recorded daily while feed intake (FI) was recorded weekly for each hen. Egg mass and feed conversion ratio (F:G) were also calculated. At the end of the experimental period, blood samples were collected from four birds from each treatment to determine protein, albumin and cholesterol. The digestibility coefficients of nutrients were calculated using four cockerels from each treatment. Semen samples were collected to determine semen-ejaculate volume, motility, sperm abnormalities. Fertility and hatchability were calculated utilising artificial insemination. The birds treated with amoxicillin showed higher egg production during the experimental period (49.21 vs 46.69 egg/hen), egg mass (29.71 vs 28.04 g/hen per day) compared to the control group and improved feed to egg mass ratio (4.26 vs 4.61), but lower FI (126.49 vs 128.85 g/day). The digestibility coefficients for dry matter (DM) (78.09% vs 81.22%), organic matter (77.73% vs 80.84%) and crude fibre (19.87% vs 23.63%) were reduced in birds receiving amoxicillin. The treated animals showed a reduction in sperm abnormalities (25.58% vs 29.28%) and dead spermatozoa (13.28% vs 17.81%) and higher sperm motility (81.07% vs 75.00%). Regarding blood analyses, treated animals showed an increase in total protein (3.86 vs 3.46 mg/100 mL) and albumin (2.52 vs 2.26 mg/100 mL). Dietary amoxicillin trihydrate at a concentration of 40 mg/kg feed (corresponding to 34.8 mg amoxicillin/kg feed) improved egg production yield in laying hens and sperm quality in cockerels, but reduced nutrient digestibility (dry matter, organic matter, crude fibre). As these results are considered contradictory for growth-promoting effects, overall, no conclusions can be drawn.

In the study of Mohammed et al. (2018), a total of 210 one-day-old male/female chickens for fattening (Ross 308) were distributed in groups of ten animals and allocated to seven dietary treatments (30 animals/treatment). Two were the relevant treatments: a control and a treatment consisting of amoxicillin (unspecified form) at a concentration of 2,000 mg/kg feed. Two-stage regime of the basal diets (starter 1–21 days and finisher 22–35 days) was used. The study lasted 35 days. Animal's weight and cumulative FI were recorded weekly and F:G calculated at the end of the experiment. The birds treated with amoxicillin showed, compared to the control group, a lower mortality (7.77% vs 11.11%). Dietary amoxicillin supplementation at 2,000 mg/kg feed did not have a growth-promoting effect in chickens for fattening.

3.3.1.3.2. Study in fish

In the study of Lee et al. (2017), a total of 360 juvenile Japanese eels (*Anguilla japonica*, 11.5 g BW) were distributed in 18 tanks in groups of 20 animals and allocated to six relevant dietary treatments. Two were the relevant treatments: a control and a treatment consisting of amoxicillin (unspecified form) at a concentration of 10,000 mg/kg feed. The study lasted 12 weeks. The total number and weight of fish in each tank were measured for the calculation of weight gain, specific growth rate (SGR), feed efficiency and survival rate. Blood samples were collected from five fish per tank to determine aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, total proteins, lysozyme and superoxide dismutase (SOD). Three fish from each tank were sacrificed to analyse whole-body proximate composition (moisture, protein, ether extract, ash). The fish treated with amoxicillin showed, compared to the control group, higher relative weight gain (122% vs 108%), SGR (1.11% vs 1.02%/day), and feed efficiency (68.7% vs 59.6%). Survival rate during the trial was higher for amoxicillin-treated eels (91.7% vs 81.7%). The treated animals showed a higher body content of protein (190 vs 163 g/kg) and ash (25.1 vs 19.6 g/kg) and an increase in SOD and lysozyme activities in blood. Dietary amoxicillin supplementation at 10,000 mg/kg feed showed growth-promoting effects in juvenile Japanese eels.

3.3.1.4. Discussion

From the studies examined, the test item has been described as (i) 'amoxicillin trihydrate' (one study) or (ii) 'amoxicillin' (unspecified form; two studies). Therefore, for the case (ii), an uncertainty on the exact product used/concentration applied has been identified.

A detailed analysis of the uncertainties for amoxicillin is included in Appendix B (Table B.1) of this document, and the Section 3.3 of the Scientific Opinion Part 1 (see also the Virtual Issue).

The three studies considered as suitable for the assessment covered two animal categories within poultry (chickens for fattening and layer hens/cockerels), and one species in aquatic animals (juvenile

Japanese eels). In the assessed studies, treatments contained groups of animals treated with only one amoxicillin concentration and did not allow dose-related effects to be assessed.

3.3.1.4.1. Poultry

In the two studies assessed in poultry, amoxicillin was supplemented with the diets of chickens for fattening (Mohammed et al., 2018) and layer hens-cockerels (Abaza et al., 2006). The dietary addition of amoxicillin at 2,000 mg/kg feed did not have a growth-promoting effect in chickens for fattening (Mohammed et al., 2018). The study of Abaza et al. (2006) in layer hens and cockerels did not allow to reach a conclusion.

3.3.1.4.2. Fish

The study in juvenile Japanese eels (Lee et al., 2017) showed that dietary supplements with amoxicillin at 10,000 mg/kg feed had a growth-promoting effect.

3.3.1.5. Concluding remarks

It is judged 33–66% certain ('about as likely as not') that amoxicillin has growth-promoting/increase yield effects in juvenile Japanese eels at a concentration of 10,000 mg/kg complete feed (one study).

No data are available in the scientific literature showing effects of amoxicillin on growth promotion/increased yield when added (i) to juvenile Japanese eels' feed at concentrations below 10,000 mg/kg or (ii) to feed of any other food-producing animal species or categories.

3.3.2. Penicillin V

3.3.2.1. Literature search results

The literature search, conducted according to the methodology described in Section 2.2.2.1 of the Scientific Opinion Part 1 (see also the Virtual Issue), resulted in 1,619 papers mentioning penicillin plus any of the food-producing animal species considered,[3] and any of the performance parameters identified as relevant for the assessment of the possible growth promoting effects of penicillin.[4,6] After removing the reports not matching the eligibility criteria, 34 publications were identified.

3.3.2.2. Evaluation of the studies

The 34 publications identified in the literature search were appraised for suitability for the assessment of the effects of penicillin on growth or yield of food-producing animals; this appraisal was performed by checking each study against a series of pre-defined exclusion criteria (see Section 2.2.2.2.1 of the Scientific Opinion Part 1; see also the Virtual Issue).[5] A total of 30 publications were not considered suitable for the assessment because of several shortcomings identified in their designs or in the reporting of the results. The other four publications were assessed but not further considered because it was not stated unambiguously if the antimicrobial used was 'Penicillin V' – as requested by the mandate – or any other form of penicillin. The list of excluded publications and their shortcomings are presented in Appendix A.2 (Table A.2).

3.3.2.3. Concluding remarks

Owing to the lack of suitable data, levels of penicillin V in feed which may have a growth promotion/production yield effect in any food-producing animal species could not be identified.

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 concentrations of amoxicillin and penicillin V in non-target feed below which there would not be emergence of, and/or selection for, resistance in the large intestines/rumen?

[6] It was assumed that, since only oral administration of the antimicrobial had been applied, only Penicillin V had been used as test item in the studies examined.

- Due to the lack of data on the parameters required to calculate the Feed Antimicrobial Resistance Selection Concentration (FARSC) corresponding to the concentrations of those antimicrobials in non-target feed below which there would not be expected to be an effect on the emergence of, and/or selection for, resistance in microbial agents relevant for human and animal health, it is not possible to conclude until further experimental data are available

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

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

With regard to amoxicillin:

- It is judged 33–66% certain ('about as likely as not') that amoxicillin has growth-promoting/increased yield effects in juvenile Japanese eels at a concentration of 10,000 mg/kg complete feed (one study).
- No data are available in the scientific literature showing the effect of amoxicillin on growth promotion/increased yield when added (i) to juvenile Japanese eels' feed at concentrations below 10,000 mg/kg or (ii) to feed of any other food-producing animal species or categories.

With regard to penicillin V:

- Owing to the lack of suitable data, levels of penicillin V in feed which may have a growth promotion/production yield effect in any food-producing animal species could not be identified.

The results from these assessments for the different animal species are summarised in Annex F (Tables F.1 and F.2) of EFSA BIOHAZ Panel, 2021a - Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (see also the Virtual Issue).

5. Recommendation

To carry out studies to generate the data that are required to fill the gaps which have prevented calculation of the FARSC for amoxicillin and penicillin V.

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Abbreviations

ALT	alanine aminotransferase
AQ	Assessment question
AST	aspartate aminotransferase
bw	body weight in toxicity studies
BW	body weight
DM	dry matter
EUCAST	European Committee on Antimicrobial Susceptibility testing
F:G	feed conversion ratio or feed to gain ratio
F	fraction of the antimicrobial that is absorbed from the digestive tract to the blood
FARSC	Feed Antimicrobial Resistance Selection Concentration
FI	feed intake
GE	fraction of the antimicrobial that is secreted back into the intestinal tract for elimination, after initially being absorbed into the bloodstream
I	fraction of the antimicrobial present in the digestive tracts that would be inactive on the microbiota

MIC	minimum inhibitory concentration
MIC_{lowest}	minimum 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}	minimum inhibitory concentration of the resistant strain
MIC_{susc}	minimum inhibitory concentration of the susceptible strain
MIC_{test}	minimum inhibitory concentration of the susceptible isolate used in the competition experiments to calculate the MSC
MSC	minimal selective concentration
NAM	*N*-acetylmuramic acid
NAG	*N*-acetylglucosamine
PBPs	penicillin-binding proteins
PK	pharmacokinetic(s)
PMSC	predicted MSC
SGR	specific growth rate
SOD	superoxide dismutase
ToRs	Terms of Reference

Appendix A – List of excluded publications and their shortcomings

A.1. Amoxicillin

The publications excluded from the assessment of the effects of amoxicillin on growth promotion/increased yield following the criteria defined in Section 2.2.2.2.1 of the Scientific Opinion Part 1 (see also the Virtual Issue) are summarised in Table A.1.

Table A.1: Publications not relevant for the assessment of the effects of amoxicillin on growth promotion/increased yield and excluding criteria

Author (year)	Species	Excluding criteria								
		Combination of substances administered to the animals	Antimicrobial used different from the one under assessment	Administration via route different from oral	Use of the antimicrobial with a therapeutic scope	Animals subjected to challenges with pathogens	Animals in the study sick or not in good health	Zootechnical parameters not reported	Insufficient reporting/ statistics	Other (indicate)
Banerjee et al. (2018)	Poultry	X						X		
Bosi et al. (2011)	Pigs								X	
Candotti and Cossettini (2010)	Pigs	X								X(1)
da Costa et al. (2011)	Poultry	X			X					
Darwish and Hobbs (2005)	Fish					X		X(2)		
Eid et al. (2020)	Poultry					X				
Jamin et al. (2012)	Pigs									X(3),(4)
Khatun et al. (2017)	Poultry									X(5)
Koutoulis et al. (2015)	Poultry				X		X			X(1)
Marien et al. (2006)	Poultry					X		X		

Author (year)	Species	Excluding criteria								
		Combination of substances administered to the animals	Antimicrobial used different from the one under assessment	Administration via route different from oral	Use of the antimicrobial with a therapeutic scope	Animals subjected to challenges with pathogens	Animals in the study sick or not in good health	Zootechnical parameters not reported	Insufficient reporting/ statistics	Other (indicate)
Marien et al. (2007)	Poultry					X		X		
Oliveira et al. (2018)	Pigs	X							X	
Piva et al. (2007)	Pigs	X							X	X(1)
Roth et al. (2019)	Poultry	X								
Schokker et al. (2017)	Poultry								X	
Soler et al. (2018)	Pig	X								
Thymann et al. (2007)	Pig	X		X(6)			X			
Verner-jeffreys et al. (2004)	Fish	X								
Wisselink et al. (2017)	Poultry								X	

(1): No negative control.
(2): Only survival rate assessed.
(3): Small number of animals (6 per treatment at the end of the trial) to get information on growth performance.
(4): Suckling piglets used as animal models for human infants. The study did not aim at exploring the effect of amoxicillin in animal feeding/nutrition.
(5): No replicates.
(6): The combined treatment was given both in feed and as intramuscular injection.

A.2. Penicillin V

The publications excluded from the assessment of the effects of penicillin V on growth/production yield following the criteria defined in Section 2.2.2.2.1 of the Scientific Opinion Part 1 (see also the Virtual Issue) are summarised in Table A.2.

Table A.2: Publications not relevant for the assessment of the effects of penicillin V on growth promotion/production yield and excluding criteria

Author (year)	Species	Excluding criteria								
		Combination of substances administered to the animals	Antimicrobial used different from the one under assessment	Administration via route different from oral	Use of the antimicrobial with a therapeutic scope	Animals subjected to challenges with pathogens	Animals in the study sick or not in good health	Zootechnical parameters not reported	Insufficient reporting/ statistics	Other (indicate)
Antoniou and Marquardt (1982)	Poultry									X(1)
Blake and Hess (2013)	Poultry									X(1)
Bowen and Sullivan (1971)	Poultry	X								
Bridges et al. (1954)	Pig								X(2)	
Burnell et al. (1988)	Pig	X								
Cho et al. (2006)	Pig	X							X	
Cornelison et al. (2006)	Poultry									X(3)
Creech and Couch (1957)	Poultry								X(4)	
Harper et al. (1983)	Pig	X			X					
Hathaway et al. (1996)	Pig	X								
Hathaway et al. (1999)	Pig	X							X	

Author (year)	Species	Excluding criteria								
		Combination of substances administered to the animals	Antimicrobial used different from the one under assessment	Administration via route different from oral	Use of the antimicrobial with a therapeutic scope	Animals subjected to challenges with pathogens	Animals in the study sick or not in good health	Zootechnical parameters not reported	Insufficient reporting/ statistics	Other (indicate)
Hathaway et al. (2003)	Pig	X								
Holme and Robinson (1965)	Pig								X(5)	
Hu and McDougald (2002)	Poultry				X	X				
Ilori (1984)	Pig	X							X(5)	
Jiraphocakul et al. (1990)	Poultry	X								
Jukes and Jukes (1973)	Several		X(6)							
Karimi et al. (2010)	Poultry									X(3)
King (1966)	Rabbit							X(7)		
Li et al. (2019)	Ruminant	X								
NCR-89 (1984)	Pig	X			X					
Nyachoti et al. (2012)	Pig	X			X	X			X	
Oplinger et al. (2015)	Fish	X		X(8)	X					
Pimentel and Cook (1988)	Poultry									X(1)
Powley et al. (1981)	Pig	X								
Radecki et al. (1988)	Pig	X								
Rollins et al. (1976)	Pig	X							X	

Author (year)	Species	Excluding criteria								
		Combination of substances administered to the animals	Antimicrobial used different from the one under assessment	Administration via route different from oral	Use of the antimicrobial with a therapeutic scope	Animals subjected to challenges with pathogens	Animals in the study sick or not in good health	Zootechnical parameters not reported	Insufficient reporting/ statistics	Other (indicate)
Roura et al. (1992)	Poultry	X					X			
Stutz and Lawton (1984)	Poultry									X(1)
Swinkels et al. (1988)	Pig	X								
Thaler et al. (1989)	Pig	X								X(9)
Unno et al. (2015)	Pig	X								X(9)
Veum et al. (1980)	Pig	X								
Wallgren et al. (1999)	Pig				X	X				

(1): The product tested is not unambiguously described as 'Penicillin V'.
(2): Old study (1954). Small number of animals tested (7/group), no description of statistical methods, insufficient reporting.
(3): The study was conducted with Penicillin G.
(4): Old study (1957). No mention of statistical methods or of statistical significance of findings.
(5): While p values are given, there is no mention of the statistical method used.
(6): An old (1973), albeit well-done for its time, review on toxicological and allergenic hazards to humans and animals related to low levels of antimicrobials. The focus is given to aureobomycin and tetracyclines rather than to penicillin.
(7): Old study (1966) mainly focused on changes in organ weight; the body weight was recorded each 15 days, but no record of feed intake was taken.
(8): Exposure of salmon eggs through water.
(9): The study focused on sequencing the microbiota.

Appendix B – Table of uncertainties

Uncertainties associated to the Growth promotion assessment

Table B.1: Potential sources of uncertainty identified in the levels of amoxicillin in feed which have growth promotion/increase yield effect and assessment of the impact that these uncertainties could have on the conclusion

Source of the uncertainty	Nature or cause of uncertainty	Impact of the uncertainty on the conclusion on the level(s) which have growth promotion/increase yield effect
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. In summarising the results, the concentrations have been reported as for 'base' substance when the form of the antimicrobial is not specified (conservative assumption).	Underestimation of the concentration which may have shown growth-promoting effect.
Evidence synthesis and integration	As described in Section 2.2.3 of the Scientific Opinion Part 1 (see also the Virtual Issue), the low number of studies retrieved prevented evidence synthesis.	Underestimation/Overestimation