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Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 3: *Amprolium*

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Abstract

The specific concentrations of amprolium 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 for amprolium, it was not possible to conclude the assessment. To address growth promotion, data from scientific publications obtained from an extensive literature review were used. Levels of amprolium in feed that showed to have an effect on growth promotion/increased yield were reported. The lack of antibacterial activity at clinically relevant concentrations for amprolium suggests that further studies relating to bacterial resistance are not a priority.

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

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1. Introduction

The European Commission requested 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 and/or the 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 amprolium.

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

Amprolium hydrochloride (1-[(4-amino-2-propyl-5-pyrimidinyl)methyl]-2-methylpyridinium chloride monohydrochloride) is a small synthetic molecule consisting of a quaternised derivative of pyrimidine ($C_{14}H_{19}ClN_4$) that belongs to the class of organic compounds known as methylpyridines, containing a pyridine ring substituted at two positions by a methyl group. This thiamine (vitamin B1) analogue interferes with thiamine metabolism, blocking thiamine uptake, thus preventing synthesis of carbohydrates in coccidia (Bauchop and King, 1968).

Amprolium has coccidiostatic activity at low doses but is coccidiocidal at higher levels, although significant antibacterial activity is not considered to occur. It can be used for prevention and treatment of coccidiosis in a range of animals, especially poultry, as it blocks the thiamine transporter mechanism in meronts of *Eimeria* spp. interfering with intracellular metabolism. This inhibits the development of coccidial merozoites and second-generation meronts. Amprolium also has some activity against the sexual stages of *Eimeria* and potentially also inhibits the development of sporozoites (Duszynski et al., 2018; Noack et al., 2019).

[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]

Amprolium hydrochloride is marketed solely for veterinary use, either as a single compound or combined with ethopabate; a p-aminobenzoic acid analogue that inhibits folic acid biosynthesis. It can also be administered in combination with sulfonamides or pyrimethamine (Duszynski et al., 2018). It is mainly used in poultry, cattle and sheep. Amprolium has been used for many years in some countries to control coccidiosis in sheep and cattle. It is mainly used orally for the treatment of animals showing clinical illness but may also be used for prevention by inclusion in feed (Baker et al., 1972; Norcross et al., 1974; Talmon et al., 1989).

Amprolium is also used for the treatment and prevention of coccidiosis in chickens, including laying hens and turkeys (Osman et al., 2020). For treatment, it can be administered via the birds' drinking water or in the feed. Amprolium is authorised as a feed additive for poultry according to Council Directive 70/524/EEC[3]; for poultry at concentrations in the range of 62.5–125 mg/kg of compound feed; use is prohibited from laying age onwards and for at least 3 days before slaughter (EMEA/CVMP, 2001; EFSA FEEDAP Panel, 2018, 2021). Amprolium is included in Table 1 of Commission Regulation (EU) No 37/2010[4] as not requiring a maximum residue limit (MRL) for meat and offal, or eggs. It is also sometimes used by the ethanol industry to manage yeast cultures and has been found in yeast residues used as feed ingredients (Hoff et al., 2021).

1.3.3. Main pharmacokinetic data

When administered orally to chickens, one study reported a very low bioavailability (around 2–3% of amprolium) (Hamamoto et al., 2000) whereas another study reported a bioavailability of 66% (El-Sayed et al., 1995). This discrepancy prevents the use of any of these values for calculations.

In one study, 8 h after dosing of chickens with amprolium, the residue concentrations were 46 and 74 mg/kg in the caecum in birds treated with 10 or 20 mg/kg body weight (bw), respectively (EMEA/CVMP, 2001). No information on the nature of the residues (parent drug or metabolites) is provided.

In rats, faecal excretion is the major route of elimination of radioactivity after oral administration of ^{14}C-labelled amprolium, with mean values of around 82% (EMEA/CVMP, 2001). Parent amprolium was detected in faecal samples at levels from 45% to 64% (EMEA/CVMP, 2001).

1.3.4. Main resistance mechanisms

Resistance develops rapidly in *Eimeria* spp. following the introduction of anticoccidials in the field. The molecular mechanisms involved in resistance are unclear, although mating experiments suggest that resistance may be transferable between coccidial gametes, which may supplement the main mechanism of clonal expansion of resistant strains (Jeffers, 1974; Chapman, 1997; Abbas et al., 2011a). *E. acervulina* and *E. maxima* are more likely to be intrinsically resistant than *E. tenella* (EFSA FEEDAP Panel, 2018, 2021).

Since, unlike the ionophore anticoccidials, amprolium is not considered to have antimicrobial activity other than its anticoccidial properties, selection of resistant bacteria or promotion of antimicrobial resistance (AMR) gene transfer would not be expected to be a relevant hazard (EMEA/CVMP, 2001; VKM, 2015; EFSA FEEDAP Panel, 2018, 2021). However, one old *in vitro* study did show that it was possible to inhibit the growth and to induce resistant mutants of *Lactobacillus fermenti* by sequential exposure to very high (175–1,400 μg/mL) concentrations of amprolium (Kishi et al., 1971).

2. Data and methodologies

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

[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 Regulation (EU) 2019/4.

[3] Council Directive 70/524/EEC of 23 November 1970 concerning additives in feeding-stuffs. OJ L 270, 14.12.1970, p. 1–17.

[4] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, p. 1–72.

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 amprolium: examples

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

No MICs are available for amprolium. No studies describing bacterial resistance development using sub-MIC concentrations are available.

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

No MICs are available for amprolium. No studies describing effects on bacterial HGT and virulence of sub-MIC concentrations are available.

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 the FARSC value for amprolium 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). However, no minimal selective concentration (MSC) data required to do the calculations is available.

Due to the lack of PMSC, no FARSC could be calculated. If PMSC was available, the FARSC 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 amprolium.

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

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

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.

The reported oral bioavailability of amprolium in chickens is uncertain and there is no value for other species. There are also no data on the fate of amprolium after absorption and especially on the metabolism before gut elimination in the food-producing animal species.

In consequence, no pharmacokinetic (PK) parameter value, that could be used to calculate FARSC for amprolium, was proposed.

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 amprolium:

i) MSC data: no data for MSCs are available.
ii) MIC data: no MIC data for bacteria are available.
iii) Bioavailability: data were only available for chickens but seem uncertain.
iv) Fraction eliminated in gut: an elimination of amprolium as inactive metabolites was described in rats. However, there are no quantitative data for other animal species to consider this process.
v) Inactive fraction: no data on the possible binding of amprolium in digestive tract are available.
vi) Ruminants: no data are available for amprolium administered to ruminants by the oral route.

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 antimicrobial concentrations in feed which have an effect in terms of growth promotion/increased yield

3.3.1. Amprolium

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 199 papers mentioning amprolium and any of the food-producing animal species considered[5] and any of the performance parameters identified as relevant for the assessment of the possible growth-promoting effects of amprolium.[6] After removing the reports not matching the eligibility criteria, 55 publications were identified.

3.3.1.2. Evaluation of the studies

The 55 publications identified in the literature search were appraised for suitability for the assessment of the effects of amprolium on growth or yield of food-producing animals; this appraisal was performed by checking each study against a series of predefined exclusion criteria (see Section 2.2.2.2.1 of the Scientific Opinion Part 1; see also the Virtual Issue).[7] A total of 52 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 (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 amprolium on growth performance and yield

Three publications were considered suitable for the assessment of the effects of amprolium 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 amprolium used – either as the base or as any specific form/commercial preparation – and the concentration(s)

[5] 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.

[6] (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.

[7] 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)'.

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. Study in ruminants

In the study of Silzell et al. (2001), a total of 96 mix-breed cattle for fattening were distributed in four dietary treatments (24 animals/treatment), each including four pens with six animals. Two controls and two treatment groups, which involved the dietary administration of amprolium (unspecified chemical form; Amprolium 1.25% Cattle Pellets; Nutra Blend Corp) at a concentration of 5 mg/kg initial body weight (BW) (equivalent to 250 mg/kg dry matter (DM), using the default value 20 g DM intake/kg BW,[8] initial BW 211 kg), with or without supplementation with thiamine at 140 mg/kg DM. The study lasted 35 days, but amprolium was only administered for the first 21 days. Steers were observed daily for signs of morbidity. BW was measured weekly and faecal samples were evaluated for coccidia oocyst counts. At the end of the trial steers treated with amprolium showed lower oocyst counts compared to controls but no effect on growth performance parameters was found regardless of the thiamine supplementation. Dietary amprolium supplementation at 250 mg/kg DM did not have growth-promoting effects in cattle for fattening.

3.3.1.3.2. Studies in poultry

In the study of Chauhan et al. (2015), a total of 168 1-day-old commercial chickens for fattening (unspecified breed/genotype) were distributed in seven experimental groups (24 birds/treatment), each including 12 birds with two replicates. The diets were either not supplemented or supplemented with different treatments (four groups were infected with *Eimeria* spp.). For the assessment only two were the relevant treatments: a control and a treatment consisting of amprolium (unspecified form) supplemented at 125 mg/kg feed without experimental *Eimeria* infection. The study lasted 30 days. Feed intake (FI) and weight gain were measured every 5 days; feed efficiency and performance index (weight gain × feed efficiency ratio) were calculated. At the end of the trial, the chickens treated with amprolium showed a decrease in cumulative FI (1,922 vs 1,998 g), cumulative weight gain (1,259 vs 1,314 g) and performance index (825 vs 865) compared to the control group. Dietary amprolium supplementation at 125 mg/kg feed had a negative effect on performance of chickens for fattening.

3.3.1.3.3. Study in fish

In the study of Manning et al. (2013), a total of 320 juvenile Channel catfish (*Ictalurus punctatus)* were distributed in four dietary treatments (80 fish/treatment) each including four replicate tanks at 20 fish each. The diets were either not supplemented or supplemented with amprolium (unspecified form) at the concentrations of 50, 100 and 200 mg per kg initial total weight of fish, corresponding to 2,000, 4,000 and 8,000 mg/kg feed, respectively; fish were given weighed diets calculated as 2.5% of total initial weight. The study lasted 10 days. Endpoints included total FI, weight gain and feed to gain ratio (F:G). Total FI and weight gain showed no differences among experimental groups. Dietary amprolium supplementation at 2,000, 4,000 and 8,000 mg/kg feed did not have a growth-promoting effect in Channel catfish.

3.3.1.4. Discussion

From the studies examined, the test item has been described as 'amprolium' (unspecified form; four studies). Therefore, an uncertainty on the exact product used/concentration applied has been identified.

A detailed analysis of the uncertainties for amprolium is included in Appendix B (Table B.1) of this document, and the Section 3.3 of the Scientific Opinion 'Part 1: Methodology, general data gaps and uncertainties' (see also the Virtual Issue).

3.3.1.4.1. Ruminants

Only one study reporting the effect of oral administration of amprolium is available for cattle for fattening (Silzell et al., 2001), from which the concentration of 250 mg/kg DM did not show a growth-promoting effect.

[8] Default values for daily feed intake scaled to body weight from the EFSA FEEDAP Guidance on the assessment of the safety of feed additives for the target species. Available online: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2017.5021

3.3.1.4.2. Poultry

Only one study reporting the effect of oral administration is available for chicken for fattening (Chauhan et al., 2015). Dietary amprolium supplementation at 125 mg/kg feed had a negative effect on the performance of chickens for fattening.

3.3.1.4.3. Fish

Only one study reporting the effect of oral administration of amprolium is available for Channel catfish (Manning et al., 2013). Dietary amprolium supplementation at 50, 100 and 200 mg/kg bw (2,000, 4,000 and 8,000 mg/kg feed) did not have a growth-promoting effect on Channel Catfish.

3.3.1.5. Concluding remarks

No data are available in the scientific literature showing effect of amprolium on growth promotion/ increased yield when added to feed of any food-producing animal species or categories. It is judged 33–66% certain ('about as likely as not') that amprolium has negative effects on performance of chickens for fattening at the concentration of 125 mg/kg complete feed (one study).

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

- Due to the lack of data on the parameters required to calculate the Feed Antimicrobial Resistance Selection Concentration (FARSC) corresponding to the concentrations of amprolium 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 amprolium in feed of food-producing animals that have an effect in terms of growth promotion/increased yield?

- No data are available in the scientific literature showing effects of amprolium on growth promotion/increased yield when added to feed of any food-producing animal species or categories.
- It is judged 33–66% certain ('about as likely as not') that amprolium has negative effects on performance of chickens for fattening at the concentration of 125 mg/kg complete feed (one study).

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 amprolium. However, due to the absence of antibacterial activity on the concentrations used for the prevention and treatment of coccidiosis, those studies are not a priority.

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Abbreviations

ATP	adenosine triphosphate
AQ	assessment question
BW	body weight
bw	body weight used in toxicity studies
DM	dry matter
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
F:G	feed to gain ratio
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
LPS	lipopolysaccharide
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
MRL	maximum residues limit
MSC	minimal selective concentration
PK	pharmacokinetic
PMSC	predicted MSC
rRNA	ribosomal ribonucleic acid
ToRs	Terms of Reference

Appendix A – List of excluded publications and their shortcomings

The publications excluded from the assessment of the effects of amprolium 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 amprolium 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)
Abbas et al. (2011b)	Poultry				X	X				
Abbas et al. (2011c)	Poultry				X	X				
Abou-Elkhair et al. (2014)	Poultry				X	X				
Adamu and Boonkaewwan (2014)	Poultry				X	X				
Ahad et al. (2016)	Poultry				X	X				
Ahad et al. (2016)	Poultry				X	X				
Ahad et al. (2017)	Poultry				X	X				
Ahad et al. (2018)	Poultry				X	X				
Ali et al. (2019)	Poultry				X	X				
Allam et al. (2008)	Poultry				X	X				
Amer et al. (2010)	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)
Cabel and Waldroup (1991)	Poultry	X			X	X				
Cabel et al. (1991)	Poultry				X	X				
Chand et al. (2016)	Poultry				X	X				
Chapman (1989)	Poultry	X			X	X			X	
Chauhan et al. (2017)	Poultry					X		X		
Dakpogan et al. (2019)	Poultry				X	X				
Damron (1994)	Poultry	X								
El-Ghany et al. (2007)	Poultry				X	X				
El-Ghoneimy and El-Shahawy (2017)	Rabbit				X		X			
El-Morsy et al. (2016)	Poultry				X	X				
Fitz-Coy and Edgar (1992)	Poultry	X			X	X				
Fitzgerald (1972)	Rabbit				X	X		X		
Furusawa (2001)	Poultry								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)
Galyen et al. (2020)	Ruminants	X			X		X			
Gerhold et al. (2011)	Poultry	X			X	X				
Golomazou et al. (2006)	Fish	X			X		X			
Hamed and Eladl (2011)	Poultry				X	X				
Holderread et al. (1983)	Poultry								X	X[(2),(3)]
Malik et al. (2014)	Poultry				X	X				
Malik et al. (2016)	Poultry				X	X				
McDougald (1979)	Poultry				X	X				
McDougald and McQuistion (1978)	Poultry				X	X				
McDougald and Seibert (1998)	Poultry				X	X				
Meskerem and Boonkaewwan (2013)	Poultry				X	X				
Munir et al. (2018)	Poultry				X	X				
Nabian et al. (2018)	Poultry	X			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)
Norton (1967)	Poultry				X	X		X		
Oe and Arakawa (1975)	Poultry	X			X	X				
Ogbe et al. (2008)	Poultry				X	X				X(3)
Ogbe et al. (2009)	Poultry				X	X				X(3)
Ogbuookiri (2013)	Poultry	X			X	X			X	X(4)
Ohe and Arakawa (1976)	Poultry	X			X	X				
Ojimelukwe et al. (2018)	Poultry				X	X				
Patton et al. (1984)	Poultry								X	
Pop et al. (2019)	Poultry				X	X				
Riddell and Classen (1992)	Poultry	X								X(5)
Ruff et al. (1991)	Poultry				X	X				
Selvarani et al. (2014)	Poultry				X	X			X	
Spencer and Waldroup (1985)	Poultry									X(4)
Waibel et al. (1991)	Poultry	X								X(4)

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)
Zaman et al. (2012)	Poultry				X	X				

(1): Designed to study the transfer of antibiotics to eggs.
(2): The study investigated the adverse effects of anticoccidial drugs administered to ducklings.
(3): Small number of animals per group, no replicates.
(4): Unclear number of birds/replicates per treatment.
(5): No untreated control group.

Appendix B – Table of uncertainties

Uncertainties associated with the growth promotion assessment

Table B.1: Potential sources of uncertainty identified in the levels of amprolium 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