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Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 9: *Polymyxins: colistin*

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Abstract

The specific concentrations of colistin 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 of colistin in feed that showed to have an effect on growth promotion/increased yield were reported. 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 antimicrobials.

Keywords: colistin, 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 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 polymyxin colistin.

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

Colistin is a non-ribosomally synthesised antimicrobial belonging to the polymyxins class (EMEA/CVMP/CHMP, 2016). Colistin is a cyclic heptapeptide with a tripeptide side chain acylated at the N terminus by a fatty acid tail. Colistin A (polymyxin E1) and colistin B (polymyxin E2), differing only in their fatty acid tails, have been used in clinical practice (Bialvaei and Samadi Kafil, 2015).

The exact mechanism of antibacterial activity of colistin is not completely understood. The primary target is the lipid A moiety of lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria (El-Sayed Ahmed et al., 2020). Colistin displaces Ca and Mg in the outer membrane thereby destabilising the membrane and killing the bacteria.

Colistin, a narrow spectrum antimicrobial, is active against several aerobic Gram-negative species that frequently cause infections in humans and animals, such as *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *Escherichia coli* and some *Salmonella enterica* serovars (EMEA/CVMP/CHMP, 2016). Nevertheless, some bacterial species, such as *Proteus* spp., *Providencia* spp., *Serratia marcescens*, *Morganella morganii, Edwardsiella* spp., some *Aeromonas* species, *Burkholderia cepacia* and *Vibrio cholerae*, are intrinsically colistin resistant.

[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]

There are two different forms of colistin available commercially, colistimethate sodium and colistin sulfate but only the latter is used in veterinary medicine (EMEA/CVMP/CHMP, 2016).

The main indications are enteric infections caused by Enterobacterales in pigs, poultry, cattle, sheep, goats and rabbits. Colistin can be also used for the same indication in laying hens and in ruminants producing milk for human consumption. Typically, colistin products are administered orally, in feed, in drinking water, as a drench, or through milk replacer diets (Catry et al., 2015).

Products for parenteral and intramammary administration are also available. Also, endotoxaemia or septicaemia due to Gram-negative infections in ruminants is claimed indications for treatment (Moore and Barton, 2003; Commission Regulation (EU) No 122/2013[3]).

For calves, colistin-containing products are also available for oral administration as tablets or paste for treatment of neonatal colibacillosis (EMEA/CVMP/CHMP, 2016).

1.3.3. Main pharmacokinetic data

Colistin sulfate is very poorly absorbed after oral administration to calves, pigs and rabbits, even if in chickens, residues can be detected in serum after oral administration (EMEA/CVMP, 2002).

Despite poor absorption, the dose recovered in faeces of humans is very low (EMEA/CVMP, 2002) suggesting a degradation or a binding of colistin in the digestive tract. Ziv (1981) reported that the concentrations of colistin in the contents of intestine in broiler and pig were 60 μg/g and 4 μg/g, respectively, after the oral administration at the dose of 20 mg⁄kg body weight (bw). A study conducted with simulated gastric fluid demonstrated a rapid degradation of colistin sulfate. This deterioration started quickly and reached the maximum at around 15 min after pepsin addition with 50% colistin sulfate degradation. However, no statistically significant difference in the antimicrobial activity of colistin sulfate and its degradation products was observed (Rhouma et al., 2015).

1.3.4. Main resistance mechanisms

The mechanisms of resistance to colistin are complex and could differ between different bacterial species. The colistin resistance mechanisms include: (i) modifications of the LPS moiety via the addition of cationic groups to the LPS, the most common mechanism; (ii) loss of the LPS, due to mutations transpositions events via the insertion sequence as those occurring in the genes related to lipid A biosynthesis (*lpxA, lpxC or lpxD*) of *A. baumannii*. (iii) porin mutations and overexpression of efflux pump systems, such as Sap (sensitive antimicrobial peptides) proteins, BrlR, the AcrAB-TolC complex or KpnEF; (iv) over-production of capsular polysaccharide (CPS) in some Gram-negative bacteria (e.g. in *K. pneumoniae*) hindering the colistin-binding sites and the release of CPS trapping colistin. Most of the resistance mechanisms are chromosomally encoded with a single transferable mechanism of resistance identified so far (Aghapour et al., 2019; El-Sayed Ahmed et al., 2020).

The most common mechanism of resistance is mainly by reducing the negative charge of the outer membrane, similar to that observed in bacteria with intrinsic resistance to colistin. Two-component regulatory systems, namely the mostly studied PhoPQ and PmrAB systems, have been involved in the replacing of the phosphate groups of lipid A by the cationic 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine moieties. Upregulation of these regulatory systems, by mutations in these regulatory systems or their regulators leads to the addition of cationic moieties to LPS, decreasing the net negative charge of the outer membrane. The *mcr-1* gene, encoding a phosphoethanolamine (PEA) transferase, was the first plasmid-mediated gene, responsible for horizontal transfer of colistin resistance. The PEA transferases catalyses the attachment of PEA to LPS-lipid A, leading also to a reduction of the negative charge of LPS, similarly to the chromosomal mutations mentioned above (El-Sayed Ahmed et al., 2020).

Since the report of *mcr-1*, in 2016, a few other acquired *mcr (mcr-2-10)* genes have been described mainly in Enterobacterales with scarce reports in *Acinetobacter* spp. (*mcr-1* and *mcr-4*) or

[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.

[3] Commission Regulation (EU) No 122/2013 of 12 February 2013 amending Regulation (EC) No 1950/2006 establishing, in accordance with Directive 2001/82/EC of the European Parliament and of the Council on the Community code relating to veterinary medicinal products, a list of substances essential for the treatment of Equidae. OJ L 42, 13.2.2013, p. 1–17.

Pseudomonas spp. (*mcr-1*). Several replicon types of plasmids have been reported to harbour *mcr*-like genes, although they can also be chromosomally located (EMEA/CVMP/CHMP, 2016; Poirel et al., 2017; Ling et al., 2020).

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), for the antimicrobials under assessment are shown below.

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

There are numerous publications that report colistin resistance in a range of bacteria, but very few that report on the response to exposure. Co-resistance to colistin and other antimicrobials has been identified in various Gram-negative bacteria, which can increase the spread of multidrug resistance (MDR) and associated virulence or heavy metal tolerance genes (Forde et al., 2018; Campos et al., 2016). Resistance genes are also being reported from a range of bacteria with pathogenic potential frequently carrying *mcr* genes in transferable plasmids (Long et al., 2018; Ling et al., 2020).

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

- A study using colistin (0.125 μg/mL) initial exposure followed by subsequent exposition every 2 days to double previous colistin concentration (up to a final concentration of 4 μg/mL), increased by 16-fold colistin MIC values of *Citrobacter* and *Enterobacter* strains. Only selected strains were able to adapt, suggesting that acquisition of colistin resistance is dependent upon individual strain characteristics. Mutations in PmrB for *Citrobacter* and PhoP in *Enterobacter* were attributed to this increased resistance, although it was not able to identify causative mutations in all strains (Wand and Sutton, 2020).

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

- Sub-inhibitory concentrations of colistin (1 μg/mL) drive phenotypic diversification of *P. aeruginosa* populations in an artificial sputum medium model. This may help to explain the observed diversification of *P. aeruginosa* in natural cystic fibrosis lung infections (Wright et al., 2013).
- Exposition of a *P. aeruginosa* clinical strain to sub-inhibitory concentrations of colistin (2 μg/mL) increased biofilm formation, loss in susceptibility to serum and a decrease in virulence determined with *Galleria mellonella* model of infection (Javed et al., 2021).

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 colistin 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 colistin as indicated in Table 1. However, no MSC data required to do the calculations are available for this antimicrobial.

Table 1: Calculation of colistin 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 ($MIC_{lowest}/MIC_{test}/MSC_{test}$)
Colistin	NA	NA	NA	0.064	NA

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

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

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

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.

Due to very poor absorption of colistin, F could be set to 0 for all species. However, there are too many uncertainties on the activity in the digestive tract of colistin itself or of its metabolites to estimate active concentrations after ingestion of colistin. This uncertainty would have a major impact on the value of I, thus any value for this parameter is presented.

Due to the absence of MSC and other PK data the estimation of the FARSC for colistin 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 to perform the assessment for colistin:

i) MSC data: no data for MSC are available.
ii) MIC data: MIC and MSC data should be considered with caution for colistin as it has chemical properties that make the determination of these values difficult. Colistin is a large molecule and does not diffuse in agar (agar dilution methods) and also, it easily binds to plastics, which influences *in vitro* susceptibility testing, including MIC and MSC assays.
iii) Inactive fraction: no data on the possible binding of colistin in digestive tract of animals are available. The data on the possible degradation of the drug in the digestive tract are scarce and inaccurate. Moreover, the antimicrobial activity of the degradation products was only described in one study.
iv) Ruminants: no data are available for colistin administered to adult ruminants by oral route.

3.2.2. Concluding remarks

Due to the lack of data on the parameters required to calculate the Feed Antimicrobial Resistance Selection Concentration (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. Colistin

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 490 papers mentioning colistin and any of the food-producing animal species considered[4] and any of the performance parameters identified as relevant for the assessment of the possible growth-promoting effects of colistin.[5] After removing the reports not matching the eligibility criteria, 108 publications were identified.

3.3.1.2. Evaluation of the studies

The 108 publications identified in the literature search were appraised for suitability for the assessment of the effects of colistin 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: Methodology, general data gaps and uncertainties'; see also the Virtual Issue).[6] A total of 85 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 colistin on growth performance and yield

A total of 23 publications (22 studies) were considered suitable for the assessment of the effects of colistin 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: Methodology, general data gaps and uncertainties' (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 colistin 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. When 'colistin sulfate' is reported, considering the existence of several sulfate products characterised by different colistin:sulfate ratios, and thus different molecular weights, the ratio of colistin base to colistin sulfate would vary between 0.82 and 0.92. When a precise description of the sulfate compound used is lacking, the calculation to base substance could not therefore be done.

3.3.1.3.1. Studies in pigs

In the study of Cao et al. (2019), a total of 36 piglets weaned at 21 days of age (Duroc × Landrace × Yorkshire) were individually distributed and allocated to three dietary treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (unspecified colistin:sulfate ratio) at a concentration of 100 mg/kg feed. The study lasted 28 days.

[4] 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.

[5] (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.

[6] 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)'.

Mortality, health status and faecal score were checked every day. Animals' weight and cumulative feed intake were recorded at the end of the experiment and F:G calculated. In addition, eight pigs per group were bled on days 14 and 28 to measure serum alanine aminotransferase, aspartate transaminase, alkaline phosphatase and lactate dehydrogenase. At the end of the trial, eight pigs per group were slaughtered and jejunum, ileum and colon sampled to determine integrity, apoptotic cells in small intestine, short-chain fatty acids, microbiota composition and function by high-throughput sequencing in colon. At the end of the trial, the pigs treated with colistin showed, compared to the control group, an improved average daily weight (ca. 250 vs. 210 g BW/day), F:G (ca. 1.5 vs. 2.0), and faecal score. At the end of the trial, amino alanine aminotransferase was lower in the serum of colistin-treated animals compared to controls (ca. 35 vs. 43 U/L) and similarly on day 14 aspartate transaminase (ca. 40 vs. 63 U/L). In addition, the integrity of intestinal *villi* was improved, and the number of intestinal apoptotic cells reduced in the pigs treated with colistin compared to the controls. Moreover, compared to the control group, the concentrations of isovalerate and valerate were higher in the colon of the colistin-treated pigs (125 vs. 75 mmol/L and 100 vs. 50 mmol/L, respectively), in parallel to the microbial amino acid, starch and sucrose metabolism. Dietary colistin sulfate supplementation at 100 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Conde et al. (2001), two experiments were carried out and outcomes analysed separately. In the first trial, a total of 96 piglets weaned at 21 days of age (Landrace) were distributed in 24 pens in groups of four animals and allocated to four dietary treatments. The study lasted 35 days. In the second trial, a total of 128 piglets half weaned at 22 days of age and half weaned at 32 days of age were distributed in 32 pens in groups of four animals and allocated to four dietary treatments. The study lasted 28 days. In both trials, two basal diets (pre-starter and starter) were either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin (unspecified form) at a concentration of 150 mg/kg feed. Animals' weight and cumulative feed intake were recorded at the end of the trial and F:G calculated. Dietary colistin supplementation at 150 mg/kg feed did not have a growth-promoting effect in weaned piglets.

In the study of Cui et al. (2019), a total of 50 piglets weaned at 35 days of age (Large White × Landrace) were individually housed and allocated to five dietary treatments. One basal diet was either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (Zhongnongxing Feed SCI. & Tech. Co., Ltd, Beijing, China; unspecified colistin:sulfate ratio) at a concentration of 40 mg/kg feed. The study lasted 28 days. Animals' weight and cumulative feed intake were recorded at the end of the trial and F:G calculated. In addition, at the end of the trial, faeces were sampled from six animals per treatment to determine the faecal apparent digestibility of gross energy, dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), calcium and phosphorus, as well as to sequence microbial DNA. Moreover, blood from the same animals was obtained to determine the concentrations of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and malondialdehyde (MDA) as well as blood urea nitrogen (BUN), glucose and immunoglobulin. At the end of the trial, the pigs treated with colistin showed, compared to the control group, improved digestibility of DM (84.0% vs. 82.2%), OM (87.1% vs. 85.3%), gross energy (83.3% vs. 81.1%), CP (80.5% vs. 76.5%), EE (58.4% vs. 33.3%), calcium (41.9% vs. 35.6%) and phosphorus (54.9% vs. 50.4%). With colistin, animals also had increased concentrations of serum BUN, glucose and IgG compared to the control group. In addition, the activities of antioxidant enzymes were higher in colistin-treated animals than in controls for SOD, GSH-Px, CAT, and showed lower MDA content. Moreover, supplementation of colistin sulfate decreased the alpha diversity index of microbial community, including richness estimators as Chao and observed species. Dietary colistin sulfate supplementation at 40 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Furbeyre et al. (2017), two trials were carried out and outcomes were analysed separately. In Trial 1, 72 mixed piglets weaned at 28 days of age ((Large White × Landrace) × Piétrain) were individually distributed in cages and allocated to four dietary treatments. A basal diet was either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (Acti Coli B, Biove, France, 106 IU/g; unspecified colistin:sulfate ratio) at a concentration of 2,000 mg/kg feed during the first 14 days. The study lasted 28 days. In Trial 2, the same conditions were used with a total of 24 mixed weaning piglets. The study lasted 14 days. Mortality and health status were checked every day. Animals' weight was recorded weekly and feed intake daily and F:G calculated each week. In trial 1, diarrhoea incidence was assessed daily. In trial 2, faeces and urine were pooled from days 28 to 34 and from days 35 to 41 to determine the digestibility of dry matter, gross energy, nitrogen, crude fibre and fibre fractions. In addition, at the end of the trial, all

animals were slaughtered and blood and small intestine (jejunum and ileum) sampled to quantify haptoglobin in plasma and assess mucosal morphology, respectively. At the end of the Trial 2, the pigs treated with colistin showed, compared to the control group, shorter *villi* heights in the jejunum (356 vs. 481 μm). Dietary colistin sulfate supplementation at 2,000 mg/kg feed had an adverse effect on performance of weaned piglets.

In the study of Guerra et al. (2007), two experiments were carried out and outcomes were analysed separately. In both experiments, a total of 40 piglets (unspecified breed/genotype) weaned at 21 days of age were individually distributed in cages and allocated to four dietary treatments. Two were the relevant treatments obtained from a basal diet which was either not supplemented (control) or supplemented with colistin sulfate (unspecified colistin:sulfate ratio) at a concentration of 12 mg/kg feed. Both experiments lasted 42 days. Mortality and health status were checked every day. Animals' weight and cumulative feed intake were recorded biweekly and F:G calculated. In addition, faeces were also sampled biweekly for coliform counts. At the end of the Trial 1, the pigs treated with colistin showed, compared to the control group, improved final body weights (30.8 vs. 28.9 kg BW), average weight gains (23.0 vs. 21.1 kg BW) and reduced feed intakes (32.5 vs. 33.0 kg feed) as well as improved F:G on day 14 (1.26 vs. 1.45). At the end of Trial 2, the colistin-treated animals also showed improved final body weights compared to the control group (30.0 vs. 30.4 kg BW) as well as higher average weight gains on day 28 (12.5 vs. 11.1 kg BW) and lower F:G on day 14 (1.52 vs. 1.44) than control animals. In addition, faecal coliform count reductions in the last sampling were greater in the colistin-treated animals than in the controls in both trials. Dietary colistin sulfate supplementation at 12 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Kong et al. (2007), a total of 60 mixed piglets weaned at 21 days of age (Duroc × Landrace × Yorkshire) were individually housed and allocated to three dietary treatments. Two were the relevant treatments obtained from a basal diet which was either not supplemented (control) or supplemented with colistin (unspecified form) at a concentration of 200 mg/kg feed. The study lasted four weeks. Mortality, health status, and faecal consistency were checked every day. Animals' weight and cumulative feed intake were recorded weekly and F:G calculated. In addition, on days 7, 14 and 28, a total of 5 animals per group were bled and biochemical parameters measured. On weeks 1 and 3, the piglets treated with colistin showed, compared to the control group, lower diarrhoea frequency (8.3% vs. 27.3% and 6.0% vs. 9.0%, respectively). In addition, on week 1, the colistin-treated animals showed lower concentrations of serum triglycerides and lactic acid and higher phosphorus than the controls. Similarly, on week 2, creatinine and alpha-amylase concentrations were lower in the colistin-treated pigs than in the controls. In contrast, on week 4, alpha-amylase and iron concentrations were higher in the colistin-treated pigs. Dietary colistin supplementation at 200 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Liu et al. (2016), a total of 240 pigs aged of 6 weeks (Yorkshire × (Duroc × Landrace)) were distributed in 24 pens in groups of 10 animals and allocated to three dietary treatments. The basal diets were either not supplemented or supplemented with colistin (unspecified form) at a concentration of 200 mg/kg feed. The study lasted 14 days. Animals' weight and cumulative feed intake were recorded on days 7 and 14 and F:G calculated at the end of the experiment. Blood samples were collected from eight piglets per treatment to determine serum amino acids at 7 and 14 days of the experiment. At the end of the trial, the pigs treated with colistin showed, compared to the control group, higher ADG (459.4 vs. 390.8 g/day) and improved F:G (2.41 vs. 2.73), as well as a higher lysine serum level and lower glutamate and methionine serum levels. Dietary colistin supplementation at 200 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Mazutti et al. (2016), a total of 72 weaned piglets (unspecified breed/genotype; 28 days of age) were distributed in 24 pens in groups of three animals and allocated to three dietary treatments. Two basal diets (pre-starter and starter phase) were either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin (unspecified form) at a concentration of 20 mg/kg feed. The study lasted 35 days. Animal health was checked daily. Animals' weight and feed intake were recorded weekly and F:G calculated. Faecal score was determined on a 0 (normal) to 3 (watery) scale. Blood samples were collected weekly from eight pigs per treatment (one pig per replicate) in order to evaluate the cellular immune response. At the end of the trial, the pigs treated with colistin showed, compared to the control group, higher final body weight (24.24 vs. 22.56 kg) and feed intake (885 vs. 794 g/day). The treated animals showed a reduction of faecal score (0.77 vs. 0.91). Dietary colistin supplementation at 20 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Santana et al. (2015), a total of 90 piglets (unspecified breed/genotype; 21-day-old, BW 6.35 kg) were distributed in 30 pens in groups of three animals and allocated to five dietary treatments. Two basal diets (21–35 days and 35–56 days) were either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (unspecified colistin:sulfate ratio) at a concentration of 40 mg/kg feed. The study lasted 35 days. Animals' weight and cumulative feed intake were recorded at days 0, 28, 35 and 56 of the experiment. At the end of the trial, six piglets per treatment (one per replicate) were slaughtered and organs weight (stomach, liver and caecum) recorded. The pH values of stomach, jejunum and caecum were measured. Samples of duodenum and jejunum were collected to measure *villi* height, crypts depth, and their ratio. Dietary colistin sulfate supplementation at 40 mg/kg feed did not have growth-promoting effects in weaned piglets.

In the study of Sbardella et al. (2016), a total of 200 weaned piglets (unspecified breed; 21-day-old; average BW 6.2 kg), were distributed in 40 pens in groups of five animals (two castrated males and three females) and allocated to five dietary treatments. Three basal diets (0–7, 7–21, 21–35 day) were either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin (unspecified form) at a concentration of 40 mg/kg feed. The study lasted 35 days. Animals' weight and feed intake were recorded at days 7, 21 and 35 and F:G calculated. Apparent total tract digestibility of crude protein, ether extract and gross energy were estimated on two piglets per pen over a 5-day collection period of faeces during phase 2 (lasting from day 7–21) and 3 (day 21–35) of the trial. Animals were checked daily for diarrhoea occurrences. Blood samples were collected from one pig per pen on days 4, 8, 16 and 32 to determine phagocyte activation and respiratory burst. One pig per treatment was slaughtered on days 7 and 35 and the weights of stomach, pancreas, liver, spleen and empty intestine were measured. In addition, samples of duodenum, jejunum and ileum were collected to measure the height of *villi* and the depth of crypts. The microbial diversity of ileal and faecal luminal contents was also studied. At the end of the trial, piglets receiving colistin showed, compared to the controls, higher final body weight (20.9 vs. 19.6 kg), ADG (425 vs. 387 g/day) and improved G:F ratio (0.61 vs. 0.58). Dietary colistin supplementation at 40 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Silva-Júnior et al. (2020), a total of 108 crossbred piglets (unspecified breed/genotype; 21-day-old; average BW 5.30 kg) were distributed in 36 pens in groups of three animals and allocated to four dietary treatments. Three basal diets (phase 1: days 0–14; phase 2: days 15–29 and phase 3: days 30–44 of trial) were either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin (unspecified form) at a concentration of 40 mg/kg feed. The study lasted 44 days. Two days before the beginning of the trial, pens were submitted to a sanitary challenge spraying 2 L of diluted pig manure. Animals' weight and cumulative feed intake were recorded at days 1, 14, 29 and 44 of the experiment. The presence of diarrhoea was monitored daily by visual inspection. Coefficients of apparent total tract digestibility (ATTD) for dry matter, organic matter, crude protein, ether extract and gross energy were estimated on faeces from each pen over a 3-day period during phase 1 (days 7, 8 and 9), 2 (days 22, 23 and 24) and 3 (days 36, 37 and 38). Acid insoluble ash was used as digestibility marker. At the end of phases 1 and 3, nine piglets per treatment (one per replicate) were slaughtered and organ weights were measured (stomach, liver, pancreas, small intestine, colon and caecum). Samples of duodenum and jejunum were collected to measure *villi* height and width, crypts depth, and goblet cells. The microbial diversity of faecal luminal contents was also assessed. At the end of the trial, piglets receiving colistin showed, compared to the controls, greater *villi* width in duodenum (444.8 vs. 433.6 μm) and jejunum (342.2 vs. 326.1 μm), higher crypts depth in duodenum (343.2 vs. 326.1 μm) and increased goblet cell number in jejunum (117.3 vs. 99.8). In phase 2, piglets receiving colistin showed higher ATTD of DM (75.0% vs. 68.3%), OM (78.2% vs. 72.3%) CP (67.8% vs. 59.7%) and gross energy (73.0% vs. 65.9%). Dietary colistin supplementation at 40 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Tian-Yang et al. (2013), a total of 72 growing pigs (unspecified breed/genotype; 56-day-old) were distributed in 12 pens in groups of six animals and allocated to three dietary treatments. Two basal diets (grower and finisher) were either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (unspecified colistin:sulfate ratio) at a concentration of 80 mg/kg feed. The study lasted 101 days. Animals' weight and cumulative feed intake were recorded weekly and F:G calculated. At the end of experiment, blood samples were collected from each pig to determine total cholesterol, triglycerides, HDL, LDL, urea nitrogen and total protein; pigs were slaughtered and samples of Longissimus dorsi

muscle were obtained to determine drip loss, cooking loss, shear force, pH (at 45', 24 h and 48 h post-mortem) and pork colour (at 24 and 48 h post-mortem). At the end of the trial, the pigs receiving colistin only showed a higher feed intake than the controls (2,185.5 vs. 2,097.4 g/day). Dietary colistin sulfate supplementation at 80 mg/kg feed did not show growth-promoting effects in pigs for fattening.

In the study of Wan et al. (2015), a total of 24 Duroc × Landrace × Yorkshire weaned piglets (25-day-old; average BW 7.67 kg), were individually housed and allocated to three dietary treatments. The basal diet was either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (unspecified colistin:sulfate ratio) at a concentration of 60 mg/kg feed. The study lasted 21 days (+ 5 days of adaptation). Animals were weighed at the beginning and at the end of the trial. Feed intake was recorded daily to calculate ADG and FCR. The occurrence of diarrhoea was visually assessed during the first week and scored on a 0 (normal)-to-5 scale. From 18 to 21 days, faeces were collected to determine apparent digestibility of nutrients. At the end of the trial pigs were euthanised and intestinal segments from duodenum, jejunum, ileum and caecum were sampled for morphological measurements (*villi* height-VH, crypts depth-CD and their ratio), and quantitative detection of bacteria (from content of the small intestine). Mucosa scrapings were analysed for disaccharidases activity (maltase, sucrase and lactase). Real-time PCR was run to study gene expression of tight junction proteins CLDN1, OCLN and ZO-1 in duodenum and jejunum. At the end of the trial, the pigs treated with colistin showed, compared to the controls, higher ADG (334 vs. 193 g/day), FI (513 vs. 350 g/day) and improved F:G (1.53 vs. 1.83). The colistin-treated animals showed an enhancement of *villus* height (277 vs. 217 μm, 297 vs. 217 μm and 196 vs. 160 μm for duodenum, jejunum and ileum, respectively) and of the ratio *villus* height:crypt depth (2.03 vs. 1.58, 2.15 vs. 1.56, 2.00 vs. 1.62 for duodenum, jejunum and ileum, respectively). Disaccharidase activities in the jejunum were higher for the colistin-treated animals compared with the controls. Colistin increased gene expression of CLDN1 and ZO-1 in the jejunum and of ZO-1 in the duodenum. Dietary colistin sulfate supplementation at 60 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Yang et al. (2012), a total of 200 Duroc × Landrace × Yorkshire weaned pigs (21-day-old; average BW 5.98 kg) were distributed in 20 pens in groups of 10 animals and allocated to five dietary treatments. A basal diet was either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (Zhejiang qianjiang biochemical Co., ltd., Haining, China; unspecified colistin:sulfate ratio) at a concentration of 20 mg/kg feed. The study lasted 14 days. Animals were weighed at the start and at 7 and 14 days of trial, cumulative feed intake was recorded and F:G calculated. On days 7 and 14, six pigs per treatment were bled to assess serum D-lactic acid and diamino oxidase and slaughtered to collect intestinal samples to measure *villi* height and crypts depth. Jejunal mucosal diamine oxidase activity was measured. The faecal digesta were used to enumerate bifidobacteria, lactobacilli, *E. coli* and *S. aureus*. At the end of the trial, the pigs treated with colistin showed, compared to the controls, higher ADG (185 vs. 139 g/day) and improved G:F (0.64 vs. 0.57). Dietary colistin sulfate supplementation at 20 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Yang et al. (2016), a total of 90 Duroc × Landrace × Yorkshire weaned piglets (21-day-old; average BW 7.86 kg; both genders) were distributed in 18 pens in groups of five animals and allocated to three dietary treatments. The basal diets were either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (unspecified colistin:sulfate ratio) at a concentration of 20 mg/kg feed for 14 days. The study lasted 35 days. Animals' weight and cumulative feed intake were recorded at day 21 and 35 and G:F calculated. Diarrhoea incidence was monitored and recorded daily. On day 35, six piglets from each treatment were selected for blood collection to determine D-lactate, diamine oxidase-DAO, and anti-oxidation index. Piglets were then euthanised, and the gastrointestinal tract removed for morphology analyses of the small intestine (*villi* height, crypts depth and their ratio, goblet cells and intraepithelial lymphocytes), antioxidant capacity, cytokines and mRNA expression of occludin and ZO-1. At the end of the trial the colistin-treated pigs showed a reduction of the diarrhoea rate (2.84% vs. 4.48%). Colistin-treated pigs showed decreased *villi* height (439.9 vs. 521.1 μm and 360.8 vs. 465.6 μm in duodenum and jejunum, respectively) and crypt depth (256.0 vs. 318.1 μm in duodenum) compared with the controls. Regarding goblet cells, the colistin group showed a decrease in duodenum *villi* (4.60% vs. 6.58%) but an increase in ileum *villi* (6.33% vs. 4.17%). Intraepithelial lymphocytes were decreased in the ileum of colistin-treated pigs that also showed a higher concentration of cytokine IL-10 in the duodenum and in the jejunum. IL-2 and IL-1β were higher in ileum. Antioxidant index on intestinal mucosa was higher in colistin-treated pigs. MDA of treated pigs was lower in

jejunum and ileum. In colistin-treated pigs, occludin and ZO-1 were both lower in the jejunum. As for blood parameters, serum total antioxidant capacity, SOD, D-lactate and diamine oxidase were higher in colistin-treated pigs. The controversial results identified did not allow a conclusion on the growth-promoting effect of dietary colistin sulfate supplementation at 20 mg/kg feed in weaned piglets.

In the study of Yin et al. (2009), 60 piglets of both genders [(Landrace × Yorkshire) × Duroc] weaned at 21 days (average 7.35 kg BW) were used in a growth trial (Trial 1) and 12 barrows (breed not specified) weaned at 21 days (7.64 kg BW) fitted with a T-cannula in the terminal ileum in a digestibility trial (Trial 2). Animals were individually housed and allocated to three dietary treatments. The basal diet was either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin (unspecified form) at a concentration of 200 mg/kg feed. Both trials lasted 28 days. In Trial 1 ADG, FI and F:G were measured weekly. On days 7, 14 and 28 blood samples were obtained from five pigs for the measurement of serum free amino acids concentration. In Trial 2, on days 7, 14 and 28, ileal digesta samples were analysed for apparent ileal digestibility. At the end of Trial 1 pigs treated with colistin showed, compared to the control group, higher ADG (386 vs. 364 g/day). Regarding serum-free amino acid concentration pigs receiving the colistin treatment showed increases compared to the controls for Arg, His, Iso, Leu, Lys, Met, Phe, Thr, Try, Ala, Asp, Gly, Pro, Ser and Tyr. In the Trial 2, pigs treated with colistin had higher apparent ileal digestibility coefficients for Cys, His, Lys, Thr, Try, Val, Gly, Ser and Tyr. Dietary colistin supplementation at 200 mg/kg feed had a growth-promoting effect in weaned piglets.

In the study of Yu et al. (2017), a total of 180 piglets (Landrace × Yorkshire × Duroc; both genders) weaned at 25 days (average 7.98 kg BW) were distributed in 30 pens in groups of six animals and allocated to five dietary treatments. The diets (starter, grower and finisher) were either not supplemented or supplemented with different treatments. Two were the relevant treatments: a control and a treatment consisting of colistin sulfate (unspecified colistin:sulfate ratio) at a concentration of 20 mg/kg feed. The study lasted 28 days. Mortality and health status were checked every day and diarrhoea was scored on a 0 (normal)-to-3 scale. Animals were weighed every 2 weeks, FI recorded and ADG and F:G calculated. Faecal samples from all pens were collected during the last three days to calculate ATTD of DM, CP and gross energy and to determine short-chain fatty acid and lactate concentration. Fresh faecal samples were also analysed (days 14 and 28) for microbial populations (total bacteria, *E. coli*, *Bifidobacterium* and *Lactobacillus*). Blood samples were collected from six piglets per treatment on days 14 and 28 to determine D-lactate, diamine oxidase, endotoxins concentration and cytokines. At the end of the trial, the pigs treated with colistin showed, compared to the controls, a reduction of diarrhoea incidence (8.62% vs. 12.40%) and higher values of ATTD for OM (88.7% vs. 87.6%), CP (84.8% vs. 81.9%) and gross energy (87.1% vs. 85.8%). Pigs treated with colistin showed lower D-lactate, DAO and endotoxins than the controls. The colistin-treated group showed an increase in faecal lactate, acetate and butyrate. Cytokine levels in serum were decreased for TNF-α, IL-1β, IL-6 and increased for IL-10. The treated animals showed a reduction of *E. coli* and an increase of lactobacilli counts in faeces. Dietary colistin sulfate supplementation at 20 mg/kg feed had a growth-promoting effect in weaned piglets.

3.3.1.3.2. Studies in poultry

In the study reported by Cao et al. (2017), 360 one-day-old chickens (Ross 308) were distributed in 18 pens (20 birds per pen) and allocated to three dietary treatments (six pens or replicates per treatment). A basal diet was either not supplemented (control) or supplemented with different treatments. One of the treatments consisted on colistin sulfate (unspecified colistin:sulfate ratio) at a concentration of 20 mg/kg feed. The study lasted 42 days. Body weight (BW) and cumulative feed intake were recorded weekly and F:G calculated. In addition, 18 birds per treatment (three per pen) were selected on days 1, 7, 14, 21, and 42 and euthanised to sample the faecal digesta, to measure metabolites, and to sequence microbiota by high-throughput sequencing. Up to day 14, birds treated with colistin showed, compared to the control group, increased body weight (ca. 348 vs. 305 g BW) and average daily gain (22.0 vs. 18.9 g/day). Body weight was also increased at day 35 (1,458 vs. 1,221 g BW), with no differences in average daily gain. F:G was improved in colistin treated birds at day 21 (1.51 vs. 1.76), but not over the whole experimental period. At the end of the trial, birds treated with colistin showed less counts of *Ruminococcus*, Ruminococcaceae and Erysipelotrichaceae and more Lachnospiraceae and Rikenellaceae in the faecal digesta. In addition, birds with colistin had decreased concentration of some metabolites in the caeca (oleic acid, sophorose, threitol, 2-deoxyerythrito, palmitoleic acid, 6-methyl-previtamin D, and 5-cholestan-3-one), whereas

concentrations of maltotriose and 1-monopalmitin were increased. Colistin sulfate had growth-promoting effects in chickens for fattening when added to feed at 20 mg/kg feed.

In the study reported by Chamba et al. (2014), 924 one-day-old chickens (Cobb 500) were distributed in 21 pens (44 birds per pen) and allocated to three dietary treatments (seven pens or replicates per treatment). Three basal diets (starter, grower and finisher) were either not supplemented (control) or supplemented with different treatments. One of the treatments consisted on colistin (unspecified form) at a concentration of 100,000 IU (4.88 mg)/kg BW (this would be approximately 60 mg/kg feed using FEEDAP default values for BW and feed intake). The study lasted 42 days. Mortality and health status were checked daily. Body weight and cumulative feed intake were recorded every two weeks and F:G calculated. In addition, seven birds per treatment were selected on days 14, 28 and 42 and euthanised to weight internal organs (proventriculus, gizzard, liver, spleen and pancreas); measure the length of the intestines; sample duodenum, jejunum, and ileum for histological evaluation; and collection of digesta from jejunum to enumerate *E. coli*. At the end of the trial, birds treated with colistin showed, compared to the control group, an improved body weight (2035 vs. 1984 g BW), and F:G (1.76 vs. 1.80). On day 14, jejunum, ileum, and small intestine were proportionally longer in colistin-treated birds than in the control group. In addition, on day 14, colistin-treated birds showed deeper duodenal and jejunal crypts than control animals (279 vs. 183 μm and 371 vs. 230 μm, respectively), with smaller *villus* to crypt ratios (1.7 vs. 2.47 and 1.9 vs. 3.61, respectively). On the contrary, at the end of the trial with colistin jejunal *villi* were longer (957 vs. 836 μm) and crypts depths in the ileum were reduced (450 vs. 529 μm), so that *villus* height to crypt depth ratio in the ileum was greater than in control birds (3.0 vs. 2.34). Colistin had growth-promoting effects in chickens for fattening when added to feed at 60 mg/kg.

In the study reported by El-Faham et al. (2018), 150 one-day-old broiler unsexed chickens (Arbor Acres) were distributed in 15 pens (ten birds per pen) and allocated to five dietary treatments (three pens or replicates per treatment). Two basal diets (starter and grower) were either not supplemented (control) or supplemented with different treatments. One of the treatments consisted on colistin sulfate (Colistix® - Agrovet, Perú; unspecified colistin:sulfate ratio) at a concentration of 1,000 mg/kg feed. The study lasted 35 days. Mortality and health status were checked every day. Body weight and cumulative feed intake were recorded on days 14 and 32 and F:G calculated. On day 32, three chickens per group were euthanised and breast, thigh, drumstick, wings, neck, abdominal fat and lymphoid organs weighed. On day 35, blood samples were taken from other three chickens per treatment and total lipids, triglycerides, cholesterol, high-density lipoprotein, low-density lipoprotein, alanine aminotransferase, and aspartate transaminase concentrations in plasma were determined. There were no differences between control and colistin supplemented chickens in any of the growth performance parameters. At the end of the trial, the birds receiving colistin showed, compared to the control group, smaller breast relative weight (42.5% vs. 45.0% of carcass) and higher plasma concentrations of total lipids. Colistin sulfate at 1,000 mg/kg feed had no growth-promoting effects in chickens for fattening.

In the study reported by He et al. (2019), 396 one-day-old chickens (Arbor Acres) were housed in 36 pens (11 birds per pen) and allocated to six experimental treatments following a 2 × 3 factorial design (challenged or not with a pathogen (*Salmonella*) and three dietary treatments). The only two diets of interest for the assessment were the unsupplemented control and that supplemented with 40 mg colistin sulfate (unspecified colistin:sulfate ratio)/kg feed, in both cases when not challenged with the pathogen. The study lasted 42 days. Mortality and health status were recorded daily. Body weight and feed intake were measured on days 14, 21, and 42 and F:G calculated. In addition, on days 14 and 21, six birds per treatment were euthanised and the ileum mucosa and digesta collected to measure histomorphometry, ileal permeability, activity of myeloperoxidase, host gene expression (claudin-1, occludin, mucin-2, IFN-γ, TLR2, TLR4, NF-κB, MyD88 and GAPDH genes), and the microbiota in the ileum through the high-throughput sequencing of the microbial DNA. There were no differences between control and colistin treatments in final BW or in feed intake, whereas F:G was worse in birds receiving colistin supplemented diets. The main zootechnical parameters were not affected by the antimicrobial, and thus growth-promoting effects of colistin sulfate at 40 mg/kg feed could not be demonstrated.

One study using colistin sulfate in ducks was reported in two publications (Lin et al., 2017, 2019). In this study, 275 female ducks (Linwu, 49 days of age) were distributed in 25 cages (11 birds per pen) and allocated to five dietary treatments (five pens or replicates per treatment). A basal diet was either not supplemented (control) or supplemented with different treatments. One of the treatments (used as a positive control) consisted of colistin sulfate (pure colistin sulfate manufactured by

Guangzhou Xingda Animal's Pharmaceutical Company; unspecified colistin:sulfate ratio) at a concentration of 30 mg/kg feed. The study lasted 21 days. Nothing was reported on any monitoring of mortality or health status of the ducks. Feed intake was recorded daily, ducks were weighed at the end of the trial and F:G was calculated. At the end of the trial, five birds per group were euthanised, dissecting the liver and small intestine (duodenum, jejunum and ileum) to examine the mRNA expression levels of hepatic antioxidant-related genes (by real-time PCR) and the intestinal mucosal morphology (Lin et al., 2017). In the publication of Lin et al. (2019), some additional results of the same study were reported. In this case, five birds per group were euthanised at the end of the trial and the carcass and the internal organs, abdominal fat, thigh, breast, and lean meat were weighed to determine carcass characteristics. In addition, blood samples were collected from another five animals per group to measure serum biochemistry (glucose, triglycerides, cholesterol, uric acid, urea nitrogen, creatinine, total protein, albumin, globulin, alkaline phosphatase, alanine transaminase and aspartate aminotransferase). Antioxidant and peroxidation biomarkers (concentrations of MDA, reduced glutathione, hepatic protein carbonyl and 8-hydroxy-2'-deoxyguanosine and activities of SOD, CAT, GSH-Px and total antioxidant capacity) were measured in serum and liver. The thigh and breast muscles were used for meat quality analysis, measuring meat colour, pH, drip loss, cooking loss, and shear force. Dietary supplementation with colistin had no effects on final BW, weight gain, feed intake or F:G. Compared with the control diet, supplementation with colistin sulfate reduced the percentage of abdominal fat (0.60% vs. 0.69% of eviscerated weight) and increased the percentage of lean meat (21.1% vs. 19.8% of eviscerated weight), increased the *villus* height to crypt depth ratio in the duodenum (not in the jejunum or ileum), and had some effects on colour and pH of leg muscle (but not of breast muscle). The manganese superoxide dismutase-2 mRNA expression level in the liver was greater in ducks supplemented with colistin than in those fed the control diet. Ducks fed the colistin sulfate supplemented diet showed higher levels of oxidation products in serum and liver, suggesting that long term intake of colistin caused formation of reactive oxygen species. Dietary colistin sulfate supplementation at 30 mg/kg feed had a growth-promoting effect in ducklings for fattening.

3.3.1.4. Discussion

From the studies examined, the test item has been described as (i) 'colistin sulfate' (unspecified colistin:sulfate ratio, 15 publications reporting 14 studies) or (ii) 'colistin' (unspecified form; eight studies). Therefore, in all studies an uncertainty on the exact product used/concentration applied has been identified.

A detailed analysis of the uncertainties for colistin 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).

3.3.1.4.1. Pigs

A total of 17 studies were considered suitable for the assessment of colistin as a potential growth promoter antimicrobial in pigs: 16 in weaned piglets and one in pigs for fattening.

In weaned piglets, most of the studies (12) showed that dietary colistin supplementation at 12 to 200 mg/kg feed had positive growth-promoting effects in piglets (Guerra et al. (2007), 12 mg colistin sulfate/kg feed; Mazutti et al. (2016), 20 mg colistin/kg feed; Yang et al. (2012) and Yu et al. (2017), 20 mg colistin sulfate/kg feed; Cui et al. (2019), 40 mg colistin sulfate/kg feed; Sbardella et al. (2016) and Silva-Júnior et al. (2020), 40 mg colistin/kg feed; Wan et al. (2015), 60 mg colistin sulfate/kg feed; Cao et al. (2019), 100 mg colistin sulfate/kg feed; Kong et al. (2007), Liu et al. (2016) and Yin et al. (2009), 200 mg colistin/kg feed). Only in two studies no growth-promoting effects of colistin in piglets were observed when administered in the diet (Santana et al. (2015), 40 mg colistin sulfate/kg feed; Conde et al. (2001), 150 mg colistin/kg feed). When administered at 2,000 mg colistin sulfate/kg feed, colistin showed negative effects in growth in weaned piglets (Furbeyre et al., 2017). Finally, from the study of Yang et al. (2016), no conclusions could be drawn.

In pigs for fattening, the only study assessed (Tian-Yang et al., 2013) showed that dietary colistin supplementation at 80 mg colistin sulfate/kg feed did not have growth-promoting effects.

3.3.1.4.2. Poultry

Five studies (six publications) were considered suitable for the assessment of colistin as a potential growth-promoter antimicrobial in poultry. In four studies, the antimicrobial was tested in chickens for fattening, and in the other one in ducklings for fattening.

In two of the studies, improved growth performance was observed in chickens for fattening when colistin was supplemented to feed at concentrations of 20 mg colistin sulfate/kg feed (Cao et al., 2017)

or 60 mg colistin/kg (Chamba et al., 2014). In contrast, from the other two studies, no growth promotion effects were shown in chickens for fattening when feed was supplemented with colistin at either 40 mg colistin sulfate/kg feed (He et al., 2019) or 1,000 mg colistin sulfate/kg feed (El-Faham et al., 2018)

In the single study with ducklings for fattening, growth-promoting effects were observed when birds were fed diets supplemented with colistin at 30 mg colistin sulfate/kg feed (Lin et al., 2017, 2019).

3.3.1.5. Concluding remarks

It is judged 66–90% certain ('likely') that colistin or colistin sulfate has growth-promoting/increase yield effects in weaned piglets at concentrations ranging from 12 to 200 mg/kg complete feed (12 studies); above this upper limit (200 mg colistin/kg) it is judged 33-66% certain ('about as likely as not') that colistin sulfate may exert negative effects on growth when administered at 2,000 mg/kg complete feed (one study).

In poultry, it is judged 33–66% certain ('about as likely as not') that colistin or colistin sulfate has growth-promoting effects when added to feed at concentrations ranging from 20 to 60 mg/kg in chickens for fattening (two studies) and at 30 mg colistin sulfate/kg feed in growing ducks (one study).

No data are available in the scientific literature showing effects of colistin or colistin sulfate on growth promotion/increased yield when added (i) to weaned piglets feed at concentrations below 12 mg/kg, (ii) to chickens for fattening feed at concentrations below 20 mg/kg, (iii) to growing ducks feed at concentrations below 30 mg/kg, or (iv) to feed of any other food-producing animal species or categories.

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 colistin 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 colistin 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 colistin in feed of food-producing animals that have an effect in terms of growth promotion/increased yield?

- It is judged 66–90% certain ('likely') that colistin or colistin sulfate have growth-promoting/ increase yield effects in weaned piglets at concentrations ranging from 12 to 200 mg/kg complete feed (12 studies); above this upper limit (200 mg colistin/kg) it is judged 33–66% certain ('about as likely as not') that colistin sulfate may exert negative effects on growth when administered at 2,000 mg/kg complete feed (one study).
- In poultry, it is judged 33–66% certain ('about as likely as not') that colistin or colistin sulfate have growth-promoting effects when added to feed at concentrations ranging from 20 to 60 mg/kg in chickens for fattening (two studies) and at 30 mg colistin sulfate/kg feed in growing ducks (one study).
- No data are available in the scientific literature showing effects of colistin or colistin sulfate on growth promotion/increased yield when added (i) to weaned piglets feed at concentrations below 12 mg/kg, (ii) to chickens for fattening feed at concentrations below 20 mg/kg, (iii) to growing ducks feed at concentrations below 30 mg/kg, or (iv) to feed of any other food-producing animal species or categories.

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. Recommendations

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

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Abbreviations

ADFI	average daily feed intake
AQ	assessment question
ATTD	apparent total tract digestibility
bw	body weight used in toxicity studies
BW	body weight
CAT	catalase
CP	crude protein
CPS	capsular polysaccharide
EE	ether extract
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
GE	fraction of the antimicrobial that is secreted back into the intestinal tract for elimination, after initially being absorbed into the bloodstream
F:G	feed to gain ratio
G:F	gain to feed ratio
GSH-Px	glutathione peroxidase
I	fraction of the antimicrobial present in the digestive tracts that would be inactive on the microbiota
LPS	lipopolysaccharide
L-Ara4	*N*-4-amino-4-deoxy-L-arabinose
MDA	malondialdehyde
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
MDR	multidrug resistance
MSC	minimal selective concentration
OM	organic matter
PEA	phosphoethanolamine
PMSC	predicted MSC
SOD	superoxide dismutase
ToRs	terms of reference

Appendix A – List of excluded publications and their shortcomings

The publications excluded from the assessment of the effects of colistin on growth promotion/increase 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 A.1.

Table A.1: Publications not relevant for the assessment of the effects of colistin 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)
Abdulameer and Firas (2018)	Poultry								X(1)	
Alabiso et al. (2017)	Rabbits	X								
Amer et al. (2020)	Poultry				X	X			X(2)	
Ateya et al. (2019)	Poultry					X				
Bovera et al. (2009)	Rabbits	X			X		X			X(3)
Bovera et al. (2010)	Rabbits	X					X			X(3)
Bovera et al. (2012)	Rabbits	X								X(3)
Bozorgmehri Fard (2004)	Poultry									X(3),(4)
Bruno et al. (2013)	Pigs	X								
Candotti and Cossettini (2010)	Pigs	X								X(3)
Che et al. (2017)	Pigs	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)
Choi et al. (2011)	Pigs	X						X		X(3)
Cunha et al. (2017)	Rabbits	X					X		X	X(3)
da Rosa et al. (2015)	Pigs	X				X				X(3)
Ekim et al. (2016)	Poultry					X				X(5)
Ekim et al. (2020)	Poultry					X				
Elbayoumi et al. (2017)	Poultry						X		X(2)	
García et al. (2007)	Poultry	X								
Grecco et al. (2018)	Pigs	X							X(2)	X(3)
Hamid et al. (2018)	Poultry	X								
Hamid et al. (2019)	Poultry	X								
Han et al. (2016)	Pigs	X								
Hassan (2020)	Poultry	X								
Herawati et al. (2020)	Poultry									X(4)
Huang et al. (2015)	Pigs	X								
Jang et al. (2004)	Poultry									X(3)

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)
Jin et al. (2008a)	Pigs	X								
Jin et al. (2008b)	Pigs	X								
Jin et al. (2009)	Pigs	X								
Kim et al. (2011a)	Ruminants	X								X(3)
Kim et al. (2011b)	Ruminants	X								
Kuang et al. (2015)	Pigs	X								X(3)
Li et al. (2008)	Pigs	X				X				
Li et al. (2012)	Pigs	X								
Li et al. (2017)	Pigs	X								
Li et al. (2018a)	Poultry	X								
Li et al. (2018b)	Pigs	X								X(3)
Lipinski et al. (2010)	Pigs						X			
Liu et al. (2020)	Poultry	X								
Long et al. (2016)	Pigs	X								
Long et al. (2018)	Pigs	X								
Luise et al. (2019)	Pigs					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)
Meurer et al. (2010)	Poultry	X								
Mohebodini et al. (2019)	Poultry					X				
Neveling et al. (2017)	Poultry	X								
Oliveira et al. (2017)	Pigs									X(3)
Pan et al. (2017)	Pigs	X				X				
Piccolo et al. (2009)	Rabbits	X							X	X(3)
Piva et al. (2007)	Pigs	X	X						X	X(3)
Romero et al. (2012)	Rabbits	X								
Savoini et al. (2002)	Pigs									X(3),(4)
Scheuermann et al. (2009)	Poultry	X								
Shi et al. (2018)	Pigs	X								
Shim et al. (2010)	Poultry		X							X(3)
Simon et al. (2016)	Poultry	X						X(6)		X(7)
Soler et al. (2018)	Pigs	X								
Song et al. (2019)	Pigs	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)
Tang et al. (2009)	Pigs					X	X			
Tang et al. (2013)	Pigs			X[8]		X	X		X	
Tawfeeq and Al Mashhdani (2020)	Poultry								X	
Teo and Tan (2006)	Poultry	X								X[3]
Tian and Piao (2019)	Pigs	X								
Torrallardona et al. (2002)	Pigs					X				
Torrallardona et al. (2003)	Pigs								X	
Torrallardona et al. (2007)	Pigs					X				
Trevisi et al. (2015)	Pigs					X				
Tummaruk et al. (2009)	Pigs									X[3]
Vilà et al. (2010)	Pigs	X								X[9]
Wang et al. (2016)	Pigs	X								X[3],[10]
Wang et al. (2017a)	Poultry					X				
Wang et al. (2017b)	Pigs									X[3]

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)
Wang et al. (2020)	Poultry					X				
Wu et al. (2012)	Pigs	X				X(11)				
Wu et al. (2018)	Poultry	X								
Xu et al. (2006)	Poultry	X								
Xu et al. (2018)	Pigs	X								
Yen et al. (2015)	Pigs								X	
Zangeronimo et al. (2011)	Pigs	X								
Zhang et al. (2014)	Poultry					X				
Zhang et al. (2016)	Poultry					X				
Zhao et al. (2019)	Pigs	X								
Zhu et al. (2017)	Pigs	X								
Zong et al. (2019)	Pigs	X								X(3)
Zou et al. (2019)	Pigs	X								X(3)
Zwirzitz et al. (2019)	Pigs	X								X(4)

(1): Antimicrobial administered via water for drinking and data on water consumption not available.
(2): No mention made of statistical analyses in Methods, Results or Tables.

(3): Absence of a control group without antimicrobial.
(4): Insufficient/unproper replication.
(5): Same results reported as in Ekim et al. (2020).
(6): Only BW reported.
(7): The authors investigated the immune response of multi-drugs treated (dysbiotic) animals.
(8): Antimicrobial administered via gavage.
(9): No antimicrobial used in Exp. 1.
(10): No antimicrobial used in Exp. 1 and 2.
(11): From day 13 of the experiment.

Appendix B – Table of uncertainties

Table B.1: Potential sources of uncertainty identified in the levels of colistin 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. Considering that several 'sulfate' products exist, with different molecular weight and a different colistin/sulfate ratio, in the absence of precise information on the form used, the calculation to the 'base' substance could not be done. Therefore, the concentrations have been reported as described in the studies.	Underestimation or overestimation 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), although meta-analysis was not applicable to the studies retrieved, evidence synthesis was done, since: • 12 studies showing consistent (positive) results in a comparable range of concentrations were available in weaned piglets. The uncertainty resulting in the process of evidence synthesis was based on 17 studies, 12 showing positive effect, 4 showing no effects and 1 showing negative effects. For chickens for fattening and growing duck the low number of studies retrieved prevented evidence synthesis.	The extent of the underestimation or overestimation on the levels which shown growth-promoting effect is modulated by the consistency of the results.