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Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 13: *Diaminopyrimidines: trimethoprim*

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Abstract

The specific concentrations of trimethoprim 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. The FARSC for trimethoprim was estimated. Uncertainties and data gaps associated to the levels reported were addressed. To address growth promotion, data from scientific publications obtained from an extensive literature review were used. No suitable data for the assessment were available. It was recommended to perform further studies to supply more diverse and complete data related to the requirements for calculation of the FARSC for trimethoprim.

Keywords: Trimethoprim, antimicrobial resistance, sub-inhibitory concentration, Feed Antimicrobial Resistance Selection Concentration (FARSC), growth promotion, yield increase, food-producing animals

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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, 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 trimethoprim.

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

Trimethoprim is a folate pathway antagonist discovered in 1956 (Anderson et al., 2012). It is a synthetic antimicrobial that can be produced through various routes (Anderson et al., 2012). Trimethoprim enters bacterial cells by passive diffusion due to its sufficiently small size and lipophilic properties. Once inside the bacterial cell, it inhibits dihydrofolate reductase (DHFR), which reduces dihydrofolate to tetrahydrofolate (O'Grady, 1975). This blocks the synthesis of folate which is an essential co-factor in the biosynthesis of thymidine and thus in DNA synthesis (Anderson et al., 2012). As bacteria cannot take up folate from the environment, disruption of this metabolic pathway results in inhibition of bacterial growth. Over the years, trimethoprim has been mainly administered in combination with sulfamethoxazole and many other sulphonamides in veterinary medicine as the main hypothesis was that sulphonamides potentiate the action of trimethoprim (Minato et al., 2018). More recently, it has been determined that: (i) trimethoprim-sulfamethoxazole synergy is driven by mutual potentiation of the action of each drug by the other (Minato et al., 2018) and (ii) although there are several clinical indications supporting administration of trimethoprim alone, this practice is unusual in veterinary medicine, especially for feed administration, and is nearly exclusively applied in human medicine in selected geographical areas (Anderson et al., 2012; Giguère et al., 2013).

[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.

1.3.2. Main use[2]

Trimethoprim belongs to the class of diaminopyrimidines and is commonly used in combination with sulfonamides in veterinary medicine in Europe (EMEA/CVMP, 1997). The combinations trimethoprim-sulfonamides are used in most animal species. The spectrum is broad, including Gram-negative and Gram-positive bacteria (including Enterobacterales, *Pasteurella* spp., *Staphylococcus* spp., *Streptococcus pneumoniae*) and many protozoans (e.g. *Histoplasma*, *Toxoplasma* and coccidia) for both constituents, so many bacterial respiratory infections, urinary tract and soft tissues infections and protozoan intestinal infections can be treated by this antimicrobial. The main indications for trimethoprim in combination with sulfonamides in food-producing animals are for the curative and metaphylactic treatment of respiratory (e.g. *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Streptococcus* spp. and *Haemophilus parasuis)* and gastrointestinal (e.g. *Escherichia coli*) infections in cattle, swine and poultry. Other treated infections include atrophic rhinitis when associated with *Bordetella bronchiseptica,* streptococcal meningitis caused by *Streptococcus suis* and coryza caused by *Avibacterium paragallinarum.*

1.3.3. Main pharmacokinetic data

Trimethoprim, by the oral route, has a bioavailability of 67% in horses (Van Duijkeren et al., 1996) higher than 90% in pigs (Nielsen and Gyrd-Hansen, 1994), 80% in broilers (Baert et al., 2003) and 100% in Atlantic salmon at 10°C (Horsberg et al., 1997). No value for the bioavailability of trimethoprim by oral route is available for rabbits. In accordance with this high bioavailability in monogastric species, a study of Peeters et al. (2016) showed that the administration of 2.5 mg of trimethoprim per kg feed to pigs led to intestinal and faecal concentrations lower than the limit of quantification (LOQ) of 0.016 mg/kg (Peeters et al., 2016). In another study from the same group, the administration of a far higher dose of 2.5 mg/kg body weight (bw) to pigs (standard dose) by oral route also led to concentrations of trimethoprim below the LOQ of 0.025 mg/kg in the rectum and faeces. The trimethoprim concentrations decreased from proximal to distal intestines with the maximum concentrations of around 1 mg/kg in the jejunum and concentrations between the LOQ and 0.5 mg/kg in ileum and caecum (De Smet et al., 2017). These data also suggested a low level of secretion through the epithelium (De Smet et al., 2017).

In calves, the absorption of trimethoprim decreased with age. In an old study using drug quantification by microbiological assay, trimethoprim was not detectable in serum (concentrations lower than 0.1 μg/mL) after oral administration of a combination of trimethoprim and sulfadiazine to 6 or 12 week-old calves fed with grain-fibre-based feed (Shoaf et al., 1987). For ruminants, it was also shown that trimethoprim is extensively degraded by ruminal microbiota (partly explaining the very low bioavailability in these animals despite the high lipophilicity of the antimicrobial) (Nielsen and Dalgaard, 1978; Shoaf et al., 1987).

Hepatic metabolism appears to be the main elimination pathway in pigs (Friis et al., 1984; Nouws et al., 1991; Giguère et al., 2013) and cows (Giguère et al., 2013).

Van Duijkeren et al. (1996) estimated that trimethoprim at concentrations lower than 10 μg/mL was, *in vitro*, bound at 69–92% (mean: 83%) to hay and at 52–70% (mean: 65%) to pelleted feed for horses. In caecal contents of horses, the trimethoprim was bound at 26–73% with a mean of 55%. No data were found for other species.

1.3.4. Main resistance mechanisms

Resistance to trimethoprim in bacteria can be mediated by different mechanisms including alteration, overproduction or replacement of the target and impaired cell permeability (Eliopoulos and Huovinen, 2001). Trimethoprim resistance is widespread in Gram-positive and Gram-negative bacteria. Replacement of the target by acquisition of exogenous genes encoding trimethoprim-resistant DHFR is the most common mechanism observed in Gram-negatives. More than 100 genes/gene variants have been described to date, generally in associations with various transposons and plasmids. This resistance mechanism also occurs in Gram-positive bacteria, but at a lower frequency compared to chromosomal mutations leading to amino acid changes and thus alteration of DHFR (Anderson et al., 2012). Notably,

[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.

several anaerobic bacteria including species of the gut microbiome are intrinsically resistant to trimethoprim, mainly due to insensitive or absent DHFR (Huovinen, 1987) and in *Campylobacter jejuni* absence of *folA,* coding for dihydrofolate reductase, so they do not offer any target for the antifolate, conferring intrinsic resistance to this antimicrobial (Myllykallio et al., 2003).

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-minimum 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 horizontal gene transfer (HGT), etc. for the antimicrobials under assessment are shown below.

3.1.1. Resistance development/spread due to sub-MIC concentrations of trimethoprim: examples

Trimethoprim has been used as an antimicrobial in human and veterinary medicine since the late 1960s, with rapid and extensive spread of resistance genes and/or resistant bacteria from diverse environments.

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

- Several publications report a correlation between trimethoprim use and the emergence of resistance in humans, with earlier studies after trimethoprim initial use demonstrating a clear increase in trimethoprim-resistant *E. coli* and *Shigella* spp. (Huovinen et al., 1995). The complexity of factors accounting for the success of an acquired antimicrobial resistance complicates the current extraction of evidences for different bacterial groups.
- Minimal selective concentration (MSC) has been determined for trimethoprim in one study using competitions between susceptible and trimethoprim resistant (due to the presence of a *dfr* gene) (Gullberg et al., 2014). MSC was 0.002 mg/L and wild type had a MIC of trimethoprim of 0.2 mg/L, showing that the MSC was 100-fold lower than the MIC.
- Sulfonamide + trimethoprim administration via liquid feeding pipelines for pigs selected for sulfonamide + trimethoprim-resistant bacteria in the biofilm lining the pipelines. This may cause contamination of liquid feed for extended periods and represent a source of resistance genes for the gastrointestinal microbiota of the pigs (Heller et al., 2017).
- No statistically significant correlation between trimethoprim usage and resistance was reported in a large European study involving multiple food animal species (Ceccarelli et al., 2020).
- Sub-inhibitory concentrations of trimethoprim increased mutation frequency in *Streptococcus pneumoniae* (*dinB*-DNA polymerase IV gene- positive isolates only) (Henderson-Begg et al., 2006).

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

- Data on concentrations of trimethoprim having secondary effects on, e.g. HGT frequencies or induction of virulence are very limited. One study (Jutkina et al., 2018) found that trimethoprim had no effect on HGT rates at sub-MIC concentrations (up to 0.25 mg/L). Another study found that trimethoprim at 20 mg/L increased the expression levels of genes involved in the conjugative transfer of a trimethoprim, sulphonamide and tetracycline resistance plasmid from *Aeromonas hydrophila*, which is expected to result in increased conjugation frequency (Cantas et al., 2012).

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 trimethoprim for different animal species followed a two-step approach as described below.

The first step was the calculation of the predicted minimal selective concentration (PMSC) for tetracycline as indicated in Table 1.

The minimal selective concentration (MSC) has been determined for trimethoprim in one study (Gullberg et al., 2014) and was 0.002 mg/L (wild type MIC 0.2 mg/L). Accordingly, the ratio MIC_{test}/MSC_{test} was 100 (Table 1).

The PMSC for trimethoprim, calculated using the lowest MIC value available in the EUCAST MIC distribution database (MIC_{lowest}) as described in Bengtsson-Palme and Larsson (2016), multiplied by the MIC_{test}/MSC_{test} factor (as described in Section 2.2.3.2 of the Scientific Opinion Part 1), was 0.00016 mg/L (Table 1).

Table 1: Calculation of the trimethoprim predicted minimal selective concentration (PMSC)

All values in mg/L	MIC_{test} values	MSC_{test} values	MIC_{test}/MSC_{test} ratios	MIC_{lowest}	Predicted MSC (PMSC) for most susceptible species ($MIC_{lowest}/MIC_{test}/MSC_{test}$)
Trimethoprim	0.2 (*E. coli*)	0.002 (*E. coli*)	100	0.016	0.00016

MIC: minimum inhibitory concentration. MSC: minimal selective concentration. MSC_{test}: MSC experimentally determined. MIC_{lowest}: lowest MIC data for trimethoprim 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.

From the PMSC for trimethoprim, the FARSC ($FARSC_{intestine}$ and $FARSC_{rumen}$) corresponding to the maximal concentrations in feed was 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 trimethoprim.

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

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

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.

From the study of Van Duijkeren et al. (1996) in horses, I was set to 0.45 for monogastric animals. A worse scenario was also simulated with I equal to 0.3. For ruminants, due to extensive degradation by ruminal microbiota (Nielsen and Dalgaard, 1978; Shoaf et al., 1987), I was set to 0.8. Due to the lack of quantitative data, other simulations were performed with I equal to 0.45 and 0.9.

According to the literature, the bioavailability (F) of trimethoprim was set to 0.9 for pigs (Nielsen and Gyrd-Hansen, 1994), 0.8 for broilers (Baert et al., 2003) and 0.7 for horses (Van Duijkeren et al., 1996). From the low secretion through the epithelium demonstrated by De Smet et al. (2017), *GE* was set to 0.1. Since no quantitative data were reported in the previous study, other simulations were performed with GE equal to 0 and 0.2 for monogastric animals.

The bioavailability was described as very low for ruminants after weaning. The potential sources for this low bioavailability such as the absence of absorption or extensive hepatic first-pass effect were not further investigated, and no quantitative data are available. Thus, the bioavailability of trimethoprim was set to 0 for ruminants (sheep, goats and cattle). A slightly higher value of 0.1 was also used for the calculations.

The different values of the parameters used for the calculations are summarised in Table 2 and the estimated FARSC values are reported in Table 3. There is no value for the bioavailability in veal calves

and rabbits. The first set of values (scenario 1) corresponds to the average of published values while scenario 2 corresponds to scenario that would lead to lower FARSC and scenario 3 to scenario that would lead to higher FARSC. The lowest FARSC (scenario 2) were obtained from lowest published values of *I* (lower inactivation of the drug resulting in higher activity on bacteria), lowest published values of *F* (lower absorption resulting in more drug in the intestines) and highest values of *GE* (higher elimination in intestines resulting in more drug in the intestines).

Table 2: Predicted minimal selective concentration (PMSC) and pharmacokinetic (PK) values used for the calculation of Feed Antimicrobial Resistance Selection Concentration (FARSC) of trimethoprim (TMP) for the different animal species

Trimethoprim data	Scenario #1	Scenario #2	Scenario #3
PMSC (mg/L)		**0.00016**	
Inactive fraction (*I*)	0.45	0.3	0.45
Inactive fraction (adult ruminant)	0.8	0.45	0.9
Bioavailability (*F*) (pig)	0.9	0.8	0.9
Bioavailability (*F*) (ruminants)	0	0	0.1
Bioavailability (*F*) (broiler)	0.8	0.7	0.8
Bioavailability (*F*) (horse)	0.7	0.5	0.8
Bioavailability (*F*) (salmon)	0.99	0.9	0.99
Gastrointestinal elimination (*GE*) (Pig/broiler/horse/salmon)	0.1	0.2	0

PMSC: Predicted minimal selective concentration (PMSC). 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. 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. The fraction remaining in the digestive tract and that could be available for the bacteria is equal to $(1 - F + F \times GE)$, thus $(1 - F)$ in Scenario 3.

Table 3: The Feed Antimicrobial Resistance Selection Concentration of trimethoprim corresponding to the maximum concentration of trimethoprim residues in non-target feed that would not develop resistance in the large intestine bacteria ($FARSC_{intestine}$)

Animal category[(a)]	Body weight (kg)[(a)]	Daily Feed Intake (kg DM/ animal per day)[(a)]	Daily output of fresh faeces (kg FM/animal per day)[(b)]	FARSC ($\times 10^{-3}$ mg drug/kg feed) Scenario 1	FARSC ($\times 10^{-3}$ mg drug/kg feed) Scenario 2	FARSC ($\times 10^{-3}$ mg drug/kg feed) Scenario 3
Sow lactating	175	5.28	7.7	2.23	0.93	4.24
Piglet	20	0.88	0.88	1.53	0.63	2.91
Pig for fattening	60	2.2	2.64	1.84	0.76	3.49
Dairy cows	650	20	55.71	11.73	2.25	**44.57**
Veal calf (milk replacer)	100	1.89	2.36	–	–	–
Cattle for fattening	400	8	18.89	9.94	1.91	37.78
Goat	60	1.2	1.73	6.07	1.16	23.07
Sheep	60	1.2	1.47	5.16	0.99	19.60
Chicken for fattening	2	0.158	0.133	0.87	0.44	1.22
Laying hen	2	0.106	0.16	1.57	0.78	4.39
Turkey for fattening	3	0.176	0.109	0.64	**0.32**	0.90
Horse	400	8	8.33	0.82	0.40	1.51
Rabbit	2	0.1	0.053	–	–	–
Salmon	0.12	0.0021	0.00238	3.02	0.93	32.97

DM: dry matter: FM: fresh matter; FARSC: Feed Antimicrobial Resistance Selection Concentration.
(a): EFSA FEEDAP Panel (2017), as indicated in Section 2.1.1.3 of the Scientific Opinion Part 1.
(b): Estimated data, obtained as indicated in Section 2.1.1.3.1 of the Scientific Opinion Part 1.

The values of $FARSC_{intestine}$, for the species with available data, ranged in the first scenario using averaged published values from 0.64 × 10^{-3} mg/kg feed in turkeys for fattening to 11.73 × 10^{-3} mg/kg feed in dairy cows. From other simulations (scenario 2 and scenario 3) made with a wider range of values for the data used in the calculation, $FARSC_{intestine}$ would range from 0.32 to 0.90 × 10^{-3} mg/kg feed in turkeys for fattening, from 0.93 to 32.97 × 10^{-3} mg/kg feed in salmon and from 2.25 to 44.57 × 10^{-3} mg/kg feed in dairy cows. In general, for the different species, the $FARSC_{intestine}$ for trimethoprim ranged from 0.32 to 44.57 × 10^{-3} mg/kg feed.

For the estimation of Feed Antimicrobial Resistance Selection Concentration of trimethoprim in rumen ($FARSC_{rumen}$), I was also set to 0.8 due to extensive degradation by ruminal microbiota (Nielsen and Dalgaard, 1978; Shoaf et al., 1987). However, due to the lack of quantitative data, other simulations were performed with I equal to 0 and 0.9.

The different values of the parameters used for the calculations are summarised in Table 4 and the estimated $FARSC_{rumen}$ values are reported in Table 5.

Table 4: Predicted minimal selective concentration (PMSC) and values for inactive fraction used for the calculation of Feed antimicrobial resistance selection concentration in rumen ($FARSC_{rumen}$) of trimethoprim (TMP) for the different animal species

Trimethoprim	Scenario #1	Scenario #2	Scenario #3
PMSC (mg/L)		**0.00016**	
Inactive fraction (I) ruminant	0.8	0	0.9

PMSC: Predicted minimal selective concentration (PMSC). Inactive fraction (I) is the fraction of antimicrobial that would not have any activity on bacteria.

Table 5: The Feed Antimicrobial Resistance Selection Concentration ($FARSC_{rumen}$) of trimethoprim (TMP) corresponding to the maximum concentration of TMP residues in non-target feed that would not develop resistance in the rumen bacteria

Animal category[(a)]	Body weight (kg)[(a)]	Daily Feed Intake (kg DM/ animal per day)[(a)]	Volume of rumen content (L)[(b)]	FARSC (× 10^{-3} mg drug/kg feed) Scenario 1	FARSC (× 10^{-3} mg drug/kg feed) Scenario 2	FARSC (× 10^{-3} mg drug/kg feed) Scenario 3
Dairy cows	650	20	90–180	3.60–7.20	0.72–1.44	**7.20**–14.40
Cattle for fattening	400	8	60–120	6.00–12.00	1.20–2.40	12.00–24.00
Sheep/Goat	60	1.2	9–18	6.00–12.00	1.20–2.40	12.00–**24.00**

DM: dry matter; FARSC: Feed Antimicrobial Resistance Selection Concentration.
(a): EFSA FEEDAP Panel (2017), as indicated in Section 2.1.1.3 of the Scientific Opinion Part 1.
(b): Source of data indicated in Section 2.1.1.3 of the Scientific Opinion Part 1.

The values of $FARSC_{rumen}$ ranged from 3.6 to 12 × 10^{-3} mg/kg feed with the first scenario. By considering that the trimethoprim is totally active in the rumen (I = 0), the values of $FARSC_{rumen}$ ranged from 0.72 to 2.4 × 10^{-3} mg/kg feed whereas inactivation of 90% of the trimethoprim would led to values of $FARSC_{rumen}$ from 7.2 to 24 × 10^{-3} mg/kg feed. In general, for the different species, the $FARSC_{rumen}$ for trimethoprim ranged from 1.20 to 24.00 × 10^{-3} mg/kg feed.

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 trimethoprim to perform the assessment:

i) MSC data: MSC is available only for *E. coli* (Gullberg et al., 2014).
ii) Impact of complexity on determined MSC: only available in single species experiment (Gullberg et al., 2014).
iii) Inactive fraction: for monogastric species, the data only come from one study conducted in horses. For ruminants, the degradation was described as extensive. However, this observation is only derived from two cows studied in 1978.

iv) Bioavailability: very few publications per species were available. No values were available for rabbits and for veal calves.
v) Intestinal secretion: the results of one study suggest that there is a low intestinal secretion of trimethoprim, but no quantitative data are available for the value of *GE*.

A detailed analysis of the associated uncertainties for trimethoprim is included in Appendix A (Table A.1) of the current, and the Section 3.3 of the Scientific Opinion Part 1.

3.2.2. Concluding remarks

The FARSC for trimethoprim (for large intestine and/or rumen in the case of adult ruminants after weaning) ranges, for the different species, from 0.32 to 44.57 $\times$ 10^{-3} mg/kg feed. No FARSC was determined for veal calves and rabbits.

–[0.93–4.24] $\times$ 10^{-3} mg/kg feed for lactating sows
–[0.63–2.91] $\times$ 10^{-3} mg/kg feed for piglets
–[0.76–3.49] $\times$ 10^{-3} mg/kg feed for pigs for fattening
–[0.72–44.57] $\times$ 10^{-3} mg/kg feed for dairy cows ($FARSC_{intestine}$ and $FARSC_{rumen}$)
–[1.2–37.78] $\times$ 10^{-3} mg/kg feed for cattle for fattening ($FARSC_{intestine}$ and $FARSC_{rumen}$)
–[1.16–24.00] $\times$ 10^{-3} mg/kg feed for adult goats ($FARSC_{intestine}$ and $FARSC_{rumen}$)
–[0.99–24.00] $\times$ 10^{-3} mg/kg feed for adult sheep ($FARSC_{intestine}$ and $FARSC_{rumen}$)
–[0.44–1.22] $\times$ 10^{-3} mg/kg feed for chicken for fattening
–[0.78–4.39] $\times$ 10^{-3} mg/kg feed for laying hens
–[0.32–0.90] $\times$ 10^{-3} mg/kg feed for turkeys for fattening
–[0.40–1.51] $\times$ 10^{-3} mg/kg feed for horses
–[0.93–32.97] $\times$ 10^{-3} mg/kg feed for salmon

The probability that trimethoprim concentrations below the lowest FARSC value for an animal species 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).

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

3.3.1. Trimethoprim

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: Methodology, general data gaps and uncertainties' (see also the Virtual Issue), resulted in 1598 papers mentioning trimethoprim 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 trimethoprim.[4] After removing the reports not matching the eligibility criteria, 39 publications were identified.

3.3.1.2. Evaluation of the studies

The 39 publications identified in the literature search were appraised for suitability for the assessment of the effects of trimethoprim 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

[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.

Section 2.2.2.2.1 of the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties'; see also the Virtual Issue).[5] None of the publications was considered suitable for the assessment because of several shortcomings identified in their designs or in the reporting of the results. The list of excluded publications and their shortcomings are presented in Appendix A (Table A.1).

3.3.1.3. Concluding remarks

Owing to the lack of suitable data, levels of trimethoprim 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 trimethoprim in non-target feed below which there would not be emergence of, and/or selection for, resistance in the large intestines/rumen?

- The Feed Antimicrobial Resistance Selection Concentration (FARSC, for large intestine and/or rumen in the case of adult ruminants after weaning) corresponding to the concentrations of trimethoprim in 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 ranges, for the different species, from 0.32 to 44.57 $\times$ 10^{-3} mg/kg feed. No FARSC was determined for veal calves and rabbits.
- For each animal species, the FARSC obtained ranged:
  - [0.93–4.24] $\times$ 10^{-3} mg/kg feed for lactating sows
  - [0.63-2.91] $\times$ 10^{-3} mg/kg feed for piglets
  - [0.76–3.49] $\times$ 10^{-3} mg/kg feed for pigs for fattening
  - [0.72–44.57] $\times$ 10^{-3} mg/kg feed for dairy cows ($FARSC_{intestine}$ and $FARSC_{rumen}$)
  - [1.2–37.78] $\times$ 10^{-3} mg/kg feed for cattle for fattening ($FARSC_{intestine}$ and $FARSC_{rumen}$)
  - [1.16–24.00] $\times$ 10^{-3} mg/kg feed for adult goats ($FARSC_{intestine}$ and $FARSC_{rumen}$)
  - [0.99–24.00] $\times$ 10^{-3} mg/kg feed for adult sheep ($FARSC_{intestine}$ and $FARSC_{rumen}$)
  - [0.44–1.22] $\times$ 10^{-3} mg/kg feed for chicken for fattening
  - [0.78–4.39] $\times$ 10^{-3} mg/kg feed for laying hens
  - [0.32–0.90] $\times$ 10^{-3} mg/kg feed for turkeys for fattening
  - [0.40–1.51] $\times$ 10^{-3} mg/kg feed for horses
  - [0.93–32.97] $\times$ 10^{-3} mg/kg feed for salmon
- The probability that concentrations of trimethoprim below the lowest FARSC value for an animal species 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).

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

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

- Owing to the lack of suitable data, levels of trimethoprim 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] 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)'.

5. Recommendations

To perform further studies to supply more diverse and complete data to reduce uncertainties around the calculation of the FARSC for trimethoprim.

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Abbreviations

AQ	assessment question
bw	body weight in toxicity studies
DM	dry matter
DHFR	dihydrofolate reductase
EUCAST	European Committee on Antimicrobial Susceptibility testing
F	fraction of the antimicrobial that is absorbed from the digestive tract to the blood
FARSC	Feed Antimicrobial Resistance Selection Concentration
FM	fresh matter
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
LOQ	limit of quantitation
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
PK	pharmacokinetic(s)
PMSC	predicted MSC
TMP	trimethoprim
ToRs	terms of reference

Appendix A – Uncertainty analysis for trimethoprim

The uncertainty analysis specific for trimethoprim with regards to the FARSC calculation is presented below.

Table A.1: Potential sources of uncertainty identified in the estimation of the maximum concentrations of trimethoprim in 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 conclusion

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)
Estimation of the maximum concentrations of antimicrobials in feed that would not select for antimicrobial resistance in the rumen and large intestines		
Estimation of PMSC data	Limited MSC data from competition experiments. MSC data is available for *E. coli* only	This limitation was overcome by the PMSC approach. Nevertheless, this could lead to an overestimation of FARSC if a bacterium with a lower MIC is described.
	Impact of bacterial community complexity on the MSCs values. It is a reasonable assumption to consider that MSCs are similar if the different antimicrobials within a class share an identical mechanism of action and resistance. There is insufficient data to assess the likely impact of complex bacterial communities on resistance selection in a single targeted member of the community, or in any other bacterium that may be present.	If this assumption is not correct, the PMSC, and accordingly the FARSC, could either be over or underestimated, depending on the specific species and the targeted community.
Antimicrobial pharmacokinetic and degradation data	The percentage of active drug in large intestines was extracted from horses data and applied to other monogastric species	The percentage of inactive drug can be higher or lower depending on the digestive content leading to potential over or underestimation of FARSC. So, other simulations were made with other values for binding to determine the range of FARSC that could be obtained.
	The average values for bioavailability were extracted from literature for each species	The complete range of possible individual values for bioavailability was not explored even if additional simulations were performed. These values could be higher or lower and thus, the FARSC could be over or underestimated.
	For trimethoprim, the description of the extensive degradation for ruminants is based on an observation in only two cows in a publication from 1978. Simulations were performed considering absence (0), $I = 0.8$ and $I = 0.9$.	The assumption used for inactivation determinations might lead to underestimation or overestimation of FARSC if inactivation would occur at different levels than the ones considered.
	Trimethoprim is always combined with a sulfonamide for feed administration to animals	A combination of antimicrobials may reduce selection for resistance. The FARSC for trimethoprim could be over or underestimated.

FARSC: Feed Antimicrobial Resistance Selection Concentration; MSC: minimal selective concentration; I: fraction of the drug present in the digestive tracts that would be inactive on the microbiota. MSC: minimal selective concentration; PMSC: predicted minimal selective concentration.

Appendix B – List of excluded publications and their shortcomings

The publications excluded from the assessment of the effects of trimethoprim on growth promotion/increased yield following the criteria defined in Section 2.2.2.2.1 of the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (see also the Virtual Issue) are summarised in Table B.1.

Table B.1: Publications not relevant for the assessment of the effects of trimethoprim on growth promotion/increased yield according to the established 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)
Abba et al. (2015)	Ruminants	X			X		X			X(1)
Abraham et al. (2017)	Fish	X				X				
Adanir and Turutoglu, 2007)	Fish	X		X	X		X	X		X(2)
Agunos et al. (2017)	Poultry	X						X		X(3)
Andree et al. (2013)	Fish	X		X	X		X	X		X(4)
Arnold et al. (2004)	Pigs	X						X		X(5)
Berge et al. (2009)	Ruminants	X			X		X			
Chadfield and Hinton, 2003)	Poultry				X	X		X		
Chair et al. (1991)	Fish	X						X		
Craven, 1995)	Poultry				X	X		X		
Duijkeren et al. (1994)	Equines	X			X			X		X(6)
El-Abasy et al. (2016)	Rabbits	X			X	X			X	X(2)
Fu et al. (2016)	Other	X			X	X		X		
Goodnough and Johnson, 1991)	Poultry				X	X		X	X	X(2)
Goren et al. (1984)	Poultry				X	X			X	X(7)

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)
Grave et al. (1996)	Fish	X			X			X		X(8)
Groothuis and Miert, 1987)	Ruminants				X	X		X		
Herdt et al. (1993)	Poultry				X	X		X		
Kamini et al. (2016)	Poultry	X						X		
Kawano and Hirazawa, 2012)	Fish				X	X		X		
Levesque et al. (2017)	Pigs	X					X	X	X	X(2)(9)
Löscher et al. (1990)	Poultry	X						X	X	
Lundén and Bylund (2000)	Fish	X			X			X		
Lundén et al. (2002)	Fish	X			X			X		
Mengelers et al. (2000)	Pigs				X		X	X	X	X(2)
Mosleh et al. (2016)	Poultry	X				X				X(2)
Neveling et al. (2017)	Poultry	X								
Nordmo et al. (1998)	Fish	X			X	X		X		
Nordmo et al. (1994)	Fish	X			X		X	X		X(9)
Okerman et al. (1990)	Rabbits	X			X	X	X			X(2)
Riggs et al. (2003)	Equines	X			X		X	X		X(1)
Smith and Tucker (1975)	Poultry	X				X		X		
Torkelson (2002)	Equines	X			X		X	X		X(1)

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)
Veiga-Gómez et al. (2019)	Ruminants							X		X(10)
Waaij et al. (1974)	Other	X			X		X			X(11)
Williams (2005)	Poultry	X			X	X				
Yildiz and Altunay (2011)	Fish	X		X				X		
Yilmaz et al. (2018)	Fish	X			X	X				X(9)
Zanchi et al. (2008)	Pigs	X								X(9)

(1): The study describes a case report of one animal.
(2): Small number of animals per group.
(3): The article regards a surveillance of antibiotics use in Canada in 2013–2015.
(4): The study describes a case report from confiscated trafficked eels.
(5): The study analysed prescriptions in Switzerland to find out whether the ban of antimicrobial growth promotion had caused an increase in orally administered antibiotics in pigs.
(6): Review paper on trimethoprim/sulfonamide combinations in horses.
(7): No replicates.
(8): Review study on the use of antimicrobial drugs in Norway in 1980–1994.
(9): No untreated control group.
(10): The study described an investigation on uncontrolled intake of drugs.
(11): Not farm animals (Guinea pigs).