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## **Risk for the development of Antimicrobial Resistance (AMR) due to feeding of calves with milk containing residues of antibiotics**

Antonia Ricci, Ana Allende, Declan Bolton, Marianne Chemaly, Robert Davies, Pablo Salvador Fernández Escámez, Rosina Girones, Kostas Koutsoumanis, Roland Lindqvist, Birgit Nørrung, et al.

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## Risk for the development of Antimicrobial Resistance (AMR) due to feeding of calves with milk containing residues of antibiotics

EFSA Panel on Biological Hazards (BIOHAZ),  
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### Abstract

EFSA was requested to: 1) assess the risk for the development of antimicrobial resistance (AMR) due to feeding on farm of calves with colostrum potentially containing residues of antibiotics; 2) assess the risk for the development of AMR due to feeding on farm of calves with milk of cows treated during lactation with an antibiotic and milked during the withdrawal period, and 3) propose possible options to mitigate the risk for the development of AMR derived from such practices. Treatment of dairy cows during the dry period and during lactation is common in the EU Member States. Penicillins, alone or in combination with aminoglycosides, and cephalosporins are most commonly used. Residue levels of antimicrobials decrease with the length of the dry period. When the interval from the start of the drying-off treatment until calving is as long as or longer than the minimum specified in the Summary of Product Characteristics of the antimicrobial, faecal shedding of antimicrobial-resistant bacteria will not increase when calves are fed colostrum from treated cows. Milk from cows receiving antimicrobial treatment during lactation contains substantial residues during the treatment and withdrawal period. Consumption of such milk will lead to increased faecal shedding of antimicrobial-resistant bacteria by calves. A range of possible options exist for restricting the feeding of such milk to calves, which could be targeting the highest priority critically important antimicrobials.  $\beta$ -Lactamases can reduce the concentration of  $\beta$ -lactams which are the most frequently used antimicrobials in milking cows. Options to mitigate the presence of resistant bacteria in raw milk or colostrum are mainly based on thermal inactivation.

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**Keywords:** antimicrobial resistance, antimicrobial residues, waste milk, colostrum, calves

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**Amendment:** The following changes in section 1.4.5 on page 11 were made:

- (i) The first sentence at the beginning of 1.4.5.1 was separated into two sentences for which new references were provided: 'The microbial load of waste milk may contribute to calf disease (e.g. diarrhoea) (Godden et al., 2012). Mastitis pathogens and other bacteria, such as *Mycobacterium avium* subsp. *paratuberculosis* (MAP), may be present (Gonzalez and Wilson, 2003; Houser et al., 2008)'.
- (ii) Two additional references were added to support the following sentence in 1.4.5.1: 'For this reason, pasteurisation of milk has been advocated by a number of studies to prevent spread of such bacteria to replacement stock (Donahue et al., 2012; Godden et al., 2012)'.
- (iii) Reference Al Mawly et al., 2015 was moved from 1.4.5.1 to 1.4.5.2: 'Feeding of waste milk potentially containing antimicrobials has been associated with reduced shedding of *Campylobacter* spp., a zoonotic pathogen (Klein et al., 2013) and a reduced incidence of clinical disease (Brunton et al., 2014; Al Mawly et al., 2015)'.
- (iv) New references "Gonzalez and Wilson, 2003"; "Houser et al., 2008" were included in the reference list.

An editorial correction was carried out that does not materially affect the contents or outcome of this scientific output. To avoid confusion, the older version has been removed from the EFSA Journal, but is available on request, as is a version showing all the changes made.

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## Summary

Following a request from the European Commission (EC), the European Food Safety Authority (EFSA) was asked to deliver a Scientific Opinion on the Risk for the development of antimicrobial resistance (AMR) due to feeding of calves with milk containing residues of antibiotics. EFSA commissioned the Scientific Panel on Biological Hazards (BIOHAZ) to work in delivering the above Opinion. EFSA was asked to: 1) assess the risk for the development of antimicrobial resistance (AMR) due to feeding on farm of calves with colostrum potentially containing residues of antibiotics (ToR 1); 2) assess the risk for the development of AMR due to feeding on farm of calves with milk of cows treated during lactation with an antibiotic (ToR 2), and 3) propose possible options to mitigate the risk for the development of AMR associated with such practices, if relevant (ToR 3).

Due to lack of quantitative data, the BIOHAZ Panel decided to conduct a qualitative evaluation of the risk. To assist in the formulation of the Opinion, the published information available was reviewed, including relevant scientific literature, data from national surveillance and monitoring programmes and the information collected through a Questionnaire sent by the EC to the European Union (EU) Member States (MSs). The evidence was collected and assessed by expert opinion, and related conclusions were formulated in a qualitative way.

The assessment was structured as follows:

- 1) current farming practices in the EU countries in relation to treatment of dairy cows with antimicrobials and the use of the milk potentially containing antimicrobial residues available on dairy farms (colostrum and post-colostral milk from cows treated at the beginning of the dry period as well as to non-marketable milk from cows treated during lactation, waste milk) as feed for calves;
- 2) antimicrobial residues present in milk and colostrum from treated cows;
- 3) antimicrobial-resistant bacteria present in colostrum and milk from treated cows;
- 4) description of experimental studies providing direct evidence of the effect of feeding waste milk containing antimicrobial residues to calves by analysing data collected: a) on farms where waste milk was routinely fed; b) on farms where waste milk was fed to a group of calves compared to a control group; and c) by carrying out experimental studies where milk with known concentrations of antimicrobials was fed to calves in comparison with a control group.
- 5) discussion of the data collected in relation to the knowledge available in the scientific literature on consequences of exposure of bacteria to antimicrobial residues for development of resistance;
- 6) potential feeding management and mitigation options to reduce the risk of the development of antimicrobial-resistant bacteria resulting from calf feeding practices.

Intramammary treatment of dairy cows for prevention and/or treatment of udder infections during the dry period is common in the EU, mainly using penicillins, alone or in combination with aminoglycosides, or 1st- and 2nd-generation cephalosporins. In some MSs, 3rd- and 4th-generation cephalosporins are also very commonly used. Results from scientific literature on the time needed between treatment and parturition indicated that the antimicrobial residue levels in colostrum samples are expected to be low. Only two studies presented results on the antimicrobial residue levels in field colostrum samples showing a low number of samples with levels above the maximum residue levels (MRLs) or detection limit. Feeding calves with colostrum from dairy cows treated with antimicrobials at the beginning of the dry period is a common practice in the majority of dairy farms in the EU. One observational study showed that feeding calves' colostrum from cows treated with penicillins and aminoglycosides at the beginning of the dry period does not increase the faecal shedding of antimicrobial-resistant *Escherichia coli*.

Treatment of dairy cows during lactation is common in the EU and mainly penicillins and 1st- and 2nd generation cephalosporins are used. In some MSs, also 3rd- and 4th-generation cephalosporins are very commonly used. Feeding calves with milk from dairy cows treated with antimicrobials during lactation and milked during the withdrawal period is a common practice, occurring in the majority of dairy farms in Europe. An observational study showed a comparable effect on the faecal shedding of resistant *Escherichia coli* when feeding calves waste milk only during the withdrawal period, and when feeding calves' milk obtained during both the treatment and the withdrawal period. The contribution of antimicrobial-resistant bacteria present in waste milk fed to calves on the faecal shedding of antimicrobial-resistant bacteria and genes cannot be quantified with the existing data. This route is

considered to be less important compared to the presence of antimicrobial residues in waste milk. In a single experimental study, pasteurisation of waste milk did not reduce the level of faecal shedding of AMR *E. coli* in calves.

**For ToR 1** 'Assess the risk for the development of AMR due to feeding on farm of calves with colostrum potentially containing residues of antibiotics', the assessment focused on colostrum and transition or post-colostral milk (from day 1 to 5). It was concluded that when the interval from the dry-off treatment until calving is as long as or longer than the minimum specified in the Summary of Product Characteristics of the antimicrobial product, faecal shedding of antimicrobial resistant bacteria will not increase when calves are fed colostrum from treated cows. When cows calve earlier than the minimum withdrawal period specified in the Summary of Product Characteristics for the antimicrobial product, the levels of antimicrobial in the colostrum are higher, and therefore, there is an increased probability of shedding of antimicrobial-resistant bacteria by calves receiving the colostrum. However, the available evidence is insufficient to quantify this increase, and no effect was observed in the single observational study dealing with this subject.

**For ToR 2**, 'Assess the risk for the development of AMR due to feeding on farm of calves with milk of cows treated during lactation with an antibiotic and milked during the withdrawal period', the assessment focused on milk (excluding colostrum and transition milk included above) from cows treated with antimicrobials during lactation. This included both systemic treatment with antimicrobials that have a withdrawal period for milk and/or local intramammary treatment. It was concluded that milk from cows receiving antimicrobial treatment during lactation contains substantial residues during the treatment and withdrawal period. Consumption of such milk will lead to increased faecal shedding of antimicrobial-resistant bacteria by calves.

**For ToR 3**, 'Propose possible options to mitigate the risk for the development of AMR derived from such practices if relevant', it was concluded that there are three principal approaches for reducing the risk for development of AMR derived from feeding waste milk or colostrum containing antimicrobial residues to calves.

- 1) Measures in feeding management when feeding calves colostrum and milk potentially containing residues of antimicrobials. The following options for restricting the feeding of milk and/or colostrum from treated cows to calves were discussed: 1) completely prohibiting the use of milk from treated cows; 2) prohibiting the use of milk from cows treated with antimicrobials of specific importance in human health care, i.e. the highest priority critically important antimicrobials (CIAs); 3) prohibiting the use of milk when the level of residues is expected to be high, i.e. during treatment or after intramammary administration.
- 2) Measures to destroy antimicrobial residues before feeding. Most of the approaches focus on the reduction of  $\beta$ -lactams (penicillins or cephalosporins) which are the most degradable and frequently used antimicrobials in milking cows. Less evidence is demonstrated in the literature against other antimicrobials used in dairy cows.  $\beta$ -Lactams (penicillins and cephalosporins) can be degraded to a level below the detection limit by direct incubation with specific  $\beta$ -lactamases. Fermentation with  $\beta$ -lactamase-producing microorganisms can efficiently reduce penicillin and cefquinome as was reported in two studies. The main drawbacks are an increase in microbial load and the lack of knowledge of the mechanism of action. Combination of ultrafiltration and permeate washes and electrochemical oxidation are not easily applicable on the farm level but have the potential to reduce a broader spectrum of antimicrobials. Increasing the pH in the milk to 10 has the potential to efficiently reduce the concentration of certain antimicrobials (at least cefquinome) as was shown in one study. The effectiveness for a broader range of antimicrobials and the suitability of this milk to be fed to calves needs further consideration.
- 3) Measures to eliminate antimicrobial-resistant bacteria. Options to mitigate the presence of the resistant bacteria in raw milk or colostrum are mainly based on thermal inactivation. Heat treatment at suitable temperature/time combinations will kill the vegetative bacteria (including antimicrobial-resistant ones) in raw milk and colostrum. Non-thermal options (e.g. microfiltration, centrifugation) are less effective and are not easily practicable at the farm level.

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## 1. Introduction

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

Combating antimicrobial resistance is a priority for the EC which launched in 2011 a 5-year Action Plan against the rising threats from AMR, based on a holistic approach, in line with the 'One Health' initiative. The plan introduced a set of rigorous measures to fight against AMR. Any use of antimicrobials, either in human or veterinary medicine, might result in the development of AMR and has an impact on human and animal health, although the specific impact has not been quantified to date.

Calves may be fed with milk originating from the same farm which potentially contains residues of antibiotics. Two cases have to be differentiated:

- Colostrum of cows treated with long-acting antibiotics at the start of their dry period
- Milk of cows that have been treated during their lactation with an antibiotic and which have been milked during the withdrawal period.

The potential risk for development of AMR due to such practices is unclear. In September 2014, the Commission sent a questionnaire to the Member States with a view to clarify the situation. The summary of the responses and the raw data received from the Member States will be sent to the EFSA secretariat by direct mail.

#### Legal situation:

- Regulation (EC) No 767/2009 on the placing on the market and use of feed requires that feed must be safe.
- Withdrawal periods as laid down in Directive 2001/82/EC on the Community code relating to veterinary medicinal products are established to define from which point in time the animal product is fit for human consumption.
- Directive 2002/32 on undesirable substances in animal feed establish limits for coccidiostats and histomonostats but not for antibiotics.
- Regulation (EC) No 1069/2009 laying down health rules as regards animal by-products and derived products not intended for human consumption contains the following provisions:
  - Art 2(2)(e) excludes raw milk, colostrum and products derived therefrom which are obtained, kept, disposed of or used on the farm of origin, from the provisions of the Regulation.
  - Art 9(c) categorising animal by-products containing residues of authorised substances or contaminants exceeding the permitted levels as referred to in Article 15(3) of Directive 96/23/EC, as category 2 materials (prohibited for placing on the market for animal feeding).

Therefore, feeding of calves with milk containing residues of antibiotics on the farm of origin is not harmonised and subject to national rules.

The EC requests the European Food Safety Authority (EFSA) to:

- Assess the risk for the development of AMR due to feeding on farm of calves with colostrum potentially containing residues of antibiotics;
- Assess the risk for the development of AMR due to feeding on farm of calves with milk of cows treated during lactation with an antibiotic and milked during the withdrawal period;
- Propose possible options to mitigate the risk for the development of AMR derived from such practices if relevant.

### 1.2. Interpretation of the Terms of Reference

The above terms of reference (ToR) have been further discussed and clarified by EFSA and the European Commission (EC), the requestor of the mandate. Each individual ToR is further examined below and its interpretation in the framework of this Scientific Opinion is presented.

ToR 1: 'Assess the risk for the development of AMR due to feeding on farm of calves with colostrum potentially containing residues of antibiotics'

ToR 1 will focus on colostrum and transition or post-colostral milk (from day 1 to 5).



ToR 2: 'Assess the risk for the development of AMR due to feeding on farm of calves with milk of cows treated during lactation with an antibiotic and milked during the withdrawal period'.

ToR 2 will focus on milk (excluding colostrum and transition milk included above) from cows treated with antimicrobials during lactation. It will include both systemic treatment with antimicrobials that have a withdrawal period for milk and local intramammary treatment.

The starting point of the assessment of ToR 1 and ToR 2 is the presence of antimicrobial residues and/or bacteria resistant to antimicrobials in milk and colostrum from cows treated with antimicrobials. This assessment does not address the medication policies in place in the EU or any aspects covering the marketing authorisation of veterinary drugs. It will also not cover the factors influencing the emergence of AMR in bacteria in dairy cows due to the use of antimicrobials, nor the role of 'prudent use' of antimicrobials in reducing the emergence of resistance. Most of these subjects were addressed in a recent EMA–EFSA Joint Scientific Opinion of the EFSA (EMA/EFSA, 2017).

Depending on the data available, for both residues and AMR, the Opinion will focus on the most commonly used antimicrobials for treatment of dairy cows.

This mandate is relevant from the public health point of view. However, the Opinion does not do a full farm-to-fork risk assessment on AMR development, and stops at the level of the development and the excretion of resistant bacteria from calves fed with either colostrum or milk potentially contaminated with antimicrobial residues or antimicrobial-resistant bacteria. The impact on human health of the practices indicated in the ToRs is not covered by the Opinion.

ToR 3: 'Propose possible options to mitigate the risk for the development of AMR derived from such practices if relevant'.

Different mitigation options will be considered, and the advantages and disadvantages of each option will be analysed. The measures used to treat the milk both aiming to destroy the residues and/or resistant bacteria will be evaluated. The Opinion will mainly focus on strategies performed at the farm level.

Animal health and welfare considerations as well as environmental risks that may originate from feeding calves with milk or colostrum of cows treated with antimicrobials or by disposal of these milk and colostrum into the environment are outside the scope of this mandate.

### 1.3. Definitions

Relevant definitions are provided in the Glossary included at the end of the Opinion.

### 1.4. Framework of the situation where milk from cows treated with antimicrobials may be used

#### 1.4.1. Waste milk

Waste milk is milk that cannot be marketed for human consumption, and may include milk from cows treated with antimicrobials but also milk from cows that cannot be marketed for other reasons, e.g. use of other types of drugs, increased somatic cell counts or post-colostral transition milk. Its use as feed may occur in different groups of calves that have a different future, i.e. being sold for meat production purposes (veal or beef), being raised as replacement stock or being kept on the farm for the production of veal or beef. Apart from the risk of spread of AMR as addressed in this Opinion, the milk may also contribute to the spread of pathogens from the cow herd to the calves. This feature is shared with other raw milk. Finally, milk containing antimicrobials may have other effects on the calves in addition to increasing the levels of AMR in bacteria in the gut of the animal.

The importance of different antimicrobials that may be contained in waste milk differs from a public health perspective. The World Health Organization (WHO) has categorised antimicrobial substances concerning their importance (WHO, 2012, 2016; Collignon et al., 2016) and updates are available on WHO website ([http://www.who.int/foodsafety/areas\\_work/antimicrobial-resistance/cia/en/](http://www.who.int/foodsafety/areas_work/antimicrobial-resistance/cia/en/); see Table A.1 in Appendix A). Most of the antimicrobials that are licensed for use in dairy cattle are from substance classes that are also important in human medicine. Highest priority is given to 3rd and 4th generation cephalosporins, fluoroquinolones and macrolides that are licensed for the use in dairy cattle in the EU. Likewise, penicillins are widely used in both dairy cattle and humans (see Section 3.2).

The waste milk may include milk from cows immediately post-calving (colostrum), milk from cows with clinical udder infections and it may furthermore include milk from cows after treatment with

antimicrobials or other medicines within the defined withdrawal period. In the latter, drug residues in excess of maximum residue limits (MRLs, see Section 3.2) may be present. Those residues can be antimicrobials and other substances depending on the pharmaceuticals used in the animals. The following list gives an overview of the different kinds of waste milk occurring on dairy farms. Only waste milk included under the point No. I listed below is relevant for the purpose of this Opinion. Waste milk included under No. II–V may be added to the waste milk from point No. I. Potential risks arising from non-antimicrobial residues in waste milk will not be addressed in this Opinion.

- I) Milk not marketable after treatment with antimicrobials
  - a) Colostrum of dairy cows that have received dry cow treatment (waste colostrum)
    - 1) 1st colostrum (first milking waste colostrum)
    - 2) Transition or post-colostral milk (day 1–5)
    - 3) During the withdrawal period after day 5 in cows calving earlier than expected
  - b) Milk from cows treated with antimicrobials during lactation
    - i) Systemic treatment with antimicrobials that have a withdrawal period for milk
      - 1) During treatment (waste milk during treatment)
      - 2) > 1 day after last treatment (waste milk during withdrawal period for human consumption)
    - ii) Local treatment (intramammary)
      - 1) During treatment
      - 2) > 1 day after last treatment
- II) Milk not marketable after treatment with other – non-antimicrobial – drugs
- III) Milk not marketable because of clinical disease (clinical mastitis) but without treatment
- IV) Milk not marketable because of subclinical mastitis (high somatic cell count)
- V) Milk not marketable within the first 5 days in lactation (untreated colostrum, transition milk from untreated cows).

The level of residues in the waste milk depends on numerous factors including the type of drug, the dosage and timing of administration relative to the milking, and the route of administration. The type of drug and its chemical properties among other things define its half-life in the animal and also its transmission from the blood to the milk via the blood–milk barrier and hence its potential concentration in the milk. For the purpose of this study, milk from cows harvested after the end of the withdrawal period will generally be considered as safe. For colostrum and transition milk, the length of the dry period is of relevance. Products for the treatment of cows at dry-off foresee a defined minimum length of the dry period. The length of this dry period differs between the products. When the required minimum length of the dry period is met, residues of the product in the colostrum will be minimal. If cows calve earlier than expected, and the minimum length of the dry period is not reached, residues in first colostrum and in transition milk may be higher.

#### 1.4.2. The production cycle of the dairy cow

Dairy cows undergo regular cycles during their productive life. They are commonly bred for the first time at the age of 15–18 months and will calve after a gestation period of 280+/- 5 days. The first milk after calving is called colostrum and differs from other milk by increased contents of protein and other solids, especially immunoglobulins. Those are of vital importance for the calf that is born practically agammaglobulinaemic. The udder secretion harvested after the first milking until 5 days into lactation is called transition milk or post-colostral milk. The change from colostrum to milk is a gradual process. The length of this interval varies in literature between three and 6 days but for the purpose of this Opinion 5 days will be considered. This milk may not be marketed for human consumption. The length of the lactation period varies between cows and is mainly determined by the time when the cow is successfully bred, as the gestation length is biologically fixed. Prior to the next parturition, cows are commonly dried-off, i.e. not milked for a certain period of time; the so-called 'dry period'. After the last milking, cows are often treated intramammarily with a long-acting antimicrobial, a so-called 'dry cow product'. The content of these products may vary, but many of the licensed dry cow products are either based on penicillins or cephalosporins or may also include an aminoglycoside. Likewise, the dry

period length that is foreseen in the Summary of Product Characteristics of these products may differ between 4 and 8 weeks. Their withdrawal period is commonly expressed as a minimum length of the dry period plus a variable number of days into lactation. If the cow calves earlier than foreseen, the number of days after calving before milk may be marketed is increased by the period between actual and expected calving.

#### 1.4.3. Calves from birth to weaning

During the first days of life, the calf depends entirely on liquid feed consisting of milk or milk replacers. Uptake of other feeds commonly starts at about 1 week of age and the contribution of this type of feed increases over time until weaning. The duration of milk feeding to calves depends on the management type of the farm. In veal calf production, liquid feed is used throughout the whole lifespan of the animals. Calves raised as replacements for the dairy herd or for producing beef are commonly weaned at the age of 6–10 weeks.

Calves may not be transported before 14 days of age. During those 14 days, all living male and female calves will almost entirely be fed on milk or milk replacer on their farm of birth and therefore fall within the scope of this Opinion. After 14 days, many calves are sold to other units. This may not only include beef and veal farms but also farms specialised in the raising of replacement stock for the dairy herds. These animals are no longer fed on the farm of origin and will therefore not be exposed to waste milk, as waste milk may not be marketed as feed for animals. The majority of the calves on dairy farms that are older than 2–4 weeks will be raised as female replacement animals either for their herd of origin or for other herds. On some dairy farms, some calves will be raised for meat production (beef or veal) as an additional source of income and may therefore also be fed with waste milk from the dairy herd.

#### 1.4.4. Microbial colonisation of the bovine gut

At birth, the calves' intestinal tract contains only very limited numbers of bacteria. After birth, colonisation with bacteria from the environment occurs rapidly. During the first few days and weeks, while the calf is suckling, the oesophageal groove mechanism causes most of the milk to pass directly to the abomasum, thus ensuring an efficient digestion and absorption of nutrients (Church, 1988; Davis and Drackley, 1998). Nonetheless, some of the milk passes into the developing rumen, providing nutrients for the microorganisms that are present from day 1 of life onwards (Fonty et al., 1987; Morvan et al., 1994). The bovine calf is born with a very rudimentary rumen, which develops over a period of weeks in response to increasing consumption of solid food and increasing microbial fermentation until the animal develops into what is commonly called the 'functional ruminant' (Heinrichs, 2005). The establishment of a microbial community in the rumen is essential for the growth and well-being of the calf. Furthermore, evidence is emerging that the microbial community that develops in early life determines the subsequent adult rumen microbial community, with implications for health and productivity in later life (Yáñez-Ruiz et al., 2015). Early research into the emergence of microbial communities in the rumen of newborn animals revealed rapid colonisation of the rumen by aerobic and facultative anaerobic microbial taxa close to birth, which decreased gradually to a constant level at between 6 and 8 weeks of age, being gradually replaced by exclusively anaerobic taxa (Bryant et al., 1958; Fonty et al., 1987; Minato et al., 1992). The adult rumen community consists almost entirely of strictly anaerobic bacteria, mainly Bacteroidetes and Firmicutes.

Diet is one of the main factors that influences the composition of gut microbiota and may also play an important role in the observed temporal changes of the rumen microbiome in neonatal calves (Malmuthuge et al., 2015).

Less attention has in general been paid to the post-ruminal microbiota in cattle than to the ruminal microbiota, and this is particularly true of its colonisation and development. One study in the preweaned calf indicated that the diversity present in the large intestine was lower than in the rumen (Malmuthuge et al., 2014); the main phyla were, like in the rumen, Bacteroidetes and Firmicutes, with a generally higher abundance of Proteobacteria. The species are distinct compared to those from the rumen. The diversity of the microbiome in faecal samples from calves increased with increasing age (Edrington et al., 2012a; Klein-Jöbstl et al., 2014) and undergoes substantial changes during the preweaning period (Klein-Jöbstl et al., 2014). As in the adult animal (Ozutsumi et al., 2005; Dowd et al., 2008; Shanks et al., 2011), *Bacteroides* rather than *Prevotella* was the predominant Bacteroidetes genus.

In both ruminal and intestinal bacterial communities, horizontal gene transfer occurs widely between bacteria of different phylogenetic origins (Shterzer and Mizrahi, 2015). These genes include antimicrobial resistance genes.

#### 1.4.5. Effects of feeding waste milk other than those associated with AMR

##### 1.4.5.1. Microbial load of waste milk

The microbial load of waste milk may contribute to calf disease (e.g. diarrhoea) (Godden et al., 2012). Mastitis pathogens and other bacteria, such as *Mycobacterium avium* subsp. *paratuberculosis* (MAP), may be present (Gonzalez and Wilson, 2003; Houser et al., 2008). For this reason, pasteurisation of milk has been advocated by a number of studies to prevent spread of such bacteria to replacement stock (Donahue et al., 2012; Godden et al., 2012). Because these pathogens may also be present in marketable milk and are not unique to milk potentially containing antimicrobial residues, this will not be considered in this Opinion.

##### 1.4.5.2. Altering calves gut microbiota – influence on calves' and cows' metabolism and health

Besides the potential effect of feeding milk that contains residues of antimicrobials on the development of AMR, these antimicrobials may have further effects.

Feeding of waste milk potentially containing antimicrobials has been associated with reduced shedding of *Campylobacter* spp., a zoonotic pathogen (Klein et al., 2013) and a reduced incidence of clinical disease (Brunton et al., 2014; Al Mawly et al., 2015).

Other potential effects of antimicrobial residues in milk on long-term calf health are poorly understood and studies on the topic have so far only been carried out in other animal species than cattle. These potential effects are acknowledged and need further study but are outside the scope of this Opinion. Nevertheless, they are briefly discussed in Section 3.7.

## 2. Data and methodologies

### 2.1. Data

#### 2.1.1. European Commission Questionnaire

In September 2014, the EC sent a questionnaire concerning 'Feeding of calves with the milk (colostrum) containing residues of antimicrobials from lactating cows after their treatment by antimicrobials' to the MSs (see Appendix B). The questionnaire contained the questions listed below:

- 1. Country concerned:
- 2. In your country, are there any (national or regional) rules, legislation or guidelines on practice of feeding calves on farm with milk that might contain antibiotic residues?
  - (a) If yes, please provide the reference and a description of the rules/legislation/guidelines.
  - (b) Provide also the result of the (official) controls on the implementation of these rules/legislation/guidelines and the body in charge of these controls.
- 3. Please provide information about:
  - (c) The percentage of herds in your country that receive regularly an antibiotic treatment (prophylactic treatment) at the beginning of or during the dry period.
  - (d) The percentage of herds in your country that are screened (diagnostic) before receiving a treatment at the beginning of or during the dry period.
  - (e) The percentage of cows that are treated with an antibiotic during lactation (systemic or intramammary antimicrobial treatment).
  - (f) The percentage of farms in your country that feed the milk from the treated cows to their calves?
  - (g) If the farm is not feeding their calves with milk from treated cows, what is the destination of the milk in these farms? Please specify the other ways of disposal of that milk in practice and if possible percentage of farms applying them.
- 4. Would you support an EU initiative for a harmonised approach on this issue?

The summary of the responses and the raw data received by the EC from the 24 MSs that answered the Questionnaire were sent to EFSA. The data were reviewed by the members of the Working Group (WG), and used for different sections of the Opinion, if relevant. The letter sent by the EC to the MSs containing the questions posed by the EC can be found in Appendix B. The raw data can be provided by EFSA upon request.

### 2.1.2. Data on Antimicrobial Resistance from surveillance and monitoring Programmes

Data on bacterial AMR generated from monitoring or surveillance programmes at the national level for different countries were reviewed by the members of the WG and, if appropriate, used for the present Opinion.

### 2.1.3. European Medicines Agency (EMA) Maximum Antimicrobial Residue Limits (MRL) assessment of antimicrobials

Data on antimicrobial concentrations found in milk after antimicrobial administration in lactating cows obtained from the EMA MRL assessment reports were reviewed by the members of the WG. The extracted data, when appropriate, were used to assess the potential contamination of milk and to estimate the concentration of antimicrobial residues in milk after antimicrobial treatment in dairy cows.

### 2.1.4. Antimicrobials sales at European and national level

Regarding the risk of calves being exposed to colostrum and/or milk potentially containing antimicrobial residues, different data on antimicrobial use in dairy cows are of interest:

- Proportion of cows treated with antimicrobials at drying-off and during lactation.
- Proportion of parenteral *versus* intramammary antimicrobial treatment.
- Estimation of the most commonly used antimicrobials in parenteral and intramammary treatment.

To this end, data on sales of antimicrobials in Europe obtained from the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) reports (ESVAC, 2016) were reviewed by the members of the WG and if appropriate used in the present Opinion for evaluating the use of antimicrobials in dairy cows in the EU.

The ESVAC project provided a harmonised approach to the collection and presentation of data on antimicrobial sales in European countries. The main limitation is that the data are not presented by animal species and it is not possible to make a specific extraction of the data concerning lactating cows. However, the ESVAC report presents specific monitoring of sales of intramammaries for all species combined which can be extrapolated to bovine species as the main target for these products.

Data on sales of antimicrobials at national level were also obtained from scientific literature or from national monitoring programmes.

The data extracted from the ESVAC reports, national monitoring programmes and scientific literature were used in the present Opinion to define the antimicrobials most commonly used in dairy cows in order to identify which substances display higher risk in terms of exposure but also to assess the extent of different types of treatments.

## 2.2. Methodologies

### 2.2.1. Data and literature searches

Several methods have been used to gather scientific publications, reports and official documents relevant for this Opinion. In general, the qualitative evaluation by means of literature reviews were carried out based on the knowledge and expertise of the WG members. In these cases, the experts in the WG selected relevant references starting from review papers, books chapters, non-peer-review papers known by the experts themselves or retrieved through non-systematic searches, and increasing the number of papers through 'footnote chasing' (White et al., 1992) until reaching a coverage of the subject considered sufficient by the WG. For specific areas, it was considered necessary to do more specific literature searches to identify relevant literature (see below). This was used to support the expert review in these areas.



Relevant data from a number of official EU publications and Agencies, reports from the EMA, EFSA and specific reports from the MSs were used as appropriate. Links for such reports are given in the reference section. Finally, considerable use was made of ongoing citation input by WG members and information about relevant publications provided by members of the EFSA Biological Hazards (BIOHAZ) Panel.

For the area of 'Antimicrobials used in EU for treatment of dairy cows' searches were conducted in PubMed using the keywords: Antibiotic AND Antimicrobial AND Usage AND Dairy cattle. The search included publications from 2012 till now. The references retrieved were screened for studies of interest.

For the area of 'Antimicrobial residues concentration found in colostrum' searches were conducted in PubMed using the keywords: Antibiotic OR Antimicrobial AND residue AND colostrum; dry cow therapy AND residue. The references retrieved were screened for studies of interest.

For the area of 'Antimicrobial resistance of mastitis pathogens in Europe' searches were conducted in PubMed using the keywords: Antimicrobial resistance AND bacteria AND mastitis. The search was restricted to European countries and divided in recent papers (from 2013 and a review paper from 2012). The references retrieved were screened for studies of interest.

For the area of 'Routines for antibiotic treatment at dry-off of dairy cows in European countries' searches were conducted in PubMed using the keywords: Dry cow AND therapy AND 2010–2016. The references retrieved were screened for studies of interest.

For the area of 'Current practices regarding use of milk from dairy cows treated with antimicrobials' searches were conducted in PubMed using the keywords: Milk AND waste AND dairy AND feed AND cattle OR cow. The references retrieved were screened for studies of interest.

### 2.2.2. Risk assessment methodology

Due to lack of quantitative data, it was not possible to conduct a quantitative evaluation of the risk (Codex Alimentarius Commission, 1999) of AMR in faecal bacteria from calves caused by feeding of calves with milk or colostrum containing residues of antimicrobials. Therefore, a qualitative evaluation (Codex Alimentarius Commission, 1999) was performed, considering the relevant scientific literature, data from national surveillance and monitoring programmes and the information from the background documents provided by the EC (Questionnaire) mentioned in Section 1.1.

In this qualitative risk assessment, the data assessment was structured as follows:

- The farming practices in the EU MSs in relation to treatment of dairy cows with antimicrobials and the use of waste milk as feed for calves (Section 3.1).
- The antimicrobial residues present in milk and colostrum from treated cows (Section 3.2).
- The antimicrobial resistant bacteria present in colostrum and milk from treated cows (Section 3.3).
- Studies presenting results on the effect of feeding calves milk and/or colostrum containing antimicrobial residues on the faecal shedding of antimicrobial-resistant bacteria (Section 3.4).
- Discussing the observations summarised in Section 3.4 in relation to the scientific literature on consequences of exposure of bacteria to antimicrobials for development of resistance. Data on antimicrobial residues (Section 3.2) and antimicrobial-resistant bacteria (Section 3.3) present in milk and/or colostrum of treated animals were taken into account to reach conclusions on the effect of feeding calves milk and/or colostrum from treated animals.
- Possible management and mitigation options to reduce the risk for the development of AMR derived from feeding calf practices (Section 3.6).

For each of these subtopics the evidence was collected, judged on the basis of expert knowledge and the summarising remarks conclusions were expressed in a qualitative way.

Results collected in Sections 3.1, 3.2, and 3.3, delivered indirect evidence for the effect on the faecal antimicrobial-resistant bacteria when calves were fed contaminated milk and/or colostrum. The studies in Section 3.4 provided direct evidence of this effect by analysing data collected 1) on farms where waste milk was routinely fed, 2) on farms where waste milk or colostrum was fed to a group of calves compared to a control group and 3) by carrying out experimental studies where milk spiked with known concentrations of antimicrobials was fed to calves in comparison with a control group.



### 2.2.3. Uncertainty

Throughout the development of the risk assessment, the evidence leading to the summary remarks at the end of each section or subsection are provided, where adequate. In Section 3.4, the limitations and uncertainties related to each individual study are detailed as well as the contribution of each study to the summary remarks of that section.

The evidence on which the main conclusions are based is clearly indicated. For the overall conclusions, the answers were related to the conditions of a certain scenario when results were limited to one study. In some cases, conclusions were expressed relative to another risk and quantification of the overall uncertainty was not performed.

## 3. Assessment

Milk from dairy cows treated during lactation or at drying-off could contain antimicrobial residues and antimicrobial-resistant bacteria. The antimicrobial residues result from cows treated during lactation or at drying-off. The antimicrobial-resistant bacteria may be related to the cow's treatment, e.g. for mastitis. These resistant bacteria could also be due to environmental and/or faecal contamination of the waste milk during harvest and storage. Because in the latter case, the resistant bacteria may equally be present in marketable milk for human consumption, the assessment focuses mainly on resistant bacteria related to mastitis. However, bacteria detected in field samples of waste milk that were considered in some studies, may likewise originate from the environment and may be exposed to selective pressure in the waste milk due to the contained antimicrobial residues. Several observational and experimental studies report shedding of resistant faecal bacteria when calves are fed milk from treated cows. These studies are assessed in detail and discussed regarding their relevance in relation to the terms of reference. Mitigation options to reduce the risk related to feeding this contaminated milk or colostrum are assessed and several scenarios are discussed.

### 3.1. Farming practices: treatment of dairy cows with antimicrobials and use of waste milk as feed for calves

This section collects information on treatment of dairy cows with antimicrobials and on the use of milk from treated cows as feed for calves in Europe. The section builds on information from scientific literature. It also considers reports and documents on antimicrobial resistance and on use of antimicrobials in animals. Relevant information from the Commission Questionnaire (SANCO/WT/jd/2014/2646653) 'Feeding of calves with the milk (colostrum) containing residues of antimicrobials from lactating cows after their treatment by antimicrobials' is also used.

#### 3.1.1. Summary of answers and raw data from the MSs addressing the Commission Questionnaire mentioned in the Mandate

As stated in Section 2.1.1, the EC sent a questionnaire (SANCO/WT/jd/2014/2646653; Appendix B) concerning 'Feeding of calves with the milk (colostrum) containing residues of antimicrobials from lactating cows after their treatment by antimicrobials' to the MSs in September 2014.

Twenty-four MS replied to the questionnaire (Austria – AT, Belgium – BE, Bulgaria – BG, Cyprus – CY, the Czech Republic – CZ, Denmark – DK, Estonia – EE, Spain – ES, Finland – FI, France – FR, Croatia – HR, Hungary – HU, Ireland – IE, Italy – IT, Lithuania – LT, Luxembourg – LU, Latvia – LV, Malta – MT, the Netherlands – NL, Poland – PL, Sweden – SE, Slovenia – SI, Slovakia SK and the United Kingdom – UK). Four MS (Germany – DE, Greece – EL, Portugal – PT and Romania – RO) did not provide any response.

The main information from the replies to the questions in the questionnaire regarding the legal situation on use of waste milk and the actual destination of waste milk, as well as information on antimicrobial treatment during the dry period and lactation, are summarised in Table 1. To make the summary more comprehensible, some information considered irrelevant to the current issue has been omitted from the table.

**Table 1:** Overview of the results collected through the EC Questionnaire (SANCO/WT/jd/2014/2646653)

	Legal situation on feeding waste milk to calves	Destination of waste milk when not fed to animals	Waste milk fed only to male calves		Waste milk fed to male and female calves		Prophylactic antimicrobial treatment during dry period		Antimicrobial treatment after diagnosis in dry period		Antimicrobial treatment during lactation		Comments
			(% of farms)	(% of farms)	% of farms	% of cows	% of farms	% of cows	% of farms	% of cows	% of farms	% of cows	
AT	Restricted in national regulation (see comment)	<ul style="list-style-type: none"> <li>In an authorised landfill</li> <li>Incinerated</li> <li>Used as organic fertilisers or soil improvers</li> <li>Composted or transformed into biogas</li> <li>Applied to land without processing</li> </ul>	No data	No data	No data	No data	No data	No data	No data	No data	No data	Milk from a treated cow can be fed to its suckling off-spring	
BE	No specific legislation but administration recommendations	Mainly in manure and spread on land	(See comment)	(See comment)	(See comment)	(See comment)	No data	No data	No data	No data	No data	Illegal in BE but no active control	
BG	No specific legislation	Burial on farm	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done		
CY	No specific legislation	<ul style="list-style-type: none"> <li>Large quantities incinerated, used as organic fertiliser, composted or biogas</li> <li>Small quantities spread on land</li> </ul>	100	95-97	–	–	–	40	–	–	2-4		
CZ	No information	Not specified	No data	No data	No data	–	46 (see comment)	No data	No data	No data	90	Unclear if this is blanket dry cow therapy or selective dry cow therapy	
DE	No information released from questionnaire	No information released from questionnaire	–	–	–	–	–	–	–	–	–		

Legal situation on feeding waste milk to calves	Destination of waste milk when not fed to animals	Waste milk fed only to male calves		Waste milk fed to male and female calves		Prophylactic antimicrobial treatment during dry period		Antimicrobial treatment after diagnosis in dry period		Antimicrobial treatment during lactation		Comments
		(% of farms)	(% of farms)	(% of farms)	(% of farms)	% of farms	% of cows	% of farms	% of cows	% of farms	% of cows	
DK	No specific legislation	Mainly in manure or in water routes on the farm	–	25 (see comment)	0	–	–	No data	–	≈ 33	–	Not specified if to male or female calves
EE	No specific legislation	Mainly in manure and spread on land	No data	No data	No data	–	–	No data	–	No data	–	–
EL	No information released from questionnaire	No information released from questionnaire	–	–	–	–	–	–	–	v	–	–
ES	No specific legislation	Not specified	100	–	–	96–99	–	–	70	20	–	–
FI	No specific legislation but industry recommendations	Mainly in manure and spread on land	–	100 (see comment)	0	–	–	–	22	35–38	–	During withdrawal period but not during treatment. Never after fluoroquinolone treatment. After treatment with $\beta$ -lactamase. milk from cows treated with benzylpenicillin can be used
FR	No specific legislation but administration recommendations through a Guide of good hygiene and production practices in the dairy industry	Most frequent is spreading on land, but also composting and for biogas production	–	Common practice	–	0	–	–	44	14	–	–
HR	No specific legislation	Mainly in manure and spread on land	–	40 (see comment)	19 (see comment)	–	–	9 (see comment)	–	25 (see comment)	–	Based on a survey of 35% dairy farms
HU	No specific legislation but general provisions	Mainly in manure and spread on land	100	–	100	–	–	100	–	25–30	–	–

Legal situation on feeding waste milk to calves	Destination of waste milk when not fed to animals	Waste milk fed only to male calves		Waste milk fed to male and female calves		Prophylactic antimicrobial treatment during dry period		Antimicrobial treatment after diagnosis in dry period		Antimicrobial treatment during lactation		Comments
		(% of farms)	(% of farms)	(% of farms)	(% of farms)	% of farms	% of cows	% of farms	% of cows	% of farms	% of cows	
IE	According to European ABP Regulations	No data	No data	No data	Very high (see comment)	No data	–	No data	–	–	–	Prophylactic antimicrobial treatment during the dry period is high according to a study performed in 2010 (data on sales of dry cow intramammary tubes)
IT	<ul style="list-style-type: none"> <li>• Application to land with manure 5–80%</li> <li>• Disposal in a composting or biogas plant 0–25%</li> <li>• Incineration 5%</li> </ul>	–	4–100 (see comment)	10–100 (see comment)	–	0–70 (see comment)	–	5–70 (see comment)	–	–	Region dependent	
LT	Mainly in manure	No data	No data	No data	No data	No data	–	No data	–	No data	–	
LU	Mainly in manure and spread on land	–	≈ 80	≈ 80	–	≈ 15	–	≈ 15	–	≈ 15	–	
LV	Not specified	–	Not forbidden	No data	No data	No data	–	No data	–	No data	–	
MT	Mainly in special manure to discard/destroy and/or spread on land	–	60–70	90	–	90	–	90	–	70–80	–	
NL	Mainly in manure and spread on land	–	15	0	–	70	60	70	60	Low	–	Not allowed during withdrawal period

Legal situation on feeding waste milk to calves	Destination of waste milk when not fed to animals	Waste milk fed only to male calves (% of farms)	Waste milk fed to male and female calves (% of farms)	Prophylactic antimicrobial treatment during dry period		Antimicrobial treatment after diagnosis in dry period		Antimicrobial treatment during lactation	Comments
				% of farms	% of cows	% of farms	% of cows		
PL No specific legislation but administrative Guide of good hygiene and production practices in the dairy industry	According to Regulation (EC) 1069/2009 <sup>(a)</sup> as animal by product, Cat.1 or 2	No data	No data	No data		No data		No data	–
PT No information released from questionnaire	No information released from questionnaire	–	–	–	–	–	–	–	–
RO No information released from questionnaire	No information released from questionnaire	–	–	–	–	–	–	–	–
SE No specific legislation but industry recommendations	No data, probably in the manure tank	(See comment)	–	0	–	26	18	Of 457 farms (8.6% Swedish farms): 89–85% fed milk from cows treated during dry period; 56% milk from cows during treatment in lactation; 79% during withdrawal period	–
SI No specific legislation	Mainly mixed in manure and spread on land	–	Common practice	No data	No data	No data	No data	No data	–
SK No specific legislation	Mainly mixed in manure and spread on land	–	11	68	–	31	21	–	–
UK No specific legislation	Mainly mixed in manure and spread on land	–	83 (see comment)	96 (see comment)	–	No data	28 (see comment)	From Brunton et al., 2012.	–

The data included in the Table are extracted from the original answers provided by the MSs to the EC, without any interpretation. Four MS (DE, EL, PT and RO) did not provide responses to the Questionnaire. –: no information provided by the MS.

AT: Austria; BE: Belgium; BG: Bulgaria; CY: Cyprus; CZ: the Czech Republic; DE: Germany; DK: Denmark; EE: Estonia; EL: Greece; ES: Spain; FI: Finland; FR: France; HR: Croatia; HU: Hungary; IE: Ireland; IT: Italy; LT: Lithuania; LU: Luxembourg; LV: Luxembourg; MT: Malta; NL: the Netherlands; PL: Poland; PT: Portugal; RO: Romania; SE: Sweden; SI: Slovenia; SK: Slovakia; UK: the United Kingdom.

(a): Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation). OJ L 300, 14.11.2009, p. 1–33.

### 3.1.2. Treatment of dairy cows

Lactating and non-lactating dairy cows are treated with antimicrobials for a number of different reasons. Most of these treatments are associated not only with mammary gland infections but also with infections of the genital tract or the locomotor system are frequent causes for antimicrobial treatments of dairy cows (Kreausukon, 2011; Växa Sverige, 2014; Kuipers et al., 2016; Stevens et al., 2016). Treatments for mastitis include those for subclinical cases during lactation, clinical cases during lactation and the dry period as well as treatments with long-acting antimicrobials at drying-off to cure existing infections and prevent new infections during the dry period (so called dry cow treatments) (Stevens et al., 2016).

Additionally, an intraruminal device releasing the ionophore monensin into the rumen is registered for use in dairy cows and heifers in the EU. The indication for the product is reduction in the incidence of ketosis, a non-infectious metabolic disease of cows, in the periparturient period. The effect is due to changes of the ruminal microflora which enhance production of glucose precursors and thus improving energy production in the cow. The device is administered orally 3–4 weeks prior to calving and releases monensin in the rumen during a 95-day period. The product has no withdrawal period for milk or meat as transfer of monensin from the rumen is minimal. Hence, it does not result in residues in waste milk. Monensin has no human medical use, and no co-resistance to other antimicrobials except for other ionophores is known so far. Therefore, use of monensin is not of direct relevance to public health and is not specifically considered in this Opinion.

#### 3.1.2.1. Non-lactating dairy cows (dry cows)

Prior to parturition, dairy cows are commonly not milked for several weeks. During the period, the mammary gland undergoes the process of involution and renewal. At the end of the dry period, colostrum is being produced by the mammary gland. The length of the dry period can vary between farms and debate on the optimal length of this period is ongoing. Most farms aim for a dry period of 4–8 weeks (Bertulat et al., 2015).

Intramammary antimicrobial treatment at the end of lactation has for many years been considered a cornerstone of udder health management especially in herds experiencing problems with contagious mastitis pathogens such as *Streptococcus agalactiae* and *Staphylococcus aureus*. The common recommendation has been to treat all udder quarters from all cows in a herd, using blanket dry cow therapy, to eliminate subclinical infections with those pathogens (Keefe, 2012). This procedure significantly reduces post-partum mammary gland infections with staphylococci and streptococci according to a meta-analysis carried out in 2009 (Halasa et al., 2009a,b).

In the early 2000s, dry cow therapy was also shown to be beneficial to the prevention of clinical mastitis caused by pathogens like *Streptococcus uberis* or *Escherichia coli* (Bradley and Green, 2001) and therefore substances like cefquinome (4th generation cephalosporin) were introduced for this kind of treatment. In a meta-analysis carried out in 2009, no benefit of this treatment with respect to *E. coli* was observed (Halasa et al., 2009a,b).

The recommendation for blanket dry cow therapy has recently been re-evaluated in some MS and in agreement with a more restrictive use of antimicrobials, selective dry cow therapy is now recommended (Scherpenzeel et al., 2016). In consequence, in some MS, farmers have turned to a more selective approach to dry cow therapy (MARAN, 2015; SDa, 2015). However, in line with the recommendations of blanket dry cow therapy a large proportion of farmers in some MSs (e.g. BE, CY, CZ, DE, ES, HR, HU, IE, IT, LU, MT, SK, UK) still treat most cows at the end of lactation while in other MS (e.g. DK, FI, FR, NL, SE) only cows confirmed to have mammary gland infections are treated at drying-off, i.e. selective dry cow therapy. Nevertheless, in the MSs practicing selective dry cow therapy a large proportion of cows are still treated (22–70%) (Tables 1 and 2).

Colostrum is needed to ensure a sufficient immunological status of the newborn calf. One of the key issues is therefore that on farms practising blanket dry cow therapy there is no colostrum from adult cows that are not treated with antimicrobials.



**Table 2:** Routines for antimicrobial treatment at drying-off of dairy cows in the European MSs: proportion of herds where dry cow therapy is practised or proportion of dairy cows receiving dry cow therapy

MS	Blanket dry cow therapy		Selective dry cow therapy		No dry cow therapy		Alternative treatment		Year	Number of herds/cows	Reference
	Herds (%)	Cows (%)	Herds (%)	Cows (%)	Herds (%)	Cows (%)	Herds (%)	Cows (%)			
BE	78.9	–	21.1	–	–	–	–	–	2012–2013	57 herds	Stevens et al. (2016)
DE	79.6	–	–	–	9.7	–	10.7	–	2013	98 herds	Bertulat et al. (2015)
DE	85.0	–	15.0	–	–	–	–	–	2010	60 herds	Kreausukon (2011)
IE	–	92.7	–	–	7.3	–	–	–	2010	≈ 1,000,000 cows	More et al. (2012)
DK	–	Not done	–	≈ 25	–	–	–	–	2014	563,000 cows	DANMAP (2014)
SE	Not done	–	70.1	–	29.9	–	–	–	2011	284 herds	Nielsen and Emanuelson (2013)
SE	–	Not done	–	≈ 26	–	–	–	–	2013–2014	340,000 cows	Växa Sverige (2014)
UK	81.6	–	14.4	–	4.0	–	–	–	2010–2011	577 herds	Brunton et al. (2012)

–: no information provided by the MS.

### 3.1.2.2. Lactating cows

Data on antimicrobial treatment of dairy cows during lactation collected in the EC Questionnaire show that in some of the 15 MS providing data, the percentage of lactating cows treated per year is very high but in most MS during 1 year approximately 20–30% of the dairy cows are treated (Table 1).

The main indication for antimicrobial treatment of lactating dairy cows is related to infections of the mammary gland but antimicrobials are also used to treat infections in, for example, the locomotor or genital systems. For example, in SE, 69% of antimicrobial treatments of dairy cows in 2013/2014 were related to clinical mastitis and 13% and 6% to diseases of the locomotor and reproductive systems, respectively (Växa Sverige, 2014).

Likewise, in a study of 94 dairy herds in the NL, 24% and 44% of the mean number of daily doses per cow year over an 8-year period were used for clinical mastitis and dry cow therapy, respectively, whereas 22% was used for other indications (Kuipers et al., 2016). Moreover, in a study from BE, 30% of the defined daily dosages in 57 dairy herds were intramammaries used during lactation and for which the only indication is mastitis (Stevens et al., 2016). Also in DK, mastitis is the main indication for treating dairy cows with antimicrobials (DANMAP, 2014).

The dominance of mastitis as an indication for antimicrobial treatment is a consequence of the common occurrence of such infections in dairy cows. The incidence of clinical mastitis per 100 cow years is high and has been estimated to 32.5 in the Netherlands 2013 (Santman-Berends et al., 2015), 54.0 in Ireland 2010 (More et al., 2012), and between 47 and 71 in historical data from the UK (Bradley et al., 2007). In Belgium, the incidence rate of mastitis treatments was 9.6/10,000 cow days at risk (Stevens et al., 2016) (i.e. 33.5/100 cow years<sup>1</sup>). According to statistics from Växa Sverige (2014), the incidence of antimicrobial treatment of dairy cows in Sweden, mainly for mastitis, was about 18 treatments with injectables per 100 completed/interrupted lactations in 2013/14.

The main routes for administering antimicrobials to dairy cows are either parenterally, by intramuscular, subcutaneous or intravenous injections, or locally in the mammary gland or uterus. Oral or topical administrations of antimicrobials are rarely practised in dairy cows (Merle et al., 2012).

An important aspect of antimicrobial treatment of dairy cows is intramammary treatment. Intramammary treatment is either applied during lactation to treat cases of clinical or subclinical mastitis or at the end of lactation as a so called dry cow therapy aimed to cure subclinical infections during the dry period and/or to prevent new infections during the early dry period.

Since the major indication for antimicrobial treatment of dairy cows is infections in the mammary gland, a large proportion of the treatments are administered via the intramammary route. In a Belgian study, this treatment route accounted for 64% of all treatments but there were substantial differences between herds (Stevens et al., 2016). About half of the intramammary treatments were administered for treating cases of clinical mastitis, the other half for dry cow therapy. In a German study, about half of

<sup>1</sup> Calculations performed: For calculating incidence rates from 10,000 cow days to 100 cow years, values need to be multiplied by 3.65. For vice versa calculations, values need to be divided by 3.65.

the treatments in dairy cows were administered via the intramammary route (Merle et al., 2012). Likewise, in a study from the Netherlands, 66% of the number of daily dosages used in dairy herds was administered by the intramammary route; 44% for dry cow therapy and 22% for treating clinical mastitis (Kuipers et al., 2016). Also, in the UK, intramammaries were used to treat mastitis on 93% of farms (Brunton et al., 2012). The common use of the intramammary route to administer antimicrobials to dairy cows is also evident from the information collected in the EC Questionnaire (Table 1).

Notably, in some MS, e.g. the Nordic countries, clinical mastitis during lactation is mainly treated by parenteral administration of antimicrobials, sometimes combined with intramammary administration. For example, in Sweden, the recommendation for treatment of clinical mastitis is parenteral administration of antimicrobials which may be supported by intramammary administration. However, only 25% of veterinarians use intramammary administration as an adjunct to parenteral administration for treating clinical mastitis (Persson Waller et al., 2016).

### 3.1.2.3. Antimicrobials used in the EU MSs for treatment of dairy cows

A wide variety of antimicrobials are used for treating dairy cows in Europe. Information from literature, national reports and also from the EC Questionnaire on the antimicrobial classes used in the EU during lactation or for dry cow therapy is summarised in Table C.1 (Appendix C).

#### *Injectables*

As represented in Table C.1 (Appendix C), there is a substantial lack of data on the use of injectable antimicrobials in dairy cows in the EU and information was only available from six MSs (BE, CZ, DK, the NL, SE and the UK). Also, the data available are not directly comparable because categorisation of antimicrobials and different units of measurements are used in the different datasets. Also, in some of the data sets, dairy cows are not separated from other cattle categories. Two of the data sets (Brunton et al., 2012; Stevens et al., 2016) are from surveys in a limited number of herds and should not be considered representative for the national situation.

From the available data, it appears that penicillins are by far the most commonly used group in SE and DK (76–84%), and this antimicrobial class is also used to a large extent in BE, the CZ and the NL (27–32%) but not very often in the UK (6%). Unfortunately, the available data do not allow the separation of the penicillin class into subgroups of substances, e. g. benzylpenicillin, aminopenicillins and  $\beta$ -lactamase-stable penicillins.

In BE and the CZ, 3rd–4th generation cephalosporins are the most commonly used antimicrobials (33–36%) and more often used than penicillins. This class of antimicrobials is also used in the UK (6%) although 1st–2nd generation cephalosporins are more commonly used (14%) in this MS. In contrast, cephalosporins are rarely used in DK, the NL and SE.

Trimethoprim/sulfonamides and tetracyclines are used to a large extent in the NL (24%) and less often in the other MSs (2–9%). Fluoroquinolones are used to about the same extent in BE, the CZ, SE and the UK (3–9%) but not at all in DK and the NL. Penicillins, in combination with aminoglycosides, are frequently used in the UK (21%) but less often in BE (4%) and not at all in the CZ, SE and DK. In the data set from the NL, an unspecified combination which may be penicillin and aminoglycoside is used in 5% of the treatments. Macrolides is the single most frequently used antimicrobial class in the UK (27%) and are also used in BE (15%), the NL (9%) and the CZ (5%) but not to any appreciable extent in SE and DK. It is uncertain if the data reflect the use of injectables in other MSs and more information is needed to appreciate the overall situation in Europe.

#### *Intramammaries*

There are more data available on the use of intramammaries during lactation and the dry period than on use of injectables (Table C.1, Appendix C). Penicillins, alone or in combination with aminoglycosides, are the most commonly used antimicrobials during both the dry period and lactation in DE, DK, the NL, SE and the UK, and during the dry period in IE, accounting for 55–100% of the intramammary treatments. These antimicrobials are also frequently used during lactation in IE (36%), and in FR, during both the dry period (34%) and lactation (23%). In BE penicillins, alone or in combination with aminoglycosides are frequently used in the dry period (31%) but less often during the lactation (17%). Moreover, the 6th ESVAC report shows that penicillins, alone or in combination with aminoglycosides, are used for intramammary treatment in all the 26 MSs reporting data (ESVAC, 2016).

Aminoglycosides alone are also frequently used in DE (21%) and during lactation in IE (33%). In FR, aminoglycoside (neomycin) in combination with bacitracin and tetracycline is the single most common therapy during lactation (37%).

The 1st–2nd generation cephalosporins, alone or in combination with aminoglycosides, are commonly used in both the dry period and in lactation in BE, DK, IE, FR and the UK (16–50%) and this is the single most commonly used class for dry cow therapy in FR (42%) (Table C.1, Appendix C). The two data sets from the UK give diverging information regarding use of 1st–2nd generation cephalosporins. In a study in 557 dairy herds in England and Wales in 2010–2011, the use of these antimicrobials during lactation and the dry period was common (30% and 16%, respectively), whereas sales data from 2014 indicate a lower use (8% and 0%, respectively). In the NL, a study of 94 dairy herds in 2012 showed that this antimicrobial class was often used during lactation (37%) but in official data from 2014 no use is registered. The 6th ESVAC report shows that 1st–2nd generation cephalosporins are used in all the 26 MS reporting data except in SE where this class of antimicrobials is not used (ESVAC, 2016).

The 3rd–4th generation cephalosporins are used to a limited extent in DE, DK, IE, the NL (1–5%) and not at all in SE. This class of antimicrobials is used frequently during both lactation and the dry period in BE (31% and 48% respectively), FR (15% and 15%, respectively) and the UK (7–29.5% and 16.4%), respectively. Moreover, the 6th ESVAC report shows that intramammaries with 3rd–4th generation cephalosporins are used in all the 26 MSs reporting data to ESVAC except in FI and SE (ESVAC, 2016).

In addition, the 6th ESVAC report (ESVAC, 2016) also shows that only negligible amounts of tetracyclines, sulphonamides, trimethoprim, macrolides or polymyxins are sold as intramammaries in the EU, which is in accordance with the information shown in Table C.1 (Appendix C). The report shows that intramammaries with lincosamides are used to a greater extent than is evident from Table C.1 (Appendix C).

#### 3.1.2.4. Summarising remarks

- Intramammary treatment of dairy cows for prevention and/or treatment of udder infections during the dry period is common in the EU and in some MS the majority of cows are treated.
  - This remark is based on separate studies from five MSs (BE, DE, IE, SE and the UK) (Table 2) and by the answers from 17 MS (BU, CY, the CZ, ES, FI, FR, HR, HU, IE, IT, LU, MT, the NL, SE, SI, SK and the UK) to the EC Questionnaire (Table 1).
- For treatment during the dry period, mainly penicillins, alone or in combination with aminoglycosides, and 1st–2nd generation cephalosporins are used. Also, 3rd–4th generation cephalosporins are used to some extent, and in some MSs this antimicrobial class is commonly used.
  - This remark is based on separate studies from eight MSs (DE, DK, BE, IE, the NL, FR, the UK and SE) (Table C.1, Appendix C) and also by the 6th ESVAC report. Quantitative data from several MS are lacking.
- Treatment of dairy cows during lactation is common in the EU and in most MSs 20–30% of dairy cows are treated per year but in some MS the majority of cows are treated.
  - This remark is based on the answers from 15 MSs (CY, the CZ, DK, ES, FI, FR, HR, HU, IT, LU, MT, the NL, SE, SK and the UK) to the EU Questionnaire (Table 1).
- The main single reason for treating dairy cows during lactation is infections of the udder.
  - This remark is not only based on separate studies from SE (69%, Växa Sverige, 2014) and the NL (29%, Kuipers et al., 2016) but can also be inferred from the high proportion of intramammaries used in lactation in studies from BE (30%, Stevens et al., 2016) and the NL (22%, Kuipers et al., 2016) as well as in the Danish national report (DANMAP, 2014).
- Treatment during lactation is mainly administered by the intramammary route, but in some MSs parenteral treatment is the common practice.
  - This remark is supported by several references from literature (e.g. Kuipers et al., 2016; Merle et al., 2012; Persson Waller et al., 2016; Stevens et al., 2016).

- For treatment with intramammaries during lactation, mainly penicillins and 1st–2nd generation cephalosporins are used. Often these antimicrobials are given in combination with aminoglycosides, but they are also used as single therapy. In some MSs, 3rd and 4th generation cephalosporins are used and in some MSs these cephalosporins are used to a great extent.
  - These conclusions are supported by several sources (Table C.1, Appendix C) and also by the 6th ESVAC report. Quantitative data from several MSs are lacking.
- For treatment of dairy cows with injectables during lactation penicillins, penicillins in combination with aminoglycosides, tetracyclines, trimethoprim/sulfonamides and macrolides are the antimicrobial classes used most often. In some MSs, 3rd–4th generation cephalosporins are also used to a large extent.
  - There is a scarcity of data on the use of injectable antimicrobials in dairy cows and the available sources do not always discriminate use in dairy cows from use in non-lactating cattle or in beef cattle. Also, some of the data sets emanate from a limited number of herds and cannot be considered representative for the general situation in a MS (Table C.1, Appendix C).

### 3.1.3. Use of milk from cows treated with antimicrobials as feed for calves

Since antimicrobial treatment of dairy cows during the dry period, as well as during lactation, is common in the EU MSs, there is an abundance of milk potentially containing antimicrobial residues available on dairy farms. This applies to colostrum and post-colostral milk from cows treated at the beginning of the dry period as well as to non-marketable milk from cows treated during lactation.

The extent of the issue can be perceived by estimating the amount of milk available on dairy farms that is potentially contaminated with antimicrobial residues. Thus, the common practice of dry cow therapy implies that a large proportion of colostrum is potentially contaminated. Likewise, with an average incidence of clinical mastitis of about 35 per 100 cow years, an average number of milking days of about 300/cow and a 5-day antimicrobial treatment combined with a 4-day withdrawal period, it can be roughly estimated that around 1% of the total lactation milk yield is non-marketable and potentially fed to calves. The amount of such milk available on individual farms will vary considerably between farms due to differences in udder health.

#### 3.1.3.1. Reasons for feeding milk from cows treated with antimicrobials

Reasons to feed milk from cows treated with antimicrobials to calves include the perceived benefits of feeding whole milk for improving calf growth, immunity and the intention to reduce economic losses due to unsaleable milk. In the UK, 9% of farmers cited difficulty with disposal of waste milk as the main reason for feeding it to calves (Brunton et al., 2012).

##### *Reasons to use colostrum*

Colostrum is essential for the health of the newborn calf. Calves are born agammaglobulinaemic (Godden, 2008). Until they develop their own ability to resist disease, through exposure to the microorganisms in their surroundings, they depend entirely on the immunity acquired by the consumption of colostrum (Moran, 2016). First colostrum contains high concentrations of antibodies that offer temporary, passive immunity from infection. The antibodies are absorbed from the gastrointestinal tract (GIT), during a short period after birth when the barrier function has not yet developed. The efficiency of absorption of antibodies from the GIT declines rapidly after birth therefore provision of sufficient amounts of high quality colostrum early after birth is critical.

No further transport of immunoglobulin G (IgG) into the circulation is possible from 24 h after birth (Godden, 2008). Nonetheless, transition milk fed subsequently may have beneficial effects, as it contains a greater concentration of IgG than milk produced later in lactation (Foley et al., 1978) and antibodies remaining in the intestinal lumen may provide local immunity against enteric pathogens (Berge et al., 2009). Feeding transition milk decreased the incidence of calves suffering from disease (Conneely et al., 2014). While it has to be acknowledged that the time interval between parturition and the possibility to market milk for human consumption varies in the range of 3–6 days between studies and also in the legislation of different countries, it can be concluded that the feeding of this so called transition milk to calves is considered to be beneficial to calf health.



### *Reasons to use lactational milk harvested during the withdrawal period from cows treated with antimicrobials*

Whole milk is considered to provide calves with an optimum mixture of nutrients. In recent years, several studies have underlined the benefit of supplying calves with sufficient amounts of whole milk (Khan et al., 2011). Waste milk is sometimes considered as a valuable alternative to 'milk replacer' and can minimise to some extent the economic losses associated with unsaleable milk. Unsaleable milk is one of the major disease-related costs in dairy farming (Rollin et al., 2015). In line with that, the most common reason reported for feeding waste milk to calves in England and Wales was to save money (Brunton et al., 2012).

There is some evidence that feeding fresh waste milk to calves can improve growth performance of the calves compared to feeding milk replacer (Horton et al., 2015). A longitudinal observational study to assess the impact of feeding waste milk to calves in England in 2013 found that calves fed waste milk weighed significantly more than calves fed a powdered milk replacer at 4, 7 and 13 weeks of age (Brunton et al., 2014).

#### **3.1.3.2. Current practices regarding use of milk from dairy cows treated with antimicrobials**

Few studies in the literature have investigated how milk from cows treated with antimicrobials is used as feed for calves. Thus, knowledge of such practices in the EU is scarce and fragmented. Therefore, the EC in 2015 asked the MSs to provide information on national practices with regard to how colostrum and milk from cows treated with antimicrobials are used (SANCO/WT/jd/2014/2646653). The results of this questionnaire are presented in Section 3.1.1 and summarised in Table 1.

##### *The legal situation*

Regulation (EC) No 1069/2009 laying down health rules as regards animal by-products and derived products not intended for human consumption contains the following provisions:

- Art 2(2)(e) excludes raw milk, colostrum and products derived therefrom which are obtained, kept, disposed of or used on the farm of origin, from the provisions of the Regulation.
- Art 9(c) categorising animal by-products containing residues of authorised substances or contaminants exceeding the permitted levels as referred to in Article 15(3) of Directive 96/23/EC, as category 2 materials (prohibited for placing on the market for animal feeding).

Therefore, feeding of calves with milk containing residues of antimicrobials on the farm of origin is not generally prohibited in the EU and potential national regulations on this practice are not harmonised.

The EC Questionnaire confirmed that the legal situation is not harmonised, with restricted feeding due to national regulation in some countries (Table 1). However, also in those countries where feeding is restricted by national regulation, actual data collection indicates that farmers used waste milk as feed although this practice it is not permitted in those EU MSs (Klein-Jöbstl et al., 2015).

##### *Data from literature*

A study from the UK revealed that colostrum fed to dairy heifer calves after removal from the dam was most commonly colostrum from the calf's own dam (50.3%) followed by colostrum pooled from several dams (39%) (Brunton et al., 2012). Few farms fed powdered colostrum or a commercial substitute (3.4%). Colostrum was usually fed fresh (95%) but some farmers reported freezing (11%), refrigerating or fermenting colostrum (5%), or pasteurising colostrum (one farmer) prior to feeding calves. The age at which feeding of only colostrum to calves stopped ranged from 0 to 21 days (mean 4 days, 95% confidence interval (CI) 3.8–4.2).

The study by Brunton et al. (2012) also showed that waste milk fed to calves could contain milk or colostrum from newly calved cows and heifers that had received dry cow antimicrobials, or from lactating cows that had received antimicrobial therapy (93% and 90%, respectively). Milk from high somatic cell count cows was also included in waste milk fed to calves on over 70% of farms surveyed. The majority of respondents (99%) fed fresh waste milk to calves, while 6% let it ferment prior to feeding (see Section 3.6). Very few respondents refrigerated (1%), froze (1%) or pasteurised (0.5%) waste milk. Only 30% discarded the milk from the first milking after antimicrobial treatment, and 87% fed milk to calves from cows with mastitis. The level of exposure to waste milk was slightly lower in replacement heifer calves compared with other calves. Around a third of farmers reported that

replacement heifers were fed primarily on waste milk, while more of the farmers (nearly half) reported that other calves were fed primarily on waste milk. The reason for this practice is a presumed risk of transmitting mastitis causing pathogens to the replacement animals, a risk that is not relevant for other calves as they will never be lactating animals potentially suffering from mastitis. The age at which milk feeding usually stopped ranged from 1 to 39 weeks for all calves reared until weaning (mean 8.5 weeks, 95% CI 8.3–8.7).

In Sweden, there are no official guidelines on the feeding of milk from cows treated with antimicrobials to calves. One of the Swedish livestock associations, however, recommends that milk from cows treated during lactation should be discarded until the second day after treatment (Växa Sverige, 2014). For cows treated at drying-off, it is recommended that colostrum from the first three complete milkings after calving should be discarded. To evaluate compliance with the recommendations, a questionnaire on use of milk from cows treated with antimicrobials was sent to 1,735 dairy farmers in Sweden, of which 457 returned the questionnaire (Duse et al., 2013). The study revealed that on many farms milk from treated cows was at least occasionally used as feed for calves. Colostrum from cows treated at drying-off was used from the first milking on 89% of the farms and from the second milking to the fourth day on 85% of farms. Milk from cows treated in lactation was used during the course of treatment on 56% of the farms and during the statutory withdrawal period on 79% of the farms. Feeding milk from cows treated for mastitis was less common than feeding milk from cows treated for other diseases. Also milk from treated cows was more frequently fed to bull calves than to heifer calves. In the study (Duse et al., 2013) feeding milk from treated cows was more common on organic farms.

In a survey of calf-rearing practices on 135 dairy farms in the Czech Republic, about two-thirds (65%) of the farmers reported that they fed non-marketable milk, i.e. waste milk, to their calves (Stanek et al., 2014). Of the farms feeding waste milk, 7% pasteurised the milk before feeding and 35% acidified it.

In a similar study in Austria, 85% of 1,501 farmers reported that they used waste milk as feed for calves (Klein-Jöbstl et al., 2015). About one-third of the farmers (29%) fed waste milk to all calves, whereas a similar proportion (31%) only fed waste milk to male calves. On 25% of the farms, waste milk was fed to calves only in exceptional cases. Feeding waste milk was more common on larger farms.

#### *Data from EC Questionnaire*

In the questionnaire, there was no separation between milk from cows treated during the dry period and cows treated during lactation. Thus, in the answers from the MSs, it is not possible to differentiate if there is a difference in how colostrum and lactational milk is used.

Of the 24 MSs responding to the questionnaire, 16 provided estimates of the proportion of farms that used milk from cows treated with antimicrobials as feed for calves, whereas eight MSs provided no estimate (Table 1).

FR and SI stated that using milk from treated cows to calves was 'common practice' for both male and female calves. BG stated that no farms use waste milk as feed for calves. The CY, ES and HU estimated that milk from treated cows was used for male calves on all farms (Table 1). In DK, HR, IT, LU, MT, the NL, SK and the UK, it was estimated that milk from treated cows was used for both male and female calves on 4–100% of the farms depending on the country (Table 1).

Two MS provided more detailed information. In FI, milk from treated cows is given to both male and female calves on all farms but only after treatment has been completed, i.e. during the statutory withdrawal period for human consumption. Milk from cows treated with benzylpenicillin can be used also during treatment if it is treated with  $\beta$ -lactamase to destroy potential residues. In SE, colostrum is given to both female and male calves on almost 90% of the farms and milk from cows treated during lactation is used during treatment and during the withdrawal period on 56% and 79% of the farms, respectively.

#### **3.1.3.3. Summarising remarks**

- The practices with regard to feeding calves with milk from cows treated with antimicrobials are not harmonised in the EU and few MSs have a national legislation setting provisions for this. Some MSs report that there are certain recommended usage limitations such as to only feed waste milk to male calves, only use milk collected during the withdrawal period and not during the treatment period, and to not use milk from cows treated with fluoroquinolones.



- This remark is based on the replies from 24 MSs to the EC Questionnaire.
- Feeding calves with colostrum from dairy cows treated with antimicrobials is a common practice in the majority of dairy farms in the EU.
  - This remark is supported by the four scientific studies from AT, the CZ, SE and the UK shown above and by the answers from the MSs to the EC Questionnaire (Table 1). The scientific studies were conducted in a limited number of farms in AT, the CZ, SE and the UK and the results could be influenced by selection bias. Moreover, the issue of colostrum from cows treated during the dry period was not specifically addressed in the studies from AT and the CZ. Also, the EC Questionnaire did not specifically address the use of colostrum, and therefore, it is not certain that the replies from the MSs regarding use of milk from treated cow included colostrum. Moreover, several of the answers to the EC Questionnaire were based on rough estimations rather than on actual data. Although it is most probable that colostrum from treated cows is commonly used in the EU, given the common practice of dry cow therapy and the importance of colostrum for the health of calves, the documented background for the conclusion is poor.
- Feeding calves with milk from dairy cows treated with antimicrobials is a common practice occurring in the majority of dairy farms in Europe.
  - This remark is supported by four scientific studies from AT, the CZ, SE and the UK and by the answers from the MSs to the EC Questionnaire (Table 1). The scientific studies were conducted in a limited number of farms in AT, the CZ, SE and the UK and the results could be influenced by selection bias. Also, several of the answers to the EC questionnaire were based on rough estimations rather than on actual data.

### 3.2. Antimicrobial residues in colostrum and milk of cows treated during lactation with antimicrobials and milked during the withdrawal period

In this section, the main information related to veterinary drug residues will be reviewed; first to clearly define, according to the European guidelines, what are the concepts of MRL and withdrawal period, and second, to extract the main scientific information available related to the compounds and concentrations of antimicrobial residues found in milk or colostrum. It is important to note that in the present report mainly the publically available data were assessed. Data related to milk kinetic depletion presented during the veterinary medicine marketing authorisation procedure are not available due to confidentiality concerns.

#### 3.2.1. Antimicrobial residues in milk and withdrawal period

In the European legislation (Council Regulation (EEC) No 2377/90<sup>2</sup>), it is laid down that foodstuffs obtained from animals treated with veterinary medicinal products must not contain residues of the medicine or metabolites which might constitute a health hazard for the consumer. In order to ensure this, MRLs have to be determined for all pharmacologically active substances used in veterinary medicinal products.

Scientifically determined MRL values for various antimicrobials in milk, and other food commodities, are assessed by the Committee for Medicinal Products for Veterinary Use (CVMP) on behalf of the EMA. The CVMP/EMA derived MRLs need to be adopted and published in a Commission Regulation. The substances that may be used are listed in Table 1 of the Annex to Commission Regulation (EU) No 37/2010.<sup>3</sup> For example, in lactating cows, the values for MRL in milk for cefalexin, bacitracin, tetracyclines and ceftiofur is 100 µg/kg; for cefalonium and cefquinome, it is 20 µg/kg; for amoxicillin and penicillin, it is 4 µg/kg; for colistin, it is 50 µg/kg; and for neomycin, it is 1,500 µg/kg.

<sup>2</sup> Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. OJ L 224, 18.8.90, p. 1–8.

<sup>3</sup> 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.

The withdrawal period is the period necessary between the last administration of the veterinary medicinal product to animals under normal conditions of use and the production of foodstuffs from such animals (see [Glossary](#) and Directive 2001/82/EC<sup>4</sup>). In fact, it is the time after treatment during which the residue concentration in the milk is declining to reach a safe concentration as defined by the MRL. Its length is product specific and defined during the authorisation procedure. As a large proportion of the waste milk occurring on farms is milk from the withdrawal period after treatment, it is important to define this period. The withdrawal period unit for milk is initially calculated in number of milkings and rounded up to the first higher full number of milking days. Since the predominant milking scheme is twice a day, experiments for the determination of withdrawal periods for milk should be carried out with animals milked twice a day. Guidance for determining the withdrawal periods is found in the EMEA/CVMP/473/98-Final document (EMEA-CVMP, 2000).

### 3.2.2. Bioavailability of antimicrobial compounds in the mammary gland

Antimicrobial residues occur in colostrum and in milk of cows when treated systemically or intramammarily during the lactation or the dry period. To some extent, intramammary treatments will lead to a higher concentration of the antimicrobial in milk in comparison to systemic treatment (EMA MRL assessments reports<sup>5</sup>; Toutain, 1984; Milhaud, 1985). Some systemically administered drugs, including, e.g. the 3rd generation cephalosporin ceftiofur, have no withdrawal period for milk as explained below.

With intramammary treatment, a very large proportion of the substance administered will remain in the mammary gland and is consequently shed with the milk. This is especially relevant for lactational intramammary treatment of clinical and subclinical mastitis that will be followed by milking the cow within 12 h or less. In those cases, the amount of residue contained in the milk will depend on the time span after treatment and the number of milkings between the last treatment and the milking included in the waste milk. Furthermore, intramammary treatments, if restricted to individual quarters of the mammary gland, lead to a variable amount of residues in the untreated quarters. The amount of residues in the quarters not included in the treatment will be considerably lower than in the treated quarter and may therefore lead to dilution of the residues. For the treated quarters, most of the antimicrobial drug administered locally will remain there and will be collected with the waste milk. This should be considered when evaluating the risk from feeding waste milk.

With dry cow treatment, the amount of antimicrobial remaining in the mammary gland at the end of the dry period is commonly minimal if the length of the dry period is not substantially shorter than the one recommended in the Summary of Product Characteristics (SPC) of the corresponding veterinary medicinal product. In cows calving earlier than expected, residues may be variable. Due to the withdrawal regulations for most dry cow products, milk from cows calving early is not marketable over a period that is considerably longer than the transition milk period of 5 days.

The level of milk residue concentration within an antimicrobial class will depend on antimicrobial formulation, dosage regimen (dose and duration), drug composition (e.g. excipient) and individual pharmacokinetic variability. In cows treated for mastitis, residues after systemic treatment are also influenced by the degree of destruction of the blood/mammary barrier that minimises the transmission of the systemically applied antimicrobial in the milk. Thus, some cephalosporins intended to be used for systemic administration present a zero day withdrawal period in milk.

Administered locally, the antimicrobial will be released and will disperse more or less quickly and easily according to the excipient (hydrophilic or lipophilic and viscous) in which it is included and the chemical form (salt or ester) in which it is present in the formulation. Overall, the bioavailability and the stability of antimicrobials in milk are governed by the physicochemical properties held by the various substances (lipophilicity, acidic behaviour, thermolability, etc.). Table 3 presents an overview of the lipophilicity and acidic behaviour of the major families of antimicrobials (Toutain, 1984; Milhaud, 1985).

The data presented in Table 3 revealed that the issue of drug residue after a systemic or intramammary antimicrobial treatment is complex and multifactorial. In addition to the interindividual variability, the difference in pharmacokinetic profile within and between an antimicrobial class as well as the drug formulation could impact the behaviour of drug residue in milk. Most antimicrobials do not pass the blood/mammary barrier in high amounts when administered systemically. Exceptions include some fluoroquinolones such as marbofloxacin (Kietzmann et al., 2008) or macrolides such as tylosin.

<sup>4</sup> Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products. OJ L 311, 28.1.2011, p. 1–66.

<sup>5</sup> [http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/landing/vet\\_mrl\\_search.jsp](http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/landing/vet_mrl_search.jsp)

**Table 3:** Physicochemicals properties of some antimicrobial classes

Antimicrobials	Lipophilicity	Acido/basic properties	
<b>β-Lactams</b>	Low	Acidic	Strong
<b>Aminoglycosides</b>	Low	Basic	Strong
<b>Macrolides</b>	Moderate	Basic	Weak
<b>Fluoroquinolones 1st generation</b>	High	Acidic	Weak
<b>Fluoroquinolones 2nd/3rd generation</b>	High	Amphoteric	–
<b>Tetracyclines</b>	Moderate	Amphoteric	–
<b>Sulfonamides</b>	Moderate	Acidic	Weak

Table adapted from Toutain (1984) and Milhaud (1985).

### 3.2.3. Antimicrobial compounds found in colostrum/milk

In theory, the antimicrobial compounds found in colostrum/milk should be derived from veterinary drugs authorised to treat animals. Data related to the antimicrobial compounds found in milk, and even more in colostrum, are scarce in the literature but at the national level, some MSs have been involved in monitoring of antimicrobial residues notably in waste milk. For example, the UK Animal and Plant Health Agency (APHA) has been involved in the analysis of waste milk samples for antimicrobial residues during three different time periods between 2011 and 2013 in England and Wales. Details of the β-lactam antimicrobials detected in 103 waste milk samples during one of these time periods are presented in Table 4.

**Table 4:** β-Lactam antimicrobials in waste milk samples from 103 farms in England and Wales

Waste milk results type for antimicrobials detected	Antimicrobial detected (µg/kg)							
	AMX	AMP	CLX	PEN-G	LEX	CFL	HAP	CFQ
Number waste milks positive	7	0	4	33	6	8	3	22
Mean conc. waste milks > LOD	258	NA	80	371	132	98	882	1433
Median conc. waste milks > LOD	18	NA	43	120	25	26	140	985
Mean conc. all waste milks	18	NA	3	119	8	8	26	306
Median conc. all waste milks	< LOD	NA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Maximum conc.	1300	NA	230	2500	660	580	2500	4600
Minimum conc. > LOD	5	NA	4	5	4	6	5	6
95% of samples at/below conc.	6	4	4	700	4	18	4	2200
Detection limit	4	4	4	4	4	4	4	4
No. added <sup>(a)</sup>	14	3	8	45	13	8	3	34
Unexpected positive <sup>(b)</sup>	2	0	1	5	1	3	0	1
MRL µg/kg <sup>(c)</sup>	4	4	30	4	100	20	60	20

Table adapted from Randall et al. (2014).

AMX: amoxicillin; AMP: ampicillin; CLX: cloxacillin; PEN-G: penicillin G; LEX: cefalexin (1st generation cephalosporin); CFL: cefalonium (1st generation cephalosporin); HAP: cefapirin (1st generation cephalosporin); CFQ: cefquinome (4th generation cephalosporin); conc.: concentration; LOD: limit of detection; NA: not applicable.

(a): No. added refers to the number of farms that reported adding stated antimicrobial to the waste milk tank via milk from treated cows.

(b): Unexpected positives are where the sample was positive for stated antimicrobial but the farmer had not reported in a voluntary survey the recent use of the stated antimicrobial for cows contributing to waste milk. This does not relate to non-compliance with farm recording obligations.

(c): MRL: Maximum residue limit in milk (Commission Regulation (EU) No 37/2010<sup>3</sup>).

The data presented in Table 4 revealed that from 103 farms (one waste milk sample per farm) in England and Wales, the number of milk samples positive for penicillin G and cefquinome was higher than for the other antimicrobials tested (such as amoxicillin, ampicillin, cloxacillin, etc.). Those antimicrobials are among the most frequently used in the EU in bovine animals, especially to prevent or treat mastitis (Table C.1, Appendix C). Furthermore, data indicate that the antimicrobial concentration found in waste milk is highly variable depending on the chemical nature of the antimicrobials, as already explained in Section 3.2.1.

### 3.2.4. Antimicrobial concentrations found in colostrum/milk

Hereafter, the information extracted from the EMA MRL assessment reports and scientific literature was reviewed. The main information related to the compounds and concentrations of antimicrobial residues found in milk is summarised in the present Opinion to define the potential antimicrobial selection pressure present in waste milk.

#### 3.2.4.1. Antimicrobial concentration found in milk

Some information related to antimicrobial concentrations found in milk after treatment could be obtained from the scientific published literature and from MRLs assessment reports. When available, these data are useful to estimate the residual antimicrobial activity and then to estimate the potential selection pressure of the contaminated milk on the pathogenic and commensal flora. The concentration of antimicrobial residues found in milk is dependent on the nature of the antimicrobials, the route of administration, the administered dose and the duration of the treatment.

##### *Data extracted from EMA MRL assessment reports*

The MRL assessment reports are the result of the CVMP assessment of the data provided by a marketing holder to the EMA for the determination of an official maximal residue limit for drugs intended to be used in food producing animals. The determination of a MRL allows the setting of the withdrawal period for a veterinary drug intended to be used for foodstuffs producing animals based on antimicrobial residue kinetics depletion experiments in the food matrix (e.g. milk). As an example, in Table 5, data from an MRL assessment report related to the concentration of ceftiofur residues in milk following two intramammary administrations are presented.

**Table 5:** Concentration ( $\mu\text{g/mL}$ ) of antimicrobials residues in milk following intramammary administration

Number of antimicrobial intramammary injection	Methods	1st and 2nd injection, 12 h apart		Time (h) after the last antimicrobials administration											
		12 h	24 h	12 h	24 h	36 h	48 h	60 h	72 h	84 h	96 h	108 h	120 h	132 h	144 h
		Cephalosporin 3rd Generation	HPLC-UV <sup>(a)</sup>	22.46	1.64	21.56	1.95	0.16	0.03	< 0.015	< 0.015	< 0.015	< 0.015	< 0.015	< 0.015
Ceftiofur IMM 132 mg/quarter	HPLC-DCA <sup>(b)</sup>	40.92	83.8	45.05	12.44	4.0	1.88	0.55	0.3	0.13	0.09	0.07	0.04	0.03	0.02

HPLC: high-performance liquid chromatography; UV: ultraviolet; DCA: desfuoylceftiofur acetamide.

Table adapted from EMA MRLs assessment reports.

(a): Method for the detection of desfuoylceftiofur.

(b): Method for the detection of acetamide derivative of desfuoylceftiofur.

According to the EMA MRL assessments reports, the concentration of ceftiofur and desfuoylceftiofur-related residues (high-performance liquid chromatography-desfuoylceftiofur acetamide (HPLC-DCA) methods) in milk from dairy cows was 0.09  $\mu\text{g/mL}$  (corresponding to 90  $\mu\text{g/kg}$ ) 96 h after the last of two intramammary administrations of ceftiofur. This is below the MRL for ceftiofur in milk (100  $\mu\text{g/kg}$ ). Likewise, the concentration of ceftiofur plus desfuoylceftiofur-related residues in the kidney and liver of neonatal calves at birth and after being fed colostrum and milk from treated cows for 96 h was less than 100  $\mu\text{g/kg}$ .

##### *Data extracted from public available MRL summary reports*

Information related to concentration of antimicrobials residue in milk of dairy cows after antimicrobial treatment were extracted from the EMA MRL summary reports, public available on the EMA website.<sup>5</sup> The information extracted for lactating cows is presented below.

- Aminoglycosides:
  - Neomycin

After oral administration, the residues of neomycin in milk were low just after the antimicrobial treatment. Following an intramammary infusion of 330 mg of lincomycin hydrochloride and 100 mg of neomycin sulfate, the level of neomycin residues in milk and in udder tissues were measured by HPLC (limit of quantification, LOQ = 100  $\mu\text{g/kg}$ ) per quarter for three successive milking in 16 healthy lactating cows. When udder quarters

were analysed independently, the mean concentration of neomycin residue during the 1st, 2nd and 3rd infusion reached 22,200, 29,900 and 28,000  $\mu\text{g/L}$ , respectively. Twenty-four hours after the last administration, the mean concentration declined to 4,900  $\mu\text{g/L}$ . In a total pooled milk sample, the measured concentrations of neomycin were 4,800, 240, 200 and 120  $\mu\text{g/L}$ , respectively, 12, 60, 72 and 84 h after the last administration.

— Streptomycin

After intramuscular injection of a combination of streptomycin and dihydrostreptomycin for three consecutive days at a dose of 10 mg/kg, the residues of both antimicrobials compounds were measured by HPLC (LOQ = 50  $\mu\text{g/kg}$ ). The concentrations of streptomycin and dihydrostreptomycin residues measured are presented in Table 6.

**Table 6:** Range of streptomycin and dihydrostreptomycin concentrations ( $\mu\text{g/kg}$ ) in milk after intramuscular treatment in lactating cows

Antimicrobial	12 h	24 h	36 h	48 h	72 h
Streptomycin	173–265	85–123	51.6–67.7	< LOQ	< LOQ
Dihydrostreptomycin	166–252	74.5–104	50.6–66.7	< LOQ	< LOQ

— Kanamycin

After intramuscular injection in five lactating cows of 7.5 mg/kg of kanamycin twice a day for five consecutive days, the mean residues concentrations measured by microbiological assay (LOQ = 100  $\mu\text{g/kg}$ ) were 1,400, 840, 150  $\mu\text{g/kg}$  and below the LOQ at the 1st, 2nd, 3rd and 4th milking, respectively.

In another study, after intramuscular injection in three lactating cows, the excretion in milk of kanamycin residues was considered negligible. Indeed, only 0.03–0.05% of the initial administered dose was recovered within 12 h after the treatment.

• Polypeptides: Colistin

According to the EMA summary reports, after intramuscular injection of a solution containing colistin, residues were detectable until the 6th milking. The amount of residue after intramammary administration were significantly higher but were undetectable by the 7th milking after treatment.

In another milk residue study, 10 lactating cows (five high-yielding and five low-yielding cows) were treated using intramammary injection of 10 mg of amoxicillin and 25,000 IU of colistin per kg per day during 5 days. The mean residues concentration of colistin measured by HPLC (LOQ 10  $\mu\text{g/kg}$ ) were 50  $\mu\text{g/kg}$  during the antimicrobial treatment (range from 30 to 109  $\mu\text{g/kg}$ ), and 13  $\mu\text{g/kg}$  (range from 10 to 33  $\mu\text{g/kg}$ ), 8  $\mu\text{g/kg}$  (range from < 10 to 16  $\mu\text{g/kg}$ ) and < 10  $\mu\text{g/kg}$  (range from < 10 to 12  $\mu\text{g/kg}$ ) on day 2, 4 and 4 after the last administration, respectively.

Following oral administration with in-feed medication containing colistin equivalent to 100,000 IU/kg/day, the amount of colistin residues in all the sample of pooled milk measured by HPLC and microbiological assays were below the LOQ.

• Macrolides: Spiramycin

After intramuscular injection in 6 lactating cows of 30,000 IU/kg of spiramycin, the mean residues concentrations measured were < 1,000  $\mu\text{g/kg}$  at the 8th milking (day 4 after the treatment) and declined to 90  $\mu\text{g/kg}$  17th milking (day 8.5 after the treatment).

• Fluoroquinolones: Marbofloxacin

After subcutaneous injection of a commercial formulation of marbofloxacin at a dosage regimen of 2 mg/kg per day in eight lactating cows (four high-yielding and four low-yielding cows), the residues concentrations measured ranged from 180 to 679  $\mu\text{g/kg}$  at 1st milking after the treatment. At the 3rd milking, the marbofloxacin residues concentration ranged from 10 to 34  $\mu\text{g/kg}$  and declined below the LOQ of 10  $\mu\text{g/kg}$  after the 5th milking.

- Cephalosporins:

- Cefquinome

After intramammary administration of a cefquinome solution in lactating cows, high concentrations of residues were measured in milk after the last administration. At the 10th milking, the residue concentrations in all bucket samples were below 20 µg/kg.

- Cefalexin

Different milk residues studies were conducted in lactating cows either to determine the amount of milk contamination after antimicrobials intramuscular or intramammary administration.

After intramuscular administration in six lactating cows of a solution containing 7 mg of radiolabelled cefalexin, the residues in milk were measured by HPLC (LOQ = 10 µg/kg). At 1st milking, the concentration of cefalexin reached 74 µg/kg then declined to 10 µg/kg and < 4 µg/kg at the 4th and 8th milking, respectively.

After intramammary administration in three lactating cows of a solution containing 200 mg of radiolabelled cefalexin, the mean total radioactive residue measured in milk decreased from 5,575 µg/kg at the 1st milking to 52 µg/kg after the 5th milking after the treatment.

Another study presented the milk residues results after intramammary administration in 10 lactating cows of a solution containing 200 mg of cefalexin per quarter for 4 days, the residues in milk were measured by HPLC (LOQ = 10 µg/kg). At the 1st milking after the treatment, the concentration of residues reached 37,061 µg/kg and then declined to less than 10 µg/kg at the 13th milking.

*Data extracted from scientific literature*

In Table 7, data from literature related to the mean concentration of antimicrobial residues in milk following two intramammary administrations are presented.



**Table 7:** Mean concentration ( $\mu\text{g/mL}$ ) of antimicrobial residues in milk following intramammary administration

Antimicrobial, quantity/dose	Number of days after last antimicrobials administration (two administrations (-1 and -2) at 24 h apart)													
	-2	-1	1	2	3	4	5	6	7	8	9	10	11	12
Neomycin sulfate, 300 mg/6 g	139.7	23.1	163.4	22.8	8.36	1.51	0.31	< 0.15	-	-	-	-	-	-
Neomycin sulfate, 200 mg/6 g	63.4	6.13	53.1	8.30	0.62	0.15	< 0.15	-	-	-	-	-	-	-
Dihydrostreptomycin sulfate, 350 mg/10 mL	241.0	137.0	268.0	13.6	4.4	1.34	0.655	0.48	0.38	0.36	0.28	0.24	0.15	< 0.15
Dihydrostreptomycin sulfate, 100 mg/4 g	58.1	29.3	51.8	5.8	1.05	0.37	0.16	< 0.15	< 0.15	< 0.15	-	-	-	-
Kanamycin sulfate, 50 mg/10 mL	10.2	9.6	15.3	3.03	0.68	0.18	< 0.15	-	-	-	-	-	-	-
Gentamicin sulfate, 50 mg/9 g	6.46	3.72	5.74	1.29	0.66	0.25	0.16	0.083	0.065	0.035	< 0.025	< 0.025	-	-
Procaine penicillin G, 450,000 IU/6 g	48.5	2.65	86.08	5.16	0.548	0.08	0.011	0.002	0.001	< 0.001	-	-	-	-
Procaine penicillin G, 1,000,000 IU/10 mL	291.0	182.3	289	9.26	0.551	0.044	0.008	0.002	< 0.001	-	-	-	-	-
Sodium cloxacillin, 200 mg/9 g	35.9	6.86	37.5	1.68	0.2	0.02	0.01	< 0.01	-	-	-	-	-	-
Cefalexin monohydrate, 100 mg/10 mL	3.1	2.94	3.21	0.051	0.031	0.02	< 0.02	< 0.02	< 0.02	-	-	-	-	-

Table adapted from Moretain and Boisseau (1989, 1993). -: not measured.

After the last administration, the concentration of antimicrobial residues reaches its maximum. The depletion profile obtained from literature or MRLs assessment reports showed that the concentration of antimicrobial residue in milk follows a classic depletion kinetic. After the last administration, the residue concentration decreases in a time-dependent manner until the LOQ of the corresponding methodology. It is important to quantify all the microbiologically active compounds and metabolites derived from the parental molecule. For example, concerning  $\beta$ -lactams, it is important to quantify the parental form found in the milk but also the derivative metabolites with an intact  $\beta$ -lactam ring.

The impact of residues on antimicrobial resistance is clearly dependent on the concentration of the active compounds (see Section 3.5). This directly influences the risk of resistance development and spread. The antimicrobial concentration in milk during the first days after treatment will be higher than the minimum inhibitory concentration (MIC, see Glossary) but then declines. In a risk assessment process, it is important to consider the different concentrations scenarios in relation to the emergence of antimicrobial resistance with attention to sub-MIC concentrations.

#### 3.2.4.2. Antimicrobial concentration found in colostrum

Treatment of cows at the beginning of the dry period does not frequently result in the presence of high concentrations of antimicrobial residues in colostrum and milk samples after calving, given that the dry cow treatment is applied according to manufacturer's instructions and cows do not calve substantially earlier than expected (Johnson et al., 1977; Oliver et al., 1984, 1990; Rangel-Lugo et al., 1998; Hausler et al., 2013). The time between treatment and calving and the nature of the antimicrobial used are the two most relevant factors in this respect.

##### *Data extracted from publically available MRL summary reports*

Information related to concentration of antimicrobials residue in the colostrum of dairy cows after antimicrobial treatment were extracted from the EMA MRL summary reports, publically available on EMA website and are presented below. For dry cows:

- Cephalosporins – Cefalonium

Concerning dry cow therapy, milk residues studies were conducted with cefalonium. The results were then compared in dairy cows with a short versus a longer dry period.

After intramammary infusion of a solution of 250 mg per quarter of radiolabelled cefalexin in seven dry cows which calved either from 11 to 17 or from 29 to 37 days after treatment, the concentration of radioactive residues were measured. In the 1st milking after calving, the concentration of total radiolabelled residue was 666–6544  $\mu\text{g}/\text{kg}$ . By high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS), the concentration of cefalonium measured was less than 10–448  $\mu\text{g}/\text{kg}$ , and the total antimicrobial activity measured by microbiological assay was less than 15–269  $\mu\text{g}/\text{kg}$ .

In another study, after intramammary infusion of a solution of 250 mg per quarter of radiolabelled cefalexin in dry cows which calved 41–71 days after treatment, the concentrations of radioactive residues were measured. According to the EMA summary reports, about 40% of the initial dose was excreted in the first 7 days after intramammary infusion mainly by urine route (33–36%) and also by faeces (3–5%). Less than 3% was excreted in milk, and the residues was mainly constituted of unchanged cefalonium (parental compound).

A third study was presented in which intramammary infusion of a solution of 250 mg per quarter of radiolabelled cefalexin in 20 dry cows which calved 29–97 days after treatment, the microbiological activity of milk was measured. Cefalonium concentrations in milk from the cow with the shortest dry period of 29 days decreased from 180  $\mu\text{g}/\text{kg}$  at the 5th milking, to less than 10  $\mu\text{g}/\text{kg}$  at the 22nd milking. Cefalonium concentration and the number of detectable positive milk samples decreased with the length of dry period.

##### *Data extracted from scientific literature*

In the scientific literature, some information can be found relating to antimicrobial residue concentration found in the colostrum after treatment at the dry period. For example, Bousquet et al. (2012) did not find antimicrobial-positive colostrum 35–42 days after intramammary treatment with cefalexin of 21 cows. Neither did intramammary infusion with cloxacillin more than 7 days before calving (Oliver et al., 1992), intramammary infusion with penicillins more than 9 days (Johnson et al., 1977) nor more than 11 days (Oliver et al., 1990) result in the presence of antimicrobial residues in the colostrum or in the milk after calving. This was also the case after treatment with cefapirin benzathine, novobiocin, cloxacillin, benzathine and erythromycin more than 30 days before calving

(Rangel-Lugo et al., 1998; Hausler et al., 2013). The study of Oliver et al. (1992) showed that 75% of colostrum samples contained residues when treated 7 days before parturition with cefapirin, and 3/8 first milking colostrum samples 17 days after treatment. Treatment of 28 cows with oxacillin 28–52 days before calving resulted in five cows with 11.0–19.6 µg/kg during the first 2 days of lactation and in two cows with 12.9–18.1 µg/kg at days 3 and 4 (Oliver et al., 1992).

Dihydrostreptomycin sulfate shows an initial rapid decline after treatment, followed by persistence of a low concentration of the drug for about 5 weeks (Rangel-Lugo et al., 1998). Accidental occurrence of antimicrobial residues in the colostrum was observed by Oliver et al. (1984) and 4 of 186 colostrum samples were found positive with a Delvotest P assay and one of them (from a cow calving 50 days after infusion with cefapirin) also tested positive with a *Bacillus stearothermophilus* disk assay. One of 174 samples of colostrum or milk from a cow infused with benzathine/cloxacillin with a dry period of 61 days was found positive for antimicrobial residues (*B. stearothermophilus* disk assay) by Oliver et al. (1990).

In a recent national project from the Netherlands related to the antimicrobial resistance development in young calves (2013), Gonggrijp et al. (2015) (Appendix D) investigated the compounds and concentrations found in colostrum. Colostrum samples from 118 cows on 114 farms were analysed by liquid chromatography–mass spectrometry (LC–MS) confirmation for the presence of antimicrobials. Eighty-eight (75%) of the cows received a dry cow intramammary treatment with β-lactam antimicrobials and 30 (25%) with a combination of aminoglycosides and β-lactam.

In Table 8, the concentrations of the antimicrobials found in the colostrum samples are summarised. Sixty-seven per cent of the colostrum samples did not exceed the MRL concentration of any antimicrobial applied, 29% of the samples contained cloxacillin at a concentration above the MRL with a median concentration of 86.5 µg/kg and a mean of 229.8 µg/kg, 3% exceeded the MRL concentration of ampicillin, and 1% of penicillin (Appendix D).

**Table 8:** Concentrations (µg/kg) of the antimicrobial residues in 118 colostrum samples

Antimicrobial	Number of samples	Concentration: median, mean	MRL (µg/kg)	Samples above MRL
Cloxacillin	48	86.5 (229.8)	30	34 (29%)
Neomycin	25	250 (297.6)	1,500	0 (0%)
Ampicillin	8	3.9 (25.5)	4	3 (3%)
Penicillin	4	2.7 (26.8)	4	1 (1%)
Cefalexin	1	5 (5)	100	0 (0%)
Streptomycin	1	118 (118)	200	0 (0%)

Table adapted from Gonggrijp et al. (2015), (Appendix D).

The dry period length of 51–60 days has been widely adopted as a constant during decades of management progress (Bachman and Schairer, 2003). The comparison of the duration of the dry period revealed that a longer dry period decreased the proportion of positive samples (Table 9).

**Table 9:** Length of the dry period, the number of cows and percentage of positive colostrum samples

Days of the dry period	Number of cows	Percentage positive (%)
Shorter than 44 days	32	72
Between 44 and 62 days	59	64
Longer than 62 days	27	52

Table adapted from Gonggrijp et al. (2015), (Appendix D).

In 23 of the 118 cows (19%), an internal teat sealer was used together with the antimicrobial injection. In 48% of these cows, antimicrobials were detected in the colostrum and in 67% of the colostrum samples from cows had antimicrobials in the colostrum when not treated with an internal teat sealer. Gonggrijp et al. (2015) (Appendix D) treated on eight dairy farms 22 cows with cloxacillin (500/600 mg) and 6 with neomycin (500 mg) and benzylpenicillin (1,314 mg). Two cows did not excrete detectable antimicrobial residues in the colostrum. The total amount of cloxacillin in the first 300 mL colostrum had a median concentration of 16.7 µg/L (mean 56.7 µg/L, range 0–263 µg/L) and in the first milking a median concentration of 0.833 mg/L (mean 3 mg/L, range 0–27 mg/L) for the

cows treated with cloxacillin. The total amount of neomycin had in the first 300 mL colostrum a median of 177 µg/L (mean 267 µg/L, range 0–600 µg/L) and in the first milking a median of 2.27 mg/L (mean 3.5 mg/L, range 0.47–11.9 mg/L).

### 3.2.5. Summarising remarks

Based on these general observations, the following remarks can be made:

- Data related to antimicrobial concentrations found in milk are presented during the marketing authorisation procedure but are not available due to confidentiality concerns. In the present report, only publically available data were assessed.
- Residue concentrations in milk and colostrum follow depletion kinetics with a maximal concentration after the last administration to a minimal concentration usually represented by the limit of quantification of the applied methodology. No information was available for concentrations below the LOQ during milk residues depletion kinetic studies.
  - The data were generated following European guidelines and were assessed by an European scientific authority (EMA).
- For systemic treatments, with some exceptions (e.g. systemic cephalosporins with zero day withdrawal period), antimicrobials used to treat or prevent infections of dairy cows during lactation will lead to the contamination of the milk by antimicrobial residues. In contaminated milk, the parent product is excreted, as well as derivative metabolites with intrinsic antimicrobial activity.
  - This remark is based on scientific literature and also based on EMA data with peer-reviewed results according to the European guideline and directives.
- Milk residue contamination after antimicrobial treatment in lactating cows is higher after an intramammary treatment than after systemic treatment.
  - This remark is based on scientific literature and also based on EMA data with peer-reviewed results according to the European guideline and directives.
- The level of antimicrobial residues contamination in colostrum and milk is mainly dependent on the drug formulation, the nature of the antimicrobials and the time between treatment and calving.
  - This remark is based on scientific literature and also based on EMA data with peer-reviewed results according to the European guideline and directives.
- Results from scientific literature on the time needed between treatment and parturition indicated that the antimicrobial residue levels in colostrum samples are expected to be low. Only two studies presented results on the antimicrobial residue levels in field colostrum samples, showing a low number of samples (5/186 and 38/118 samples) with levels above the MRLs or detection limit.
- The MRL assessment reports and scientific literature show that antimicrobial residue levels decrease with the length of dry period.
  - This remark is based on scientific literature and also based on EMA data with peer-reviewed results according to the European guideline and directives.
- With a dry period length as long as or longer than the minimum specified in the Summary of Product Characteristics of the antimicrobial product the antimicrobial residue levels in the colostrum are judged to be low.
  - The data were generated following European guidelines and were assessed and accepted by the European Medicines Agency.

### 3.3. Occurrence and epidemiology of AMR in bacteria isolated from colostrum and milk of cows treated during lactation with antibiotic and milked during the withdrawal period

Fresh milk or colostrum provided to calves is usually not free of bacteria. A wide range of different bacterial species have been reported to be present in the colostrum (Fecteau et al., 2002), including

potential mastitis pathogens (Tenhagen et al., 2009), but also other environmental bacteria, which are likely to include bacteria of faecal origin, that end up in the colostrum or transition milk during milking and storage. Likewise, bacteria in waste milk may be related to mastitis but also to environmental and/or faecal contamination during milking and storage. In view of this the assessment focuses mainly on resistant bacteria related to mastitis and detected in field samples of waste milk. However, bacteria detected in field samples of waste milk that were considered in some studies, may likewise originate from the environment and may be exposed to selective pressure in the waste milk due to the contained antimicrobial residues.

### 3.3.1. Antimicrobial resistance of mastitis pathogens in Europe

It should be noted that for AMR in mastitis pathogens, the literature does not consistently state whether isolates are from treated or untreated animals. Moreover, it should be noted that cut-offs or breakpoints used to classify organisms as sensitive or resistant to an antimicrobial may vary between studies referenced in this section. A table including the different methods and criteria used in the analyses mentioned in this section can be found in Appendix E, Table E.1.

Staphylococci, streptococci and coliform bacteria are considered as the main pathogens causing mastitis in lactating cows. *E. coli*, *S. aureus* and *S. uberis* isolated from milk samples with clinical mastitis treated with antimicrobials were investigated in eight European countries via the VetPath monitoring programme (Thomas et al., 2015). The MIC<sub>50</sub> and MIC<sub>90</sub> values (MICs at which at least 50% and 90% of the isolates in a test population are inhibited, respectively) were determined for each organism (Thomas et al., 2015; Table 10)



**Table 10:** Minimum inhibitory concentration (MIC) of antimicrobials for *E. coli*, *S. aureus* and *S. uberis* from acute mastitis in dairy cows

Bacterial species	Antimicrobial agent	Susceptible <sup>(a)</sup>		Intermediate <sup>(a)</sup>		Resistant <sup>(a)</sup>		MIC <sub>50</sub> (µg/mL)	MIC <sub>90</sub> (µg/mL)
		n	%	n	%	n	%		
<i>Escherichia coli</i> (n = 280)	Amoxicillin/clavulanic acid	273	97.5	4	1.4	3	1.1	4	8
	Cefalexin	268	95.7	10	3.6	2	0.7	4	8
	Cefapirin	185	66.1	64	22.9	31	11.1	8	32
	Ceftiofur	280	100	0	0	0	0	0.25	0.5
	Cefquinome	–	–	–	–	–	–	0.03	0.06
	Enrofloxacin	–	–	–	–	–	–	0.03	0.03
	Marbofloxacin	–	–	–	–	–	–	0.03	0.06
	Neomycin	–	–	–	–	–	–	1	2
	Tetracyclines	239	85.4	1	0.4	40	14.3	2	≥ 4
	Penicillin G	160	64	–	–	90	36	0.06	2
<i>Staphylococcus aureus</i> (n = 250)	Amoxicillin/clavulanic acid	250	100	–	–	0	0	0.25	1
	Cloxacillin	250	100	–	–	0	0	0.25	0.5
	Cefalexin	248	99.2	2	0.8	0	0	2	4
	Cefapirin	250	100	0	0	0	0	0.25	0.25
	Ceftiofur	249	99.6	1	0.4	0	0	1	1
	Cefquinome	–	–	–	–	–	–	0.5	1
	Enrofloxacin	–	–	–	–	–	–	0.12	0.25
	Marbofloxacin	–	–	–	–	–	–	0.25	0.5
	Erythromycin	246	98.4	–	–	2	0.8	0.25	0.5
	Neomycin	–	–	–	–	–	–	0.5	1
Tetracyclines	237	94.8	–	–	13	5.2	0.25	0.5	

Bacterial species	Antimicrobial agent	Susceptible <sup>(a)</sup>		Intermediate <sup>(a)</sup>		Resistant <sup>(a)</sup>		MIC <sub>50</sub> (µg/mL)	MIC <sub>90</sub> (µg/mL)
		n	%	n	%	n	%		
<i>Streptococcus uberis</i> (n = 282)	Penicillin G	198	70.2	84	29.8	0	0	0.03	0.25
	Amoxicillin/clavulanic acid	282	100	0	0	0	0	≤ 0.012	0.5
	Cloxacillin	–	–	–	–	–	–	0.5	4
	Cefalexin	282	100	0	0	0	0	0.5	0.5
	Cefapirin	282	100	0	0	0	0	0.06	0.5
	Ceftiofur	280	99.3	2	0.7	0	0	0.12	2
	Cefquinome	–	–	–	–	–	–	0.03	0.25
	Enrofloxacin	–	–	–	–	–	–	0.5	1
	Marbofloxacin	–	–	–	–	–	–	1	2
	Erythromycin	225	79.8	4	1.4	53	18.8	0.06	16
	Neomycin	–	–	–	–	–	–	64	≥ 64
	Tetracyclines	201	71.3	0	0	81	28.7	0.25	64

Table adapted from Thomas et al. (2015). –: no data.

(a): According to CLSI (see Appendix E).

Earlier studies (Roesch et al., 2006; Bengtsson et al., 2009; Botrel et al., 2010; Kalmus et al., 2011; Persson et al., 2011; for, respectively, Switzerland, Sweden, France, Estonia, Sweden) were reviewed by Oliver and Murinda (2012).

Among mastitis *E. coli* isolates (n = 280), resistance to tetracyclines (14.3% of isolates) and cefapirin (11.1% of isolates) was most common (Thomas et al., 2015). The MIC<sub>90</sub> of enrofloxacin and marbofloxacin was 0.03 and 0.06 g/mL, respectively, with 0.7% of isolates displaying a deviating high MIC (Thomas et al., 2015). Also, a high percentage of isolates were resistant to ampicillin (31.3%) and to streptomycin in Estonia (21.4%) (Kalmus et al., 2011) and France (13.4%) (Botrel et al., 2010). In a Swedish study, quinolone-resistant *E. coli* were found in 3% of the milk/milk substitute and colostrum samples (Duse et al., 2016). In DE in 2012, 17.3% of *E. coli* from mastitis in a national surveillance programme were resistant to ampicillin and 10.5% to tetracycline. Of note, 5.9% were microbiologically resistant (MIC > 0.06) to ciprofloxacin (4% with clinical resistance (MIC > 0.5) and 9% to ceftiofur (MIC > 2). Moreover, resistance to ceftiofur increased substantially between 2010 and 2012 along with an increase in resistance to ampicillin (BVL, 2015).

*S. uberis* mastitis strains (n = 282) showed resistance to erythromycin (18.8% of isolates) and tetracycline (28.7% of isolates) (Thomas et al., 2015). A high percentage of isolates was also resistant to enrofloxacin (42.1%) and gentamicin (52.6%) in Switzerland; to gentamicin (28.1%) in Estonia (Kalmus et al., 2011) and to different antimicrobials such as lincomycin (15.4%), oxacillin (12.6%), spiramycin (17%) and streptomycin (17.1%) in France (Botrel et al., 2010).

*S. aureus* mastitis strains (n = 250) were resistant to penicillin G (36.0% of isolates), but susceptible to most other antimicrobials (Thomas et al., 2015). The high percentage of isolates resistant to penicillin G was linked with resistance to ampicillin (59.5% of isolates) and clindamycin (18.1% of isolates) in Estonia (Kalmus et al., 2011) and with resistance to chloramphenicol (19.6% of isolates) and gentamicin (10.9% of isolates) in Switzerland (Roesch et al., 2006). In Germany, resistance to penicillin has decreased in *S. aureus* from mastitis samples in recent years (see footnote<sup>8</sup>) with 16.1% being resistant in 2013 (BVL, 2015). In the study of Selim and Cullor (1997), 24 out of 26 *E. coli* isolates tested were resistant to erythromycin, 21 to cefalothin and 12 to tetracycline. All *S. aureus* isolates (seven) were resistant to penicillin G and ampicillin.

For amoxicillin/clavulanic acid, cefalexin, cefapirin, ceftiofur and cloxacillin, no significant differences in the percentages of resistant mastitis strains were reported between the participating countries (Thomas et al., 2015). Significant differences in the percentages of mastitis isolates resistant to certain antimicrobials were noted between countries for penicillin G, erythromycin and tetracycline as explained below:

- *E. coli*: Resistance to tetracyclines for mastitis *E. coli* was lowest (2.7% of isolates) in the UK and highest (24.4% of isolates) in Ireland (Thomas et al., 2015). This variation in the percentage of isolates resistant to tetracyclines for *E. coli* was not explained or discussed but also observed in the studies reviewed by Oliver and Murinda (2012) in Sweden (4.9% of isolates) and Estonia (22.2% of isolates) (Thomas et al., 2015).
- *S. aureus*. Resistance of mastitis *S. aureus* strains to penicillin G was lower in Germany (11.9%) compared to Ireland (65.8%) (Thomas et al., 2015). Similar differences were also observed in studies reviewed by Oliver and Murinda (2012) with lowest percentages of isolates resistant to Penicillin G in Sweden (4%, and 7.1% in Persson et al., 2011 and Bengtsson et al., 2009; respectively) and the highest percentage of resistant isolates in Estonia (61.4%) (Kalmus et al., 2011). For *S. aureus* and tetracyclines, the lowest percentage of resistant isolates was observed in the UK (0.0% of isolates) compared to the Czech Republic (15.0% of isolates) (Thomas et al., 2015).
- *S. uberis*. Resistance of mastitis *S. uberis* to erythromycin was lower (4.3% of isolates) in the Netherlands and higher (48.5% of isolates) in Italy. For tetracyclines and *S. uberis*, the lowest (12.3% of isolates) percentage resistance was observed for isolates from France compared to Italy (75.8% of isolates) (Thomas et al., 2015).

### 3.3.2. Antimicrobial resistance in bacteria from waste milk

It is likely that the cocktail of antimicrobial residues that can be present in waste milk provides a unique selective environment for resistant isolates, such as extended-spectrum beta-lactamases (ESBL)-producing organisms (Horton et al., 2016), or even colistin-resistant isolates (Brennan et al., 2016) with co-resistance to other antimicrobials. This selective environment could possibly lead to an increase in numbers of resistant bacteria as well as in the exchange of genetic material between bacteria by conjugation or transformation.

The contribution of mastitis pathogens and environmental flora (including those from bovine faeces) to the bacterial composition of waste milk is largely unknown, and will depend largely on milking hygiene and storage conditions of waste milk. However, viable bacterial counts within waste milk show that bacterial levels can be as high as  $10^9$  CFU/mL, and that most waste milk samples are contaminated with bacteria (Randall et al., 2012).

It seems reasonable to assume that feeding of waste milk contaminated with antimicrobial resistant bacteria (pathogens or commensals) or AMR-encoding genes to calves is therefore one of the possible routes for increasing the rate of resistant bacteria in the calf gut. In a recent study (Horton et al., 2016), the majority of calves were shedding faeces containing  $> 10^4$  CFU/g of presumptive CTX-M ESBL-producing *E. coli* on a farm feeding waste milk to calves, where most of the adult cattle were also positive for CTX-M- producing *E. coli*, and the waste milk was shown to contain cefquinome. In other studies, high bacterial counts and antimicrobial-resistant bacteria were found in waste milk offered to calves with predominance of *Streptococcus* species in 50.9% of samples (84/165), Enterobacteriaceae in 50.3% of samples (83/165), *E. coli* in 32% of the samples (52/165) and staphylococci including some mastitis pathogenic strains in 41.4% of samples (68/165) (Selim and Cullor, 1997). In another study, the mean bacterial count (~ maximum) without enrichment in 103 waste milk samples (from different farms) were  $\sim 10^7$  ( $10^9$ ) CFU/mL on blood agar and  $\sim 10^4$  ( $10^5$ ) CFU/mL for presumptive *E. coli* (Randall et al., 2012). For these samples, 21% of 120 isolates identified from waste milk were *E. coli* (Randall et al., 2014). Also, 5.8% of the 103 waste milk samples (from different farms) were positive for ESBL-producing Enterobacteriaceae (including 3.9% positive for CTX-M producing *E. coli*) (Randall et al., 2014).

Another study found a high prevalence of cefazolin-resistant bacteria (Beneragama et al., 2013) in waste milk and dairy manure. In the study by Randall et al. (2014), the MICs of cefotaxime, cefquinome, ceftazidime and cefalexin against isolates of *Citrobacter* spp., *E. coli*, *Enterobacter* spp., *Kluyvera* spp and *Raoultella* spp. from waste milk samples were determined. MICs to all four antimicrobials for the more resistant isolates were 4–16 to  $> 128$  mg/L. A previous poster publication of the same study also reported resistance (based on EUCAST epidemiological cut-off values (ECOFFs), see [Glossary](#)) in Enterobacteriaceae isolates ( $n = 29$ ) to amoxicillin, ceftazidime, cefotaxime, neomycin, streptomycin and sulfonamides (with MIC results also suggesting resistance to cefquinome and oxytetracycline, but ECOFFs are not available for these antimicrobials), with most of the MICs for the tested isolates between 16 and  $> 128$  mg/L (Randall et al., 2012). These results demonstrate resistance in these isolates to aminoglycosides, cephalosporins, penicillins, sulfonamides and tetracyclines, and hence multiple antimicrobial drug resistance.

### 3.3.2.1. Summarising remarks

- Mastitis pathogens, which are likely to be a significant part of the bacterial flora of waste milk, have varying levels of resistance to different antimicrobials. Where AMR data are available, most are for older antimicrobials.
  - This remark is supported by several published studies that report on the AMR in mastitis pathogens from different European MSs, and as such the data presented should be robust. However, data were limited or absent for 3rd and 4th generation cephalosporins. Also, the contribution of mastitis pathogens and environmental flora (including those from bovine faeces) to the flora of waste milk is largely unknown, and will depend largely on milking hygiene and storage conditions of waste milk.
- Isolates from waste milk show resistance to antimicrobials including to both 3rd- and 4th-generation cephalosporins, and also multiple antimicrobial resistance.
  - This remark is supported by the three scientific papers and a poster cited above. However, these studies are limited in the conclusions that can be drawn from them, as the studies that looked at antimicrobial resistant bacteria from waste milk from  $\sim 100$  different farms and longitudinally on one farm were only based in the UK, and as such may not represent the resistance patterns of isolates from waste milk in other countries. The other two studies were based on waste milk and manure collected from a single dairy farm in Japan, and as such may not reflect the resistance patterns due to antimicrobials that would be present in waste milk in different EU MSs.

### 3.4. Occurrence and epidemiology of AMR in bacteria isolated from calves fed with colostrum and milk of cows treated during lactation with antimicrobial and milked during the withdrawal period

Several studies on faecal shedding of AMR bacteria (mainly *E. coli*) have been carried out to investigate the effect of feeding calves milk and/or colostrum from cows treated during lactation and/or at dry-off. The methodology and results of these studies are summarised in the tables below.

The studies are grouped according to their experimental set up:

- 1) Observational studies on farms where waste milk was routinely fed to calves (Table 11).
- 2) Observational studies on farms where waste milk was fed to a group of calves and where one group was fed with antimicrobial-free milk as control (Table 12).
- 3) Observational studies on farms where colostrum from cows treated with a known antimicrobial during dry-off was fed to a group of calves and where one group was fed with antimicrobial-free colostrum as control (Table 13).
- 4) Experimental studies where with known concentrations of antimicrobials added to milk were fed to calves in comparison with calves fed antimicrobial-free milk (Table 14).

For each study, the uncertainties and limitations are summarised and, based on this, the individual studies were differentially taken into account in the conclusions (Table 15).

#### 3.4.1. Summary tables on studies investigating the association of feeding milk or colostrum containing antimicrobial residues on AMR in faecal bacteria from calves

In Tables 11–14, the results of different published studies on the effect of the shedding of AMR bacteria (mostly *E. coli*) are summarised. Information on the criteria applied to classify organisms as sensitive or resistant to an antimicrobial in each of the studies is provided in Appendix E.



**Table 11:** Antimicrobial resistant bacteria in faeces of calves and dairy cows on farms feeding calves milk from treated cows

	<b>Dolejšká et al. (2008)</b>	<b>Randall et al. (2014)</b>	<b>Duse et al. (2015)</b>	<b>Kyselková et al. (2015)</b>
Study design	Field study <sup>(a)</sup>	Field study <sup>(a)</sup>	Field study <sup>(b)</sup>	Field study <sup>(a)</sup>
Country	Czech Republic	England/Wales; 2011	Sweden	Czech Republic
Farm	2 dairy farms	3 dairy farms with 200–500 adult milking cows and 30–50 unweaned calves; weaning occurred at 7–9 weeks, in 2 farms only to beef calves	243 dairy farms with a median herd size of 72 cows, ranging from 28 to 1,175 cows	1 dairy farm with 200 cows
Analysis	Observational study on farms feeding calves waste milk; 183 samples from calves and 95 samples from cows analysed	Observational study on 3 farms that about 10 months previously had been positive for both cefquinome and ESBL-producing Enterobacteriaceae in waste milk. Waste milk from each farm was tested for cefquinome and ESBL-producing <i>Escherichia coli</i>	Observational study to investigate statistical correlation between treatment from questionnaire and AMR shedding; samples from 729 calves analysed	Observational study on a farm where chlortetracycline (intrauterine suppository) is prophylactically used after each calving; 56 samples from 21 cows; 12 samples from 12 heifers and calves analysed
Feeding calves	Milk and colostrum from cows treated during lactation and dry period	Calves were fed waste milk containing milk or colostrum from cows treated at dry-off, lactating cows treated with antimicrobials and for which the first milk after treatment was not discarded and milk with high cell counts	Waste colostrum and waste transition milk (from the second milking till the 4th day after calving) from cows treated with antimicrobials at dry-off Waste milk from cows treated with antimicrobials during lactation	Milk during the withdrawal period from cows treated during lactation
Antimicrobial concentration	Not determined	In samples of waste milk taken from each farm on 3 occasions mean cefquinome concentration was 3,763 µg/kg and the maximum concentration 27,000 µg/kg	Not determined Colostrum might contain only penicillins and aminoglycosides, the only antimicrobials used in dry cow therapy in Sweden	Not determined
Method and criteria applied to interpret results <sup>(c)</sup>	<i>E. coli</i> , Disk diffusion (Literák et al., 2007) in accordance with CLSI (NCCLS, 2002)	<i>E. coli</i> , plating to ESBL selective and testing representative colonies for CTX-M ESBL genes, by the method of the British Society for Antimicrobial Chemotherapy (BSAC, 2011 ( <a href="http://www.bsac.org.uk/">http://www.bsac.org.uk/</a> ))	<i>E. coli</i> , MIC broth microdilution, in accordance with CLSI, 2013a	Polymerase chain reaction (PCR) (calves n = 26; heifers n = 45; cows n = 56)
AMR	12 antimicrobials	CTX-M positive <i>E. coli</i>	12 antimicrobials	Tetracycline resistance genes: <i>tet(M)</i> , <i>tet(Q)</i> , <i>tet(W)</i>
Time/animals	Calves – cows age not specified	For each farm, samples from calves and adult cows (n = 90) taken in 2011 Calves were not classified by age, but as on milk machine, receiving colostrum; receiving milk powder; unweaned and weaned	Calves week 1–week 4	Calves (week 2; week 4–6) – heifers/cows

	<b>Dolejská et al. (2008)</b>	<b>Randall et al. (2014)</b>	<b>Duse et al. (2015)</b>	<b>Kyselková et al. (2015)</b>
Result	<ul style="list-style-type: none"> <li>Calves: 40% AMR <i>E. coli</i> (n = 183).</li> <li>Cows: 3% AMR <i>E. coli</i> (n = 95).</li> </ul>	<ul style="list-style-type: none"> <li>Presumptive/confirmed CTX-M-positive <i>E. coli</i> in 33.3% to 74.4% of all faecal samples.</li> <li>For some groups of calves from two farms 100% of faecal samples positive for ESBLs.</li> <li>A greater proportion of faecal samples from calves were positive for CTX-M-positive <i>E. coli</i> compared with faecal samples from older animals</li> </ul>	<ul style="list-style-type: none"> <li>Colostrum: no effect on AMR <i>E. coli</i>.</li> <li>Waste milk: significantly more nalidixic and streptomycin res <i>E.coli</i>.</li> <li>Treatment or withdrawal period: no ≠</li> <li>Older calves had less AMR <i>E. coli</i></li> </ul>	<ul style="list-style-type: none"> <li><i>tet</i> (M): calves week 2 log 1–2 &gt; calves week 4–6</li> <li><i>tet</i>(Q), <i>tet</i>(W): calves week 2 + week 4–6 log 1–2 &gt; heifers, cows</li> </ul>
Main relevant results as reported in abstract	<p>'The prevalence of resistant <i>E. coli</i> in calves compared to adult cattle was much higher and probably was influenced by oral antimicrobial usage in calves, feeding with milk and colostrum from treated cows, as well as mechanisms unrelated to antimicrobial drug selection'</p>	<p>'All three dairy farms fed waste milk to calves, and both cefquinome and ESBL-producing <i>E. coli</i> were detected in the waste milk samples tested. For farms A and B, 100% of faecal samples from calves classified as "weaned", "unweaned", "on milk machine" and "receiving colostrum" were positive for CTX-M <i>E. coli</i>. For farm A, a greater proportion of faecal samples from calves were positive for CTX-M-positive <i>E. coli</i> compared with faecal samples from older animals. A combination of serotyping and PFGE suggested the same strains in waste milk and in calves could occur'</p>	<p>'Feeding colostrum from cows treated with antimicrobials at drying-off did not affect the prevalence of resistant <i>E. coli</i>. In contrast, feeding milk from cows treated with antimicrobials during lactation resulted in significantly more nalidixic acid- and streptomycin resistant <i>E. coli</i>; no significant effect was seen for other resistance traits. In general, the prevalence of resistance was lower for older calves and calves on small farms. We detected no significant difference between feeding waste milk produced during the withdrawal period and feeding waste milk during both the treatment and the withdrawal period'</p>	<p>'Calves acquired the tetracyclin-R genes in their early age (1–2 weeks). The relative abundance of the <i>tet</i>(W), <i>tet</i>(Q), and <i>tet</i>(M) genes in excrements of calves was about 1–2 orders of magnitude higher compared to heifers and dairy cows, possibly due to the presence of antimicrobial residues in milk fed to calves. The occurrence and abundance of tetracycline resistance genes in fresh excrements of heifers and adult cows remained unaffected by intramammary chlortetracycline applications'</p>
Main uncertainties	<p>Relationship between AMR <i>E. coli</i> shedding and feeding waste milk is only hypothesised, not based on experimental data. Only two farms were included in the study limiting the representativeness of the results</p>	<p>Because no control group was included in the study, no conclusions can be made on the causal relationship between feeding milk containing antimicrobial residues and the shedding of CTX-M <i>E. coli</i></p>	<p>Statistical analysis of a large data set reduces the uncertainty on the obtained results. Uncertainty remains due to the design of the study, lacking experimental control groups. Also only farms, voluntarily accepting to contribute were included, creating a volunteer selection bias The conclusion on the colostrum is restricted to penicillins and aminoglycosides as these are the only antimicrobials used in dry cow therapy in Sweden. The study is restricted to antimicrobial-resistant <i>E. coli</i></p>	<p>Relationship between AMR shedding and feeding waste milk is only hypothesised, not based on experimental data; only resistance to one antimicrobial is investigated. The study is based on the presence of AMR genes detected by PCR and therefore no information is available on the identity of bacteria carrying the AMR genes. Study presents results of only a limited amount of samples taken from one dairy farm, limiting the representativeness of the results</p>

ESBL: extended-spectrum beta-lactamases; CTX-M: Cephalosporinase-type cefotaximase; MIC: minimum inhibitory concentration; AMR: antimicrobial resistance.

(a): Study on the occurrence of AMR bacteria in faeces of calves on farms feeding calves' milk from cows treated with antimicrobials. No relationship between this feeding and the induction of AMR was investigated.

(b): Study on the effect of feeding calves milk from treated cows using statistical correlation between shedding results of AMR and treatment as indicated in questionnaires to farmers.

(c): see Appendix E.

**Table 12:** Effect on faecal shedding of antimicrobial-resistant bacteria due to feeding calves waste milk in experimental trials

	<b>Aust et al. (2013)</b>	<b>Langford et al. (2003)<sup>(a)</sup></b>	<b>Brunton et al. (2014)</b>
Study design	Experimental study comparing the effect of feeding waste milk and antimicrobial-free milk to calves	Experimental study comparing the effect of feeding milk from cows treated with penicillin at different concentrations or antimicrobial-free milk	Experimental study comparing the effect of feeding waste milk and milk replacer to calves
Country	Germany	Not specified	UK
Farm	1 dairy farm	Not specified	1 ESBL positive dairy farm known to use cefquinome
Treatment	1 group fed waste milk 1 group pasteurised waste milk/1 group bulk milk/1 group pasteurised bulk milk	1 group fed milk containing different concentrations of penicillin due to dilution of the milk harvested after treating the cow; 1 control group without antimicrobials	1 group fed waste milk/1 group control
Feeding scheme	Week 1 to-week 6	3 days/week contaminated milk from day 10 till day 40	Week 1 to week 6
Antimicrobials/concentration	Residues in 17/20 waste milk samples as determined by a microbial inhibitory test	Penicillin present at concentrations of 37.5, 75, 150 and 300 µg/kg milk	Mean cefquinome concentration 746 µg/kg (ranging from 390–1,700 µg/kg)
Number of calves	114 in 4 groups: waste milk, pasteurised waste milk, bulk milk, pasteurised bulk milk	31 calves in total (16 males, 15 females); 7 calves in control group, 6 calves in each treatment group	50-cows in control group; 50 cows in treatment group
Method and criteria applied to interpret results <sup>(b)</sup>	<i>Escherichia coli</i> and <i>Enterococcus faecalis</i> from faecal swabs on Coliform Agar Chromocult and Enterococci-Agar ChromoCULT; identified colonies tested by broth microdilution, clinical breakpoint concentrations published by the CLSI, 2008	Released material from faecal swabs cultured on soy tryptase plates with penicillin disk (10 µg). Inhibition zone was measured and compared between groups; differences in resistance/susceptibility were statistically determined	<i>E coli</i> from faecal swabs on CHROMAgar supplemented with CTX, a subset of isolates confirmed by PCR for the presence of CTX-M ESBL genes
Antimicrobial resistance	Resistance to 25 antimicrobials	Penicillin resistance	Cefquinome resistance
Time animals tested for faecal shedding	Week 1, week 2, week 4, week 6	Day 10 till day 40	Weekly from week 1 till 6 weeks after weaning
Result	<ul style="list-style-type: none"> <li>From week 2–week 6, a significant higher amount of antimicrobial-resistant <i>E. coli</i> detected in calves fed waste milk and pasteurised waste milk than in the bulk milk and pasteurised bulk milk groups.</li> <li>At week 1, only 1 resistant <i>E. coli</i> was multiresistant while from week 2 till week 4 all resistant <i>E. coli</i> were multiresistant; at week 6 the % multiresistant <i>E. coli</i> declined.</li> <li>Highest proportion of resistant <i>E. coli</i> found on week 2 (day 14).</li> <li>No difference in antimicrobial-resistant <i>E. faecalis</i> observed between the 4 groups.</li> <li>No difference between calves fed waste and pasteurised waste milk</li> </ul>	<ul style="list-style-type: none"> <li>In pretreatment period, statistically significant difference between treatment groups.</li> <li>300 and 150 groups: clear decreased inhibition (increased resistance) in relation to control and pretreatment period; not in 75 and 37.5 groups.</li> <li>Resistance increased in 300 and 150 groups from day 15 (5 days after treatment) till day 40 (30 days after treatment).</li> <li>Resistance induction in 300 and 150 treatment groups remained stable over time</li> </ul>	<ul style="list-style-type: none"> <li>Week 1, week 2.5 and week 3: significant higher AMR <i>E. coli</i> counts in treatment group compared to control group.</li> <li>Not ≠ at start and 6 weeks after weaning (12 weeks of age) between both groups with slower decline in treatment group</li> </ul>

	<b>Aust et al. (2013)</b>	<b>Langford et al. (2003)<sup>(a)</sup></b>	<b>Brunton et al. (2014)</b>
Main relevant results as reported in abstract	'The proportion of resistant <i>E. coli</i> isolates was significantly higher in calves fed waste milk and in calves fed pasteurised waste milk (most pronounced for cephalosporins) than in calves receiving bulk milk. No differences in resistance were found for <i>Enterococcus</i> spp. Thus, the concerns for selecting resistant faecal bacteria by feeding waste milk seem to be justified'	'Inhibition was greatest for bacteria from calves fed milk with no penicillin and declined as the penicillin dose provided in the milk increased. In conclusion, resistance of gut bacteria to antimicrobials increased with increasing concentrations of penicillin in the milk fed to dairy calves'	'Calves in the treatment group shed greater numbers of CTX-M-positive <i>E. coli</i> than calves in the control group throughout the study, and shedding decreased at a slower rate in the treatment group. There was no difference between calves fed waste milk with antimicrobial residues or calves fed milk replacer in the proportion of <i>E. coli</i> isolates that were CTX-M-positive. Shedding of CTX-M-positive <i>E. coli</i> persisted for longer in calves fed waste milk with antimicrobial residues and persisted after weaning'
Main uncertainties and limitations of the study	The waste milk consisted of all unsaleable milk and had therefore a not standardised composition. The presence of antimicrobial activity as determined by the used microbial inhibition tests gives no information on the presence of individual antimicrobials and their concentration. Therefore, the study is not informative on the relationship between antimicrobial residue concentration and antimicrobial resistance induction. The study is limited to resistance of <i>E. coli</i> and <i>E. faecalis</i>	The differences and fluctuations in the resistance in the control group and pretreatment period (variation in baseline levels) leads to uncertainty on small effects, e.g. in the lower dose treatment groups The statistical interpretation of the data is not provided. The graphical presentation of the data indicates only a clear difference in resistance for the two highest doses penicillin and not for the lower ones which is not what the authors concluded. Therefore, it is uncertain if the lower penicillin doses (37.5 and 75 µg/kg) would induce resistance in the gut microbiota. The study is limited to penicillin resistance for bacteria cultivated on soy tryptase plates	The waste milk consisted of all unsaleable milk and had therefore not a standardised composition and a concentration of ceftinome ranging from 390–1,700 µg/kg. Therefore, the study is not informative on the relationship between antimicrobial residue concentration and antimicrobial resistance induction. The study is limited to ceftinome resistance in <i>E. coli</i> leaving uncertainty on the resistance development towards other antimicrobials/bacteria present in waste milk

ESBL: extended-spectrum beta-lactamases; PCR: polymerase chain reaction.

(a): Concentration (µg/kg) of penicillin were calculated by assuming that 1,670 IU penicillin = 1 mg.

(b): See Appendix E.



**Table 13:** Effect on shedding of AMR *E. coli* and residues of feeding calves colostrum from cows treated during dry therapy

<b>Gonggrijp et al. (2015)<sup>(a)</sup></b>	
Study design	Experimental study determining the effect of feeding colostrum from cows treated with cloxacillin 500 mg and 600 mg at dry-off
Country	The Netherlands
Farm	10 conventional dairy farms
Treatment	1 group of cow-calf couples with 500 mg cloxacillin as dry cow therapy, 1 group of couples with 600 mg; 1 control group of couples (no dry cow therapy)
Feeding scheme	Calves were fed colostrum and milk from their treated mother (cow-calf couples)
Antimicrobials/concentration	The median concentration of cloxacillin in the colostrum was 148 µg/kg (mean 218 µg/kg) and in the second till fifth milkings 67 µg/kg (mean 94 µg/kg). One sample of the control group produced colostrum with 6.3 µg/kg
Amount calves	20 cow-calf couples with 500 mg cloxacillin; 38 couples with 600 mg; 29 control couples
Method and criteria applied to interpret results	<i>Escherichia coli</i> from faecal swabs; antimicrobial residues in faecal samples; antimicrobial residues, <i>E. coli</i> and total aerobic and coliform germ count in colostrum; antimicrobial residues by LC-MS; <i>E. coli</i> , total germ count and coliforms on MacConkey agar +/- cefotaxim and disk diffusion test for cefotaxim and ceftazidime +/- clavulanic acid and cefoxitin; visual growth compared to resistant and susceptible control strain
Antimicrobial resistance	ESBL- and AmpC-producing <i>E. coli</i>
Time animals tested for faecal shedding	Faecal samples at days 1, 7, 14, 21
Result	<ul style="list-style-type: none"> <li>• Cloxacillin was detected in 60% of the colostrum samples of cows treated with cloxacillin with a median concentration of 148 µg/kg (mean 218 µg/kg) in the colostrum and of 67 µg/kg (mean 94 µg/kg) in the second until the fifth milkings. There was no difference between residue levels in the colostrum between the cows with a dry therapy of 500 or 600 mg cloxacillin.</li> <li>• On day 7 and day 14, significantly more positive faeces samples were collected than on day 1.</li> <li>• No significant association has been found between the presence of ESBL/AmpC-producing <i>E. coli</i> in calf faeces and in the colostrum and with the total aerobic germ count in colostrum. A higher amount of ESBL/AmpC-producing <i>E. coli</i> in the calves, faeces was significantly correlated with a lower total coliform count in colostrum.</li> </ul>
Main relevant results as reported in abstract	In 60% of the colostrum samples of cows treated with cloxacillin as dry cow therapy, residues were detected. Cloxacillin was not found in any of the calves faecal samples. There is no association between the prevalence of ESBL/AmpC-producing <i>E. coli</i> in faeces of young calves and the use of cloxacillin as dry cow therapy, the presence of cloxacillin residues in the colostrum of calf faeces, the presence of ESBL/AmpC-producing <i>E. coli</i> in the colostrum or in calf faeces and the total (aerobic) germ count of colostrum. A limitation of the study is that only cloxacillin therapy and ESBL/AmpC-resistant <i>E. coli</i> have been tested and that cloxacillin does not select for ESBL/AmpC-resistant isolates. Only an association was found between the prevalence of ESBL/AmpC-producing <i>E. coli</i> in faeces of young calves and the total coliform count in colostrum. Calves without ESBL/AmpC-producing <i>E. coli</i> in faeces, have significantly lower coliform counts in the colostrum compared to calves with ESBL/AmpC-producing <i>E. coli</i> in their faeces. This could be explained by the fact that a large amount of other coliform bacteria are limiting the colonisation of ESBL/AmpC-producing <i>E. coli</i> in faeces
Main uncertainties and limitations of the study	Uncertainty relates to the fact that the study was carried out on 10 different dairy farms and no analysis was provided investigating the influence of the identity of the farm on the results. Therefore, influences of farm management could not be excluded. Also no information has been provided on the time between dry cow therapy and calf birth for each of the cow-calf couples. A limitation of the study is that only cloxacillin therapy and ESBL/AmpC-resistant <i>E. coli</i> have been tested and that cloxacillin does not select for ESBL/AmpC-resistant isolates

ESBL: extended-spectrum beta-lactamases; LC-MS: liquid chromatography-tandem mass spectrometry.

(a): See Appendix D.



**Table 14:** Effect on faecal shedding of antimicrobial-resistant bacteria due to feeding calves milk artificially spiked with antimicrobials

	<b>Alali et al. (2004)</b>	<b>Berge et al. (2006)</b>	<b>Thames et al. (2012)</b>	<b>Pereira et al. (2014)</b>
Study design	Experimental study feeding milk spiked with antimicrobials	Experimental study feeding milk spiked with antimicrobials	Experimental study feeding milk spiked inoculated with antimicrobials	Experimental study feeding milk spiked with antimicrobials
Country	US	US	US	US
Farm	Two rooms in a Biosafety Level 2 facility	Calif ranch for heifer replacement, veal and dairy beef	Experimental study feeding milk spiked with antimicrobials	Experimental farm
Treatment	1 group fed spiked milk with oxytetracycline and neomycin/1 group control with no drug residue/both groups challenged with a nalidixic acid resistant <i>E. coli</i> O157:H7 strain after 1–2 weeks	1 group fed spiked milk/one group residue free milk replacer	1 group fed subtherapeutic concentrations of drug residues spiked in milk/1 group fed therapeutic concentrations in spiked milk/1 group fed residue free milk replacer	1 group fed spiked milk with drug residues (DR)/1 group control with no drug residue (NR)
Feeding scheme	Spiked milk fed from week 1 till 8 weeks after inoculation	Spiked milk fed from week 1 until week 4	Subtherapeutic group was fed spiked milk from day 1 until weaning; therapeutic group was fed spiked milk from day 37 for 14 days	Spiked milk fed from week 1 till week 6; 7.6 L per day
Antimicrobials/concentration	Oxytetracycline (200 mg/kg or 2 mg/kg body weight daily) and neomycin (400 mg/kg or 4 mg/kg body weight daily)	Tetracycline hydrochloride and neomycin sulfate at concentrations of 22 mg/kg body weight/day, each	Subtherapeutic group: neomycin sulfate and oxytetracycline hydrochloride at concentrations of 10 mg/calf per day, each. Therapeutic group: same Antimicrobials at concentrations each of 1,000 mg/calf per day	Ceftiofur, penicillin, ampicillin, oxytetracycline at concentrations of 0.1, 0.05, 0.01, 0.3 µg/mL, respectively
Amount calves	18 male calves (9 treated; 9 control)	30 treated calves/60 control calves	28 divided over three groups (treated/therapeutic/control) (amount of calves/group is not provided)	30 male calves (15 treated; 15 control) in three individual studies of each 10 calves (5 treated/5 control)
Method and criteria applied to interpret results <sup>(a)</sup>	Nalidixic acid resistant <i>E. coli</i> O157:H7 from faecal rectal material isolated on MacConkey agar with 20 µg/mL nalidixic acid and detection after enrichment	<i>E. coli</i> from faecal swabs isolated on MacConkey agar; 909 isolates; antimicrobial resistance by disk diffusion according to NCCLS (2002)	Quantitative PCR resistance genes detected in faecal samples	<i>E. coli</i> from faecal samples isolated on MacConkey agar; 270 isolates; antimicrobial resistance tested by disk diffusion according to CLSI (2008)
Antimicrobial resistance	Amount of <i>E. coli</i> O157:H7 shed was compared between treated and non-treated group	Resistance to 12 antimicrobials tested	Detection by PCR of the following resistance genes <i>tet</i> (C), <i>tet</i> (G), <i>tet</i> (W), <i>tet</i> (X) (tetracycline resistance), <i>ermB</i> , <i>ermF</i> (MLSs resistance), and <i>sul1</i> , <i>sul2</i> (sulfonamides resistance)	Resistance to 12 antimicrobials
Time animals tested for faecal shedding	Weekly, started from 2 days after inoculation to 8 weeks after inoculation	Day 1, day 14, day 28	Daily for 7 days starting at week 6 and weekly after weaning till week 12	Weekly from week 1 till week 6

	<b>Alali et al. (2004)</b>	<b>Berge et al. (2006)</b>	<b>Thames et al. (2012)</b>	<b>Pereira et al. (2014)</b>
Result	<ul style="list-style-type: none"> <li>A wide variation in the magnitude and duration of faecal shedding of <i>E. coli</i> O157:H7 was observed among individual calves.</li> <li>There was a significantly (<math>p &lt; 0.001</math>) higher proportion of the calves shedding <i>E. coli</i> O157:H7 in the antimicrobial-fed group than the no-antimicrobial group on day 6 and day 10; there was no difference later on during 8 weeks.</li> <li>There was no difference between treatment and control groups in the concentration of <i>E. coli</i> O157 in faeces.</li> <li>A comparison of the duration of faecal shedding between treated and untreated calves showed no significant difference between groups</li> </ul>	<ul style="list-style-type: none"> <li>Treated calves shed more antimicrobial-resistant <i>E. coli</i> at day 14 and day 28 compared to control calves and treated calves at day 1.</li> <li>Also in control calves a shift to an increased shedding of antimicrobial-resistant <i>E. coli</i> occurred at day 14 and day 28 compared to day 1</li> </ul>	<ul style="list-style-type: none"> <li>All genes except <i>tet(C)</i> were detected on week 6 and week 12. <i>Tet(C)</i> was not detected in any of the calf samples the control group.</li> <li>No effect of subtherapeutic antimicrobial treatment was established.</li> <li>No effect of breed, gender or week of testing was observed</li> </ul>	<ul style="list-style-type: none"> <li>46% of isolates from control group were not antimicrobial resistant and 37% were resistant to at least 3 antimicrobials.</li> <li>6% of isolates from treatment group were not antimicrobial resistant and 84% were resistant to at least 3 antimicrobials.</li> <li>For most antimicrobial resistances, a peak of resistance in treated and control calves was observed at 1–2 weeks; afterwards there was a gradual decline in resistance</li> </ul>
Main relevant results as reported in abstract	<p>'The percentage of calves shedding nalidixic acid-resistant <i>E. coli</i> O157:H7 in the feces in the antimicrobial-fed group was higher (<math>p &lt; 0.001</math>) early in the study period (d 6 and 10) compared with the control group fed no antimicrobials. There was no difference between treatment and control groups in the concentration of <i>E. coli</i> O157 in feces that were positive at quantifiable concentrations. A comparison of the duration of faecal shedding between treated and untreated calves showed no significant difference between groups. Supplementations of milk replacer with antimicrobials may increase the probability of <i>E. coli</i> O157:H7 shedding in dairy calves, but the effect appeared to be of low magnitude and short duration'</p>	<p>In-feed antimicrobials were associated with higher levels of multiple AMR in faecal <i>E. coli</i>. In calves not receiving in-feed antimicrobials, older calves had higher levels of resistance compared to day-old calves'</p>	<p>'Relative abundance (gene copies normalized to 16S rRNA genes) of <i>tet(O)</i> was higher in calves fed the highest dose of antimicrobial (therapeutic group) than in the other treatments. All genes, except <i>tet(C)</i> and <i>int1</i> were detectable in faeces from 6 weeks onward, and <i>tet(W)</i> and <i>tet(G)</i> significantly increased even in control calves'</p>	<p>'A significantly greater proportion of <i>E. coli</i> resistant to ampicillin, ceftiofur, streptomycin and tetracycline was observed in DR calves when compared to NR calves. Additionally, isolates from DR calves had a significant decrease in susceptibility to ceftiofur and ceftiofur when compared to isolates from NR calves. A greater proportion of <i>E. coli</i> isolates from calves in the DR group were resistant to 3 or more antimicrobial drugs when compared to calves in the ND group. These findings highlight the role that low concentrations of antimicrobial drugs have on the evolution and selection of resistance to multiple antimicrobial drugs in vivo'</p>

	<b>Alali et al. (2004)</b>	<b>Berge et al. (2006)</b>	<b>Thames et al. (2012)</b>	<b>Pereira et al. (2014)</b>
Main uncertainties and limitations of the study	<ul style="list-style-type: none"> <li>It is not clear from the information when the calves were inoculated with <i>E. coli</i> O157:H7 (after 1 week or after 2 weeks).</li> <li>The study was carried out in rooms with a Biosafety Level 2 label, with the consequence of a far from normal bacterial environmental challenge.</li> <li>The calves were artificially inoculated with a nalidixic acid resistant <i>E. coli</i> O157:H7 strain. The strain was also resistant to the antimicrobials supplemented in the milk with resistance to oxytetracycline (MIC &gt; 40 µg/mL) and neomycin (MIC &gt; 40 µg/mL). This resistance could have influenced the results increasing the shedding of this resistance strain due to the decrease in competitive non-resistant gut microflora. So, it is highly uncertain if the increased faecal shedding would also occur for non-antimicrobial-resistant <i>E. coli</i> strains</li> </ul>	<p>Because the body weight of the calves is not provided, it is not possible to quantify the actual doses of antimicrobials received by the calves fed with spiked milk. So no quantitative comparison with other studies is possible on the relationship between antimicrobial residue concentration and AMR induction</p>	<ul style="list-style-type: none"> <li>The culture-independent assay tested only for a subset of AMR genes and not for the AMR phenotype.</li> <li>Testing was only started at week 6–7 while in most studies a peak of resistance induction is seen at week 1–2.</li> <li>It is not clear how many animals were tested in each treatment group</li> </ul>	<p>Limitations concerning the representativeness of the results because the animals were housed in an experimental farm and only a limited amount of animals were included in the study</p>

(a): See Appendix E.

### 3.4.2. Summary analysis of the results of the studies

#### *Effect of feeding milk (including waste milk) containing antimicrobials to calves*

In all studies presented in Tables 12, 13 and 15 with the exception of the study of Thames et al. (2012), an increased proportion of antimicrobial-resistant faecal bacteria are shed when calves are fed milk containing antimicrobial residues at subtherapeutic doses. In contrast to the other studies, Thames et al. (2012) measured the occurrence of AMR genes by a culture-independent detection method using quantitative PCR. Thames et al. (2012) did not observe an effect of feeding calves with subtherapeutic concentrations of antimicrobials; this could be explained by 1) the culture-independent assay testing only for a limited subset of AMR genes rather than phenotypic AMR, 2) the limited detection limit of the method used and 3) the testing of older calves only (week 6–7 and week 12). It is clear from other studies that the main effect of feeding antimicrobial containing milk to calves is observed for calves of 2–3 weeks of age (see below).

In contrast to the increased AMR observed for *E. coli*, Aust et al. (2013) observed no increase in AMR in *E. faecalis* isolates isolated from the same faecal samples. This observation is probably due to the high baseline resistance observed for *E. faecalis* in calves, since enterococci are inherently resistant to several of the antimicrobials tested.

Because heat treatment by pasteurisation will kill vegetative bacteria (including antimicrobial resistant strains) in raw milk, the effect of this treatment on the faecal shedding of antimicrobial-resistant bacteria was investigated. Aust et al. (2013) did not find any effect of this heat treatment on the faecal shedding of antimicrobial-resistant *E. coli*.

#### *Effect of feeding colostrum containing antimicrobials to calves*

In contrast to the effect of feeding calves milk from the withdrawal period of cows treated during lactation, no effect was observed of feeding calves colostrum from cows treated with penicillins and aminoglycosides at drying-off or waste transition milk (Duse et al., 2015; Gonggrijp et al., 2015). This observation is limited to *E. coli* and to treatment with penicillins and aminoglycosides and is not confirmed by other studies with other antimicrobials. A limitation of the study of Gonggrijp et al. (2015) is that only cloxacillin therapy and ESBL/AmpC-producing *E. coli* have been tested and cloxacillin does not select for ESBL/AmpC-producing Enterobacteriaceae.

No significant association has been found between the ESBL/AmpC-producing *E. coli* in calves' faeces and 1) the ESBL/AmpC-producing *E. coli* in the colostrum and 2) the total aerobic bacterial count in colostrum. A higher amount of ESBL/AmpC-producing *E. coli* in calves' faeces was significantly correlated with a lower total coliform count in the colostrum and the milkings from day 1 to 5 (Gonggrijp et al., 2015). The authors hypothesised that this could be due to the fact that a large amount of other coliform bacteria were limiting the colonisation of ESBL/AmpC-producing *E. coli* in the gastrointestinal tract of calves.

#### *Selection for antimicrobial-resistant bacteria in calves fed milk with low concentrations of antimicrobials*

No significant differences were observed when calves were fed waste milk from lactating cows obtained during treatment compared to milk obtained during both the treatment and withdrawal period (Duse et al., 2015), indicating that low concentrations of antimicrobials in the milk may also select for antimicrobial-resistant *E. coli* isolates in the calf intestine. Langford et al. (2003), observed no effect on the shedding of penicillin resistant faecal bacteria when milk with low penicillin concentrations was fed (37.5 and 75 µg/kg). When milk with penicillin concentrations of 150 and 300 µg/kg milk was fed, a clear increase in antimicrobial-resistant isolates was observed. Pereira et al. (2014) showed an increase in AMR in *E. coli* shed by calves fed milk inoculated in separate experiments with ceftiofur, penicillin, ampicillin or oxytetracycline at concentrations of 0.1, 0.05, 0.01 and 0.3 µg/mL, respectively. The same was seen by Berge et al. (2006) with milk spiked with tetracycline hydrochloride and neomycin sulfate, each at a dose of 22 mg/kg body weight/day.

#### *Effect of the age of the calves/cows on shedding of antimicrobial-resistant *E. coli**

Several studies showed a clear effect of the feeding of milk containing antimicrobial residues on the faecal shedding of antimicrobial-resistant *E. coli* by calves in week 2 and 3 (Aust et al., 2013; Brunton et al., 2014; Pereira et al., 2014). A reduction in shedding of resistant *E. coli* was seen at week 7 (Aust et al., 2013). The shedding of antimicrobial-resistant *E. coli* declined further after weaning and no difference between the treatment and control was observed for calves at an age of 12 weeks, i.e. 6 weeks after weaning (Brunton et al., 2014).

Duse et al. (2015) and Berge et al. (2006) showed a lower prevalence of AMR in older calves irrespective of the treatment. In contrast, Gonggrijp et al. (2015), found significantly more faecal samples containing ESBL/AmpC-producing *E. coli* in calves on day 7 and day 14 after birth than on day 1. This finding was independent of dry cow treatment with cloxacillin (Gonggrijp et al., 2015).

A lower prevalence of AMR in isolates from cows compared to isolates from calves was also seen in the studies of Kyselková et al. (2015) and Randall et al. (2014). This was also observed in monitoring programmes of indicator bacteria from healthy animals, e.g. in Sweden (Swedres-Svarm, 2015) and the Netherlands (MARAN, 2015).

Alali et al. (2004) also observed increased faecal shedding of the inoculated and resistant *E. coli* O157:H7 at day 6 and day 10, but there were no difference observed after this during the 8-week study period.

It has been suggested that the age-related occurrence of AMR in intestinal *E. coli* and *Campylobacter* of young calves is a consequence of a frequent turnover of strains in the developing intestine (Edrington et al., 2012b), implying that susceptible strains over the first weeks of life are successively replaced by resistant strains and then again by susceptible strains (Hinton et al., 1985). Observations of this replacement in the absence of a selection pressure from antimicrobials has led to the suggestion that resistance in some *E. coli* strains is linked to other factors promoting colonisation of the intestine of young calves (Berge et al., 2005; Khachatryan et al., 2006). Such factors could include virulence traits or adhesion factors as suggested by Karami et al. (2006). Thus, the high shedding of resistant *E. coli* observed in young calves is likely a complex phenomenon influenced also by factors other than selection pressure from antimicrobial usages.

#### 3.4.2.1. Summarising remarks

The contribution of each of the studies presented in Tables 11–14 to each of the following remarks, is summarised in Table 15.

- 1) Feeding of milk containing antimicrobial residues results in an increase in faecal shedding of antimicrobial-resistant *E. coli*.
- 2) Pasteurisation of milk containing antimicrobial residues had no effect on the increased faecal shedding of antimicrobial-resistant *E. coli*. However, this result was only observed in one study.
- 3) In one study, the increased resistance observed for *E. coli* was not observed for *E. faecalis* isolates from the same faecal samples, which was explained by the high baseline resistance observed for *E. faecalis* in calves.
- 4) Feeding calves colostrum or transition milk from cows treated with penicillins and aminoglycosides at drying-off did not increase the faecal shedding of antimicrobial-resistant *E. coli*. The experimental studies do not support any conclusions on the effect of feeding calves colostrum or transition milk from cows treated at drying-off with antimicrobials other than penicillins and aminoglycosides.
- 5) A higher number of ESBL/AmpC-producing *E. coli* in calves' faeces was significantly correlated with a lower total coliform count in the colostrum and the milkings from day 1 until 5. Thus, a large population of other coliform bacteria may be limiting the colonisation of ESBL/AmpC-producing *E. coli*.
- 6) The most pronounced effect of the feeding of milk containing antimicrobial residues on the faecal shedding of antimicrobial-resistant *E. coli* was observed in calves of 2–3 weeks of age; a decreased effect was seen for calves at an age of 6–7 weeks, possibly correlating with weaning.
- 7) At an age of 12 weeks (6 weeks after weaning), the shedding of CTX-M-*E. coli* due to feeding of calves from birth to 6 weeks milk containing cefquinome residues reverted to the same level as the control group fed with non-treated milk replacer.
- 8) A lower level of shedding of resistant *E. coli* was observed in older calves and in dairy cows, irrespective of the treatment.
- 9) Feeding calves waste milk from lactating cows obtained during treatment has a comparable effect on the shedding of resistant *E. coli* to feeding calves milk obtained during the withdrawal period.
- 10) Low concentrations of antimicrobials (ceftiofur, cefquinome, penicillin, ampicillin, oxytetracycline, tetrahydrochloride or neomycin sulfate) in milk fed to calves may select for antimicrobial-resistant *E. coli* in the calf's intestine.



**Table 15:** Summary of the contribution of each of the studies (summarised in Tables 11–14) to each of the remarks

No. (a)	Studies supporting the conclusion	Microbial organisms (isolated from calf faeces), antimicrobials (AMs) and/or resistance mechanisms investigated
1	Dolejská et al. (2008)	<i>Escherichia coli</i> ; 12 AMs
	Kyselková et al. (2015)	<i>tet</i> (tetracycline resistance) genes by PCR
	Duse et al. (2015)	<i>E. coli</i> ; 12 AMs
	Brunton et al. (2014)	<i>E. coli</i> ; cefquinome
	Aust et al. (2013)	<i>E. coli</i> ; 25 AMs
	Langford et al. (2003)	Bacteria; penicillin
	Pereira et al. (2014)	<i>E. coli</i> ; ceftofur, penicillin, ampicillin, oxytetracycline
	Berge et al. (2006)	<i>E. coli</i> ; tetracycline hydrochloride, neomycin sulfate
2	Aust et al. (2013)	<i>E. coli</i> ; 25 AMs
3	Aust et al. (2013)	<i>Enterococcus faecalis</i> ; 25 AMs
4	Duse et al. (2015)	<i>E. coli</i> ; penicillin and aminoglycosides
5	Gonggrijp et al. (2015)	<i>E. coli</i> ; ESBL/AmpC-encoding genes
6	Kyselková et al. (2015)	<i>tet</i> (tetracycline resistance) genes by PCR
	Randall et al. (2014)	<i>E. coli</i> ; AMs and CTX-M encoding genes
	Brunton et al. (2014)	<i>E. coli</i> ; cefquinome
	Aust et al. (2013)	<i>E. coli</i> ; 25 AMs
	Pereira et al. (2014)	<i>E. coli</i> ; ceftofur, penicillin, ampicillin, oxytetracycline
	Alali et al. (2004)	<i>E. coli</i> O157:H7; nalidixic acid resistant
7	Brunton et al. (2004)	<i>E. coli</i> ; cefquinome
8	Duse et al. (2015)	<i>E. coli</i> ; 12 AMs
	Berge et al. (2006)	<i>E. coli</i> ; tetracycline hydrochloride, neomycin sulfate
9	Duse et al., 2015;	<i>E. coli</i> ; 12 AMs
10	Langford et al. (2003)	Bacteria; penicillin resistance
	Pereira et al. (2014)	<i>E. coli</i> ; ceftofur, penicillin, ampicillin, oxytetracycline
	Berge et al. (2006)	<i>E. coli</i> ; tetracycline hydrochloride, neomycin sulfate

PCR: polymerase chain reaction.

(a): The numbers relate to the numbered summary remarks.

### 3.5. Development of AMR due to feeding calves with colostrum potentially containing residues of antimicrobials and/or milk of cows treated during lactation with antimicrobials and milked during the withdrawal period

The aim of this section is to explain the observations summarised in Section 3.4 in the framework of the scientific literature on consequences of exposure of bacteria to antimicrobials for development of resistance. Driving factors for selection and spreading of AMR can be identified and the effects predicted of exposure to levels of antimicrobials expected as a result of feeding waste milk to calves. Both, selection for resistance occurring in the microbiota of the milk and in the intestines of the calves, will be discussed. Data on antimicrobial residues (Section 3.1) and antimicrobial-resistant bacteria (Section 3.2) present in milk and/or colostrum of treated animals are used to interpret results obtained in studies on the effect of feeding calves this milk (Section 3.4). The objective of this discussion is to reach conclusions on the effect of feeding calves milk and/or colostrum from treated animals on AMR levels.

#### 3.5.1. Relation between residue concentration and resistance emergence

The exposure of microorganisms to low (subinhibitory) concentrations of antimicrobials is known to select for antimicrobial resistance development (Andersson and Hughes, 2014). General principles of this process and mechanisms involved are discussed to ascertain that the observations described in Section 3.4 can be explained by the scientific insight to-date. In particular, it will be attempted to



match field observations of antimicrobial concentrations and AMR monitoring with dose–response relationships inferred from experimental studies.

Resistance to antimicrobials can be brought about in susceptible cells by three main mechanisms:

- 1) Adaptation of cellular processes at the level of gene expression (Händel et al., 2013);
- 2) Mutations in the DNA of the target organism (Händel et al., 2014);
- 3) Horizontal transfer of genes conferring resistance (Andersson and Hughes, 2014; Händel et al., 2015).

Each of these mechanisms can contribute to AMR at any non-lethal concentration. The suggestion that concentrations below the MIC would not select for resistance because cells can grow at these levels has been proven incorrect experimentally (Gullberg et al., 2011, 2014). For those antimicrobials against which resistance is caused by mutations, e.g. *gyrA* mutations and fluoroquinolone resistance, the dynamics of resistance development are best explained by the hypothesis of the 'Mutant Selection Window' (MSW). This hypothesis postulates that selective amplification of spontaneous resistant mutants occurs more often within a specific range of antimicrobial concentrations. This range can be predicted by the pharmacokinetic (PK) and pharmacodynamic (PD) properties of therapeutic agents, known as PK/PD relationships (Kallman et al., 2008; EMA-CVMP, 2016a). The MSW previously was thought to be the concentrations between the MIC and the Mutation Prevention Concentration (MPC), but since selection for AMR can also occur at levels below the MIC, this range also includes lower concentrations.

Sublethal concentrations of antimicrobials can select for resistance. In the case of fluoroquinolones, levels 230 times less than the MIC can result in an increase in resistance (Gullberg et al., 2011). Experiments analysing faeces of chickens treated under well controlled conditions showed that even a carry-over concentration of 2.5% of a therapeutic dose of amoxicillin had an effect on the presence of resistant *E. coli* strains 2 weeks after the antimicrobial had last been ingested by the animals (van der Horst et al., 2013). While it is not certain that similar concentrations in the intestines of calves will have the same effect, this cannot be excluded. Consequently, the concept of 'Minimum Selective Concentration' has been proposed as the lowest concentration of an antimicrobial that still selects for a given resistance (Sandegren, 2014). This minimum selective concentration is reached when the growth reduction in the susceptible strain by the antimicrobial is equal to the growth rate reducing effect (fitness cost) of the resistance determinant in the resistant strain. This occurs at levels below the MIC and as a consequence, the sub-MIC selective window is much larger than the traditional selective window. Exposure to low levels of antimicrobials can select for pre-existing and *de novo* resistant mutants.

There are several reasons why sub-MIC-selected resistant mutants are potentially more problematic than those selected at high concentrations (Andersson and Hughes, 2012). First, it is predicted that sub-MIC-selected resistant mutants will be more stable in bacterial populations than those selected by high concentrations due to lower metabolic costs (Andersson and Hughes, 2014). Secondly, when a mixed microbiota is exposed to relevant antimicrobials, pre-existing resistant mutants will outgrow the susceptible population at all but the lowest concentrations. Thirdly, low levels of antimicrobials have been shown to increase homologous recombination rates, stimulate horizontal gene transfer, and activate integrating genetic elements (Davies and Davies, 2010).

Resistance can be exchanged between bacteria, for example by horizontal transfer of plasmids containing resistance genes. Establishment of AMR plasmids needs two steps; first the plasmid is transferred, and once it is established in the acceptor cell, replicates of the newly resistant cell are selected by antimicrobial pressure. Transfer of plasmids carrying AMR genes occurs at higher rates in the absence of antimicrobials than in the presence, and higher concentrations inhibit transfer more than low concentrations (Schuurmans et al., 2014; Händel et al., 2015), although low concentrations may actually stimulate transfer (Davies and Davies, 2010). The selection step for the recombinant cells does not occur without exposure to antimicrobials (Davies and Davies, 2010). So the levels of antimicrobials causing most risk for AMR spreading are those that do not inhibit transfer much, but do select for resistance.

### 3.5.1.1. The bovine faecal resistome

In addition to pathogens, antimicrobial-resistant bacteria include many harmless and beneficial commensal microbes that act as a reservoir of AMR genes and related determinants called 'the resistome' (D'Costa et al., 2006; Martinez et al., 2015). Unlike for humans and experimental animal models, there is limited information concerning the effect of antimicrobials on the microbial

composition or the total resistome within the bovine gastrointestinal tract. Feeding waste milk containing antimicrobial residues to calves is associated with a decrease in the diversity of bacteria in colon and faeces (Edrington et al., 2012a). In the study performed by Klein et al. (2013), calves being fed waste milk were less likely to harbour *Campylobacter* spp.

Several studies document the effect of therapeutic and subtherapeutic administration of specific antimicrobials on the prevalence of some AMR-encoding genes in bovine faeces, see Tables 11 and 14. In calves, neomycin or oxytetracycline at subtherapeutic levels had no effect on resistance genes, while therapeutic treatment with oxytetracycline increased the abundance of tetracycline resistance genes (Thames et al., 2012).

A significant increase in resistance to ceftiofur, a 3rd generation cephalosporin (highest priority CIA, see Appendix A), and a decrease in tetracycline resistance were observed in steers that received experimental ceftiofur treatments (Kanwar et al., 2014). Subsequent chlortetracycline administration led to rapid increase in both ceftiofur and tetracycline resistance gene copies/g faeces. Ceftiofur treatment in dairy cattle caused increases in sequences associated with resistance to  $\beta$ -lactam and multidrug resistance (Caudle, 2014). Similarly,  $\beta$ -lactam resistance encoding genes increased in faeces from Holstein cows administered ceftiofur relative to control cows (Chambers et al., 2015). Although total numbers of AMR genes across all classes were unaffected by ceftiofur treatment, the treatment resulted in increases in gene sequences associated with 'phages, prophages, transposable elements, and plasmids', suggesting that this treatment also stimulated horizontal transfer of AMR-encoding genes.

Although antimicrobial exposure in calves did not appear to increase a selected panel of AMR genes in calf faeces (Thames et al., 2012), this selection was observed in other bovine experiments as follows. In the absence of comprehensive resistome information, the available data seem to indicate that antimicrobial exposure is likely to increase the reservoir of AMR-encoding genes in the colon and faeces of cattle. The AMR reservoir resides in a microbial community where horizontal gene transfer occurs widely between rumen and intestinal bacteria of different phylogenetic origins (Shterzer and Mizrahi, 2015). This transfer has been implicated in the evolution of complex multidomain gene structures that are widespread among cellulolytic bacteria (Ben David et al., 2015) and AMR genes have spread in this manner (Shterzer and Mizrahi, 2015). Mobile elements are involved in the transfer of AMR genes between unrelated genera, suggesting that the anaerobic/commensal community may be a reservoir of AMR, not only for gut pathogens but also via the shedding of faeces to the environment.

Anaerobic ruminal bacteria possess inherent resistance to a number of antimicrobials (Fulghum et al., 1968). Gram-positive ruminal bacteria were generally much more sensitive than Gram-negative bacteria to antimicrobials used as growth promoters (Nagaraja and Taylor, 1987). This is the basis of growth promotion by these compounds because Gram-positive bacteria have detrimental effects on energy and protein metabolism in the rumen (Nagaraja et al., 1997). AMR research in the large intestine and faeces has focussed mainly on pathogens, in particular *E. coli* O157 (Galland et al., 2001; Fitzgerald et al., 2003) and others including *Clostridium difficile* and *Salmonella* (Dargatz et al., 2016; DeMars et al., 2016; Thitaram et al., 2016).

The correlation between feeding calves colostrum or waste milk from cows treated with antimicrobials and increases in AMR in their faecal bacteria has been documented in several studies, as summarised in Section 3.4 (Berge et al., 2006; Thames et al., 2012; Aust et al., 2013; Brunton et al., 2014; Pereira et al., 2014). There are three possible causes for increase in AMR in the faecal bacteria: 1) bacteria from the waste milk or colostrum that are already resistant are taken up by the calf; 2) antimicrobial residues in the milk select for resistant variants in the gut microbiota, e.g. selection of plasmid mediated ESBL-producing *E. coli* and 3) antimicrobial residues may cause *de novo* development of low levels of AMR by upregulating gene expression levels and selecting for spontaneous mutations. Selection for AMR can amplify the effect of taking up resistant bacteria from the milk or colostrum. When the selection takes place in the gut, the concentration of the drugs strongly influences the final outcome. Levels above the MIC in the gut are unlikely to result from feeding waste milk (Randall et al., 2014), therefore selection will take place at lower concentrations within the sub-MIC selective window (Sandegren, 2014). The effects of exposure to low concentrations of antimicrobials depend on the combination of the drug and the microbial species and can vary widely (Andersson and Hughes, 2014; Sandegren, 2014). As a result, the acceptable residue concentration from the perspective of AMR selection has to be determined for each class of antimicrobials and possibly even for each specific compound.

### 3.5.1.2. Factors driving development of resistance due to antimicrobial residues in waste milk

In the previous Section 3.4, several observations were made that can be interpreted in the framework of the present knowledge on development and selection of AMR:

The prevalence of AMR in the faecal bacteria of calves is higher than in that of cows (Dolejská et al., 2008) and reduces with the age of the calf. Milk fed to calves invariably contains AMR bacteria (Straley et al., 2006). The presence of low concentrations of antimicrobials is enough to further select resistant strains (Feng et al., 2014) and to induce resistance *de novo* (Händel et al., 2013). Below, some documented examples on the consequences of exposure to concentrations of antimicrobials resulting from feeding waste milk are discussed.

Faecal bacteria of calves acquire tetracycline resistance genes in the first weeks of life and maintain these resistance genes at higher levels than cows (Kyselková et al., 2015). Tetracycline resistance is almost always related to genes located on mobile genetic elements (Roberts, 2011). As a result, *tet* genes can spread rapidly in environments that contain moderate levels of tetracycline or other antimicrobials (Schoormans et al., 2014). The metabolic costs of maintaining these plasmids or resistance in another form are comparatively low (Händel et al., 2013, 2015). Therefore, the antimicrobial-resistant bacteria that colonise calves when these are fed waste milk with antimicrobials, are only outcompeted by susceptible strains in the longer term. While the composition of microbiota changes, the resistome (D'Costa et al., 2006) may be retained by transfer of mobile genetic elements. Hence, tetracycline resistance genes acquired by calves' microbiota are retained for many months (MARAN, 2015). This is likely to be a result of transfer of AMR genes between successive strains of bacteria.

Feeding calves milk containing penicillin causes AMR in gut bacteria in a concentration dependent manner (Langford et al., 2003). The induction of AMR in bacteria in the gut of calves fed milk with penicillins is consistent with laboratory experiments investigating the induction of  $\beta$ -lactam resistance. Exposure to antimicrobials also selects for plasmid-mediated resistance. Sublethal concentrations influence gene expression, increasing the MIC to a level that is considered clinically resistant (Händel et al., 2013; Feng et al., 2014). In particular, genes that are involved in transport and other membrane processes are differentially regulated upon exposure to antimicrobials. This response at the expression level is very rapid and allows cells to survive moderate concentrations until mutations occur and the higher MIC values are reached. The reversal of differential expression after the antimicrobial is removed is very slow compared to the induction.

Calves fed waste milk with cefquinome shed more *E. coli* containing the ESBL gene CTX-M and for longer time than a control group (Brunton et al., 2014). Exposure of bacteria to sublethal levels of antimicrobials induces the SOS response (Bengtsson et al., 2009; López and Blázquez, 2009; Gutierrez et al., 2013; Baharoglu and Mazel, 2014; Händel et al., 2016). One of the consequences of activation of the SOS response is an increase in the rate of uptake and incorporation of mobile genetic elements, such as transposons and prophages, for example under the influence of fluoroquinolones (Bearson and Brunelle, 2015). This can also explain the retention of *E. coli* containing the ESBL gene CTX-M in calves fed waste milk with antimicrobials (Brunton et al., 2014) as described in Section 3.4. Small numbers of cells within the mutated population combine low fitness costs with increased tolerance to the antimicrobial, and therefore, antimicrobial-resistant cells will be selected upon exposure to low concentrations of antimicrobials (Martinez et al., 2009). Even after removal of the antimicrobials, the shedding continues for some time, exactly as observed on the farm (Brunton et al., 2014).

Calves fed colostrum with antimicrobials from cows treated at drying-off did not shed more antimicrobial-resistant bacteria, but calves fed milk from cows treated during lactation did (Duse et al., 2015). Experimental studies on the relation between length of exposure and build-up of resistance (van der Horst et al., 2011; Händel et al., 2014) correspond well with the reality on farms, where calves that were fed milk from treated cows shed more antimicrobial-resistant bacteria than controls, but calves fed colostrum with antimicrobials did not (Brunton et al., 2014). The concentration of residues in the colostrum is lower, as the cows were treated at the start of the dry period (Oliver et al., 1984). Milk taken directly from cows treated for infections contains amounts that are in the same order of magnitude as the regular therapeutic dose. At the end of the dry period, the levels in the colostrum and afterwards the milk, are much lower (Randall et al., 2014). As a result, the impact of colostrum from cows treated at the start of the dry period is far less than that of waste milk from cows during and immediately after treatment.

Feeding waste milk containing antimicrobials did increase the proportion of resistant *E. coli* in the faeces of calves but not that of *Enterococcus* spp. (Aust et al., 2013). The different physiological responses of *E. coli* isolates compared to *Enterococcus* spp. results in a higher proportion of *E. coli* cells becoming resistant. This corresponds to the difference in metabolic costs of acquired resistance between these species (Händel et al., 2013). The larger costs for *Enterococcus* cause a selection for the susceptible variants once the selective pressure of the antimicrobial is removed, which occurs much more slowly in *E. coli*. Once selection and enrichment of resistant strains has taken place, the resistant subpopulation persists for a long time in beef and dairy cattle (Call et al., 2008).

Exposing calves to antimicrobials by adding experimental concentrations to milk was followed by detection of AMR in faecal bacteria (Thames et al., 2012; Pereira et al., 2014). Due to the complex relationship between exposure to antimicrobials and the resulting resistance, a clear correlation between the usage of antimicrobials and prevalence of antimicrobial-resistant bacteria is not always found. Reduction in the total selection pressure by reducing the overall application of antimicrobials to an animal population, however, does not always correlate with an overall decrease in antimicrobial-resistant strains as a percentage of the total microbiota (MARAN, 2015).

### 3.5.1.3. Relationship between antimicrobial concentrations in the feed milk and levels in the intestine

Generally speaking, experimental studies are in agreement with observations on farms and in animal experiments (van der Horst et al., 2013). Feeding milk experimentally spiked with antimicrobials yielded a comparable outcome to the effects of feeding waste milk for regular farming purposes. Quantitatively, there is a lot of variation in the data depending on species of bacteria, antimicrobials and experimental conditions applied (Andersson and Hughes, 2014). So while some confidence may be derived from the matching of experimental and observational studies for the understanding of mechanisms and operational principles, using laboratory data to make quantitative predictions for on-farm practices is not yet feasible. Considering all information available from monitoring AMR in the agricultural sector, the observed increase in resistance, including that caused by feeding calves waste milk, can be explained as the cumulative effects of exposures due to repeated treatments or presence of antimicrobials in the environment.

There are at present no well-developed pharmacodynamic models to describe the fate of antimicrobials from the moment of uptake until excretion, neither for calves nor for other animals or for humans (Muller et al., 2015). Lacking that, two main routes need to be considered. The first is the effect of antimicrobials taken up into the blood and distributed throughout the body. When milk with residues of antimicrobials is fed, even the highest level that will reach microbes in other areas than the intestine is likely to be sublethal, because the milk contains less than therapeutic levels and subsequently in the gut, further dilution takes place. Antimicrobials at these concentrations thus select for resistance, unless the concentrations are so low as to be negligible. If a calf being fed milk with antimicrobials develops an infection, the chance of selecting for antimicrobial-resistant pathogens is increased. The second effect, which is more direct and the most relevant risk for public health, results from the intestinal microbiota being exposed to relatively low levels of antimicrobials. These antimicrobials select for resistance and when the faeces is disposed of, resistant bacteria and AMR genes will be spread into the environment (Chee-Sanford et al., 2009). Additionally, it is possible that antimicrobial-resistant bacteria from faeces of treated cows could contaminate the bulk milk tank and be a direct source of infection to humans via unpasteurised milk or milk products.

To predict, within the limitations outlined above, effects of feeding milk containing antimicrobials on microbiota of calves, insight in the quantitative relationship between the concentrations in the milk and in the intestine is essential. A worst-case scenario would be that this concentration is equal to the concentration in the milk. The best case scenario is that all antimicrobials are destroyed in the stomach and that only negligible levels are left in the intestines. Neither scenario is likely to be reality. In the faeces of calves antimicrobials of all classes except of  $\beta$ -lactams are often detected (Berendsen et al., 2015). No detection of  $\beta$ -lactams in the faeces does not mean they were not present in the intestines, because  $\beta$ -lactamases are often induced when bacteria are exposed to  $\beta$ -lactam antimicrobials (Händel et al., 2013, 2014). Since  $\beta$ -lactams are frequently used for treatment of cattle,  $\beta$ -lactamases are almost certainly present in the microbiota of calves' intestines.  $\beta$ -lactams are probably broken down in the intestine by  $\beta$ -lactamases produced by the microbiota of the calves and thus not detected in faeces (Berendsen et al., 2015), but breakdown products are. None of the other antimicrobials are chemically completely stable, but the extent of the decay during passage through the calf is not known.



#### 3.5.1.4. Summarising remarks

- Low concentrations of antimicrobials select for resistance to such agents, both by selecting existing and *de novo* mutations and plasmid-mediated resistance. Dose–response relationships between concentrations of antimicrobials the cell is exposed to and the development and spread of resistance vary between drug–microbe combinations and are not known quantitatively.
- The low concentrations of antimicrobials in waste milk from cows treated with antimicrobials contribute to the development of AMR in the intestinal microbiota of calves drinking this milk.
  - Quantification of the risk is not possible at the moment due to the lack of well-developed models describing the pharmacodynamics and the dose–response relationship. This risk is documented in a large number of studies.
- The outcome of experimental studies concerning development and spread of resistance due to exposure to antimicrobials corresponds well with the observations made in studies surveying AMR on farms.
- It should be noted that most studies assess the prevalence AMR from microbiological cultivation studies using indicator bacteria such as *E. coli*. AMR in the anaerobic community is not usually assessed.

### 3.6. Options to mitigate the risk for the development of AMR derived from feeding of calves with milk or colostrum potentially containing residues of antimicrobials

Mitigation methods to be considered in this section focussed on the milk already contaminated with antimicrobials (waste milk). Issues related to prevention of the contamination of milk will not be proposed. This assessment therefore excludes all aspects of infection prevention and health management of the dairy herd aiming to reduce the extent of antimicrobial use and hence the contamination of milk with antimicrobials.

#### 3.6.1. Potential measures for the feeding calves' colostrum or milk from cows treated with antimicrobials

Measures to mitigate the risk for selection and emergence of AMR in the intestinal tract of calves by feeding milk from cows treated with antimicrobials could focus on reducing the risk of exposure of calves to milk potentially contaminated with antimicrobial residues. This could be done effectively by completely prohibiting the use of milk from treated cows.

Another option could be to prohibit the use of milk from cows treated with antimicrobials of specific importance in human healthcare, i.e. the highest priority critically important antimicrobials (CIA) but to allow use of milk from cows treated with antimicrobials that are considered of lesser importance to public health. This approach would affect the type of residues in the milk, but not necessarily their amount.

A third option would be to prohibit the use of milk when the level of residues is expected to be high, i.e. during treatment or after intramammary administration, but allow use of milk produced during the statutory withdrawal period from human consumption starting from the second day after cessation of treatment. This approach is likely to reduce the concentration of residues substantially, while having a minor influence on the type of residues. Finally, reducing the level of antimicrobial residues by processing the milk before feeding it to calves is also an option.

In Tables 16 and 17, possible measures for mitigation are presented, focusing on the feeding management of the colostrum or the waste milk. The scientific evidence in support of or against each measure is listed in the tables. In addition, for each measure, the advantages and disadvantages from a general perspective are briefly summarised as well as considerations for the practical implementation on farms.



**Table 16:** Possible measures with regard to feeding calves colostrum from cows treated with antimicrobials during the dry period

Measure	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
C1 Allow use of colostrum during the withdrawal period irrespective of antimicrobial used	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Colostrum from cows treated with penicillins and aminoglycosides did not increase faecal shedding of resistant <i>Escherichia coli</i> (Duse et al., 2015) (3.4.1)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Residue levels in the colostrum are low and decrease with the length of dry period (3.2.3)</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>No evidence from studies (3.4.1)</li> </ul>	<ul style="list-style-type: none"> <li>No interference with current routines on dairy farms.</li> <li>Sufficient colostrum for calves available</li> </ul>	<ul style="list-style-type: none"> <li>Increased shedding of AMR bacteria by calves cannot be ruled out</li> </ul>
C2 Prohibit use of colostrum during the withdrawal period irrespective of antimicrobial used	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>No evidence from studies (3.4.1)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Possible presence of residues when cows calve earlier than the specified withdrawal period</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Colostrum from cows treated with penicillins and aminoglycosides did not increase the faecal shedding of resistant <i>E. coli</i> (Duse et al., 2015) (3.4.1)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Residue levels in the colostrum are low and decrease with the length of dry period (3.2.3)</li> </ul>	<ul style="list-style-type: none"> <li>Reduced risk for shedding of AMR bacteria by calves</li> </ul>	<ul style="list-style-type: none"> <li>Shortage of colostrum on farms using antimicrobials at dry-off</li> <li>Disposal of waste colostrum might increase residues in the environment if not well founded and communicated</li> </ul>

Measure	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
C3 Prohibit use of colostrum during the withdrawal period except from cows treated with $\beta$ -lactams and/or aminoglycosides. Prohibition could be limited to highest priority CIAs	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Colostrum from cows treated with penicillins and aminoglycosides did not increase faecal shedding of resistant <i>E. coli</i> (Duse et al., 2015) (3.4.1)</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>No studies supporting a distinction between antimicrobials (3.4.1)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Residue levels in the colostrum is low and decrease with the length of dry period (3.2.3)</li> </ul>	<ul style="list-style-type: none"> <li>Reduced risk for shedding of AMR bacteria by calves</li> <li>No interference with current routines on farms using penicillin and/or aminoglycosides</li> <li>To focus on highest priority CIAs targets resistance of most public health significance and could also be a lever for reducing the use of these antimicrobials CIAs</li> </ul>	<ul style="list-style-type: none"> <li>Shortage of colostrum on farms using antimicrobials other than penicillin and/or aminoglycosides</li> <li>Separation of colostrum from cows by treatment includes practicalities that might interfere with compliance</li> <li>Disposal of waste colostrum might increase residues in the environment</li> </ul>
C4 Prohibit use of colostrum if it is not treated to reduce bacterial load and/or residue levels	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Ensures that no or minimal residues and/or bacteria are present</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Colostrum from cows treated with penicillins and aminoglycosides did not increase faecal shedding of resistant <i>E. coli</i> (Duse et al., 2015) (3.4.1)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Residue levels in the colostrum are low and decrease with the length of dry period (3.2.3)</li> </ul>	<ul style="list-style-type: none"> <li>Ameliorates some disadvantages of a complete ban (C2)</li> <li>Probably higher compliance than to a complete ban.</li> <li>Treatment methods are available (3.6.2 and 3.6.3)</li> </ul>	<ul style="list-style-type: none"> <li>Practicality at the farm level is not obvious and might interfere with compliance</li> <li>Potential impact on nutritional and immunological properties of the colostrum</li> <li>A need to adapt and evaluate methods for practical use</li> </ul>

AMR: antimicrobial resistance.

CIA: critically important antimicrobial for human medicine, e.g. 3rd–4th generation cephalosporins and fluoroquinolones, according to the WHO definition (Collignon et al., 2016; WHO, 2016). See Appendix A.

**Table 17:** Possible measures with regard to feeding calves milk from cows treated with antimicrobials during lactation

Measure	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
L1 Allow use of milk during treatment and withdrawal period irrespective of antimicrobial or administration route	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>No evidence from studies (3.4.1)</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Increased shedding of AMR bacteria by calves fed waste milk in an observational study (Duse et al., 2015) and in three experimental studies (Langford et al., 2003; Aust et al., 2013; Brunton et al., 2014) (3.4.1)</li> <li>Increased shedding of antimicrobial resistant bacteria by calves fed milk spiked with antimicrobials in three experimental studies (Berge et al., 2006; Alali et al., 2004; Pereira et al., 2014) (3.4.1)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>In lactating cows, residue levels will be higher after intramammary than after systemic treatment (3.2.4)</li> <li>Antimicrobial concentrations in milk during treatment will be higher than the MIC but then decline (3.2.4)</li> </ul>	<ul style="list-style-type: none"> <li>No interference with current routines on dairy farms</li> </ul>	<ul style="list-style-type: none"> <li>Increased shedding of AMR bacteria by calves</li> </ul>

Measure	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
L2 Prohibit use of milk during treatment and withdrawal period irrespective of antimicrobial or administration route	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Increased shedding of AMR bacteria by calves fed waste milk in 1 observational study (Duse et al., 2015) and in three experimental studies (Langford et al., 2003; Aust et al., 2013; Brunton et al., 2014) (3.4.1)</li> <li>Increased shedding of AMR bacteria by calves fed milk spiked with antimicrobials in three experimental studies (Berge et al., 2006; Alali et al., 2004; Pereira et al., 2014) (3.4.1)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Antimicrobial concentrations in milk during treatment will be higher than the MIC but then decline (3.2.4)</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>No evidence from studies (3.4.1)</li> </ul>	<ul style="list-style-type: none"> <li>Reduced risk for shedding AMR bacteria by calves</li> </ul>	<ul style="list-style-type: none"> <li>Loss of waste milk as valuable feed for calves</li> <li>Low compliance if not well founded and communicated</li> <li>Disposal of waste milk could increase residues in the environment</li> </ul>

Measure	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
L3 Prohibit use of milk from cows treated with highest priority CIAs during treatment and withdrawal period	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>In a dairy herd using cefquinome to treat cows, calves receiving waste milk shed greater numbers of cefotaxime resistant <i>E. coli</i> than calves receiving milk replacer (Brunton et al., 2014)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Antimicrobial concentrations in milk during treatment will be higher than the MIC but then decline (3.2.4)</li> <li>Applied in FI and apparently is practically applicable</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>No evidence from studies (3.4.1)</li> </ul>	<ul style="list-style-type: none"> <li>Reduced risk for shedding bacteria resistant to CIAs by calves</li> <li>Ameliorates some disadvantages of a complete ban (L2)</li> <li>To focus on highest priority, CIAs targets resistance of most public health significance and could also be a lever for reducing the use of these antimicrobials</li> </ul>	<ul style="list-style-type: none"> <li>Disadvantages as for L2 on farms using CIAs</li> <li>Separation of milk by treatment includes practicalities that might interfere with compliance</li> </ul>
L4 Prohibit use of milk during treatment but allow use of milk during the withdrawal period Could be limited to highest priority CIAs	<p><u>Direct</u></p> <p>No evidence from studies (3.4.1)</p> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Antimicrobial concentrations in milk during treatment will be higher than the MIC but then decline (3.2.4)</li> <li>This measure is recommended in some MSS and apparently is practically applicable</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>In an observational study there was no difference in shedding of AMR <i>E. coli</i> by calves fed milk during treatment or withdrawal (Duse et al., 2015)</li> </ul> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>Relatively low antimicrobial residue levels may induce equal or even more resistance compared to higher levels</li> </ul>	<ul style="list-style-type: none"> <li>Reduced risk for shedding AMR bacteria by calves.</li> <li>Ameliorates some disadvantages of a complete ban (L2)</li> </ul>	<ul style="list-style-type: none"> <li>Partial loss of waste milk as valuable feed for calves</li> <li>Low compliance if not well founded and communicated</li> <li>Separation of milk fractions from individual cows includes practicalities that might interfere with compliance</li> <li>Disposal of waste milk might increase residues in the environment</li> </ul>



Measure	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
L5 Prohibit use of milk during treatment and withdrawal period from cows treated intramammary but allow use of milk from cows treated systemically. Could be limited to milk during treatment; to highest priority CIAs; or to the highest priority CIAs during treatment	<p><u>Direct</u></p> <p>No evidence from studies (3.4.1).</p> <p><u>Indirect</u></p> <ul style="list-style-type: none"> <li>In lactating cows, residue levels will be higher after intramammary than after systemic treatment (3.2.4)</li> <li>Antimicrobial concentrations in milk during treatment will be higher than the MIC but then decline (3.2.4)</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>No evidence from studies (3.4.1)</li> </ul>	<ul style="list-style-type: none"> <li>Reduced risk for shedding antimicrobial resistant bacteria by calves</li> <li>Ameliorates some disadvantages of a complete ban (L2)</li> </ul>	<ul style="list-style-type: none"> <li>Since the majority of lactating cows are treated by the intramammary route this measure would have largely similar implications as a complete ban (L2)</li> </ul>
L6 Allow use of milk after treatment to reduce residue levels	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Some milk treatment methods, mainly to remove <math>\beta</math>-lactam antimicrobials, are described (3.6.2 and Table 18)</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Evidence on activity spectrum towards different antimicrobials is lacking (3.6.2 and Table 18)</li> </ul>	<ul style="list-style-type: none"> <li>Ameliorates some disadvantages of a complete ban (L1).</li> <li>Probably higher compliance than to a complete ban</li> </ul>	<ul style="list-style-type: none"> <li>Practicality at the farm level is not obvious and might interfere with compliance</li> <li>Potential impact on nutritional and properties of the milk</li> <li>A need to adapt and evaluate methods for practical use</li> </ul>
L7 Allow use of milk after treatment to reduce bacterial load	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>No evidence from studies that AMR bacteria in waste milk increase shedding by calves (3.4.1)</li> <li>Some milk treatment methods to remove bacteria are described (3.6.3 and Table 19)</li> </ul>	<p><u>Direct</u></p> <ul style="list-style-type: none"> <li>Pasteurisation of milk containing antimicrobial residues had no effect on the increased faecal shedding of antimicrobial-resistant <i>E. coli</i>. (Aust et al., 2013) (3.4.1)</li> </ul>	<ul style="list-style-type: none"> <li>Ameliorates some disadvantages of a complete ban (L1) Probably higher compliance than to a complete ban</li> </ul>	<ul style="list-style-type: none"> <li>Practicality at the farm level is not obvious and might interfere with compliance</li> <li>Potential impact on nutritional and properties of the milk A need to adapt and evaluate methods for practical use</li> </ul>

AMR: antimicrobial resistance; FI: Finland.

CIA: critically important antimicrobial for human medicine, e.g. 3rd–4th generation cephalosporins and fluoroquinolones, according to the WHO definition (Collignon et al., 2016; WHO, 2016). See Appendix A.

### 3.6.2. Options for the removal of antimicrobials in milk

#### 3.6.2.1. Bioavailability and stability of antimicrobials in milk

The stability of antimicrobials under different conditions has been investigated over several decades in various sectors such as food production, agriculture environment, waste water treatment, and analytical chemistry (Svahn and Björklund, 2015). However, information on the stability in milk, although considered rather high for many families of antimicrobials, is not always readily available in the scientific literature.

In milk, the stability of antimicrobials is mainly investigated for two specific situations:

- Through the residue file studies that are delivered by the pharmaceutical industry applicants in the dossier released to the agencies of competent authorities to apply for market authorisation of veterinary medicinal products for intramammary treatments (EMA Guideline on the conduct of efficacy studies for intramammary products for use in cattle (1999–2016), EMA-CVMP, 2016b; EMA Note for Guidance for the determination of withdrawal periods in milk EMEA-CVMP, 2000; Guidances VICH 46 to VICH 49 (2012–2016) (EMA-CVMP, 2011a,b, 2015a,b). However, these data are included in the Market Authorization Dossier of each of the applicants for new veterinary medicines and thus are considered confidential and not available to our evaluation under the EMA confidentiality rules.
- Through the heat treatment studies implemented in the dairy industry to control the technological parameters for milk processing and prior to marketing the different milk products (milk powders, yoghurts, cheeses, etc., see below).

Overall, it is assumed that the bioavailability and the stability of antimicrobials in milk is determined by specific physicochemical properties of the various substances or by the families of substances (lipophilicity or hydrophilicity; acidic or alkaline behaviour; thermolability and tendency to metabolise and/or degrade in the biochemical conditions of milk). An overview of the lipophilicity and acidic behaviour of the major families of antimicrobials is displayed in Table 3 of Section 3.2.2.

#### 3.6.2.2. Set of treatments proposed in the literature for the removal of specific antimicrobials from raw milk

The options with their advantages and disadvantages are discussed in Table 18.

##### *Incubation with $\beta$ -lactamases<sup>6</sup>*

Penicillin-G from spiked milk (0.3 mg/L) was reduced to an undetectable amount within 18 h at 4°C by 1.0 mU/mL of crude *B. cereus*  $\beta$ -lactamase (Penicillinase type II) (Korycka-Dahl et al., 1985). In another study (Horton et al., 2015), a commercially available purified  $\beta$ -lactamase was not effective at 10°C to degrade cefquinome (4th generation cephalosporin) added to milk compared to the use of conditioned media from growth of cephalosporinase-type cefotaximase (CTX-M) producing *E. coli*.

The  $\beta$ -lactam antimicrobials (penicillins, cephalosporins and some others like carbapenems, monobactams and cephamycins) all contain a common element in their molecular structure, namely a four-atom ring known as the  $\beta$ -lactam ring. The  $\beta$ -lactamases are enzymatic molecules produced by Gram-negative bacteria that are able to confer resistance to target  $\beta$ -lactam antimicrobials by degrading the  $\beta$ -lactam ring by hydrolysis, and hence they can deactivate the antibacterial properties of these drugs. The generic name ' $\beta$ -lactamase enzymes' is given to all enzymes having an effect on  $\beta$ -lactam antimicrobials.

$\beta$ -Lactamases have been classified in four different groups and divided into several subgroups because they bear different activities against various  $\beta$ -lactams depending on their spectrum: against penicillins alone (penicillinases), against cephalosporins alone or even against penicillins, cephalosporins and other  $\beta$ -lactams (cephalosporinases, cephamynases, carbapenemases including metallo- $\beta$ -lactamases, oxacillinases, etc.). That means different subgroups of penicillinases are specifically active to a variable extent against several penicillins but have no effect against cephalosporins. In contrast, different cephalosporinases and metallo- $\beta$ -lactamases are active against different groups of cephalosporins and also to some extent against penicillins. In the past, such preparations (probably containing penicillinases only) were used in the dairy industry to be added to contaminated milk intended for the production of fermented products like yoghurt and cheese (Tamine and Robinson, 1999). It should be noted that

<sup>6</sup> It is likely that this use would fall under the definition of technological feed additives according to Regulation 1831/2013 which would trigger a premarketing authorisation based on a risk assessment.

different commercial preparations may contain  $\beta$ -lactamases capable of breaking down earlier penicillin antimicrobials, such as penicillin, amoxicillin and ampicillin, but are less likely to contain cephalosporinase-type  $\beta$ -lactamases capable of breaking down cephalosporin-type antimicrobials such as cefquinome.

#### *Treatment with conditioned medium from bacteria producing cefotaximase*

Treatment of cefquinome spiked milk samples (2  $\mu\text{g}/\text{mL}$ ) with supernatant from growth of bacteria containing a CTX-M encoding gene (ESBL-producing *E. coli*) resulted in a nearly linear degradation of cefquinome in milk at 10°C for 8 h and reached the limit of quantification at 6  $\mu\text{g}/\text{kg}$  after 24 h (Horton et al., 2015).

#### *Fermentation<sup>6</sup>*

Fermentation with *Streptococcus cremoris*–*Streptococcus lactis* starter culture of raw milk to pH 4.7 (acidic pH) was proposed for reduction of penicillin-G and although with a lower rate, this was also observed for novobiocin (Keys et al., 1979). It is not clear from the experimental data if the effect is due to the acidic pH or due to the growth of the bacteria in the raw milk.

A significant reduction of cefquinome was obtained by fermentation of unpasteurised milk at 37°C for 24 h when the starter culture was from a bulk milk tank sample (Horton et al., 2015). It was hypothesised that this degradation was due to the activity of cephalosporinase produced by the enriched populations of CTX-M *E. coli* in the fermented upasteurised milk. Total bacterial counts increased during this fermentation, but the majority of these bacteria were successfully killed at 60°C for 2 h (Horton et al., 2015). Additionally, fermentation at 37°C using two different commercially available probiotic cultures and a starter culture also resulted in a significant reduction of cefquinome (Horton et al., 2015). At 10°C, neither the starter culture nor the two probiotics caused degradation of cefquinome (Horton et al., 2015). This indicates that beside the duration of incubation, the temperature plays a crucial role for the efficacy of this measure.

#### *Combination of ultrafiltration and permeation washes*

Concentrations from 0.03 to 0.120 mg/L of penicillin-G were reduced to undetectable levels by a combination of ultrafiltration and permeate washes (Kosikowski and Jimenez-Flores, 1985). The penicillin-contaminated milks were ultrafiltered at 54°C to one-third of their original volume (membranes with a molecular weight cut-off of 20,000 Da). The retentate was washed by adding of fresh uncontaminated ultrafiltered milk permeates and the mixture was ultrafiltered again. The washing and ultrafiltration step was repeated 4 times to reduce the penicillin concentration to undetectable levels.

#### *pH increase to pH 10*

Cefquinome was stable in spiked milk (2 mg/L) at pH 1, pH 4 and pH 6–7 for the whole experimental period (168 h). A decrease in the concentration of cefquinome in the inoculated milk to below the limit of quantification (125  $\mu\text{g}/\text{kg}$ ) occurred after 8 h at pH 10 (Horton et al., 2015). The pH was adjusted to pH 10 from the natural pH of 6.6 by adding NaOH. The authors also considered further work would be required as milk treated in this way is unlikely to be palatable to calves and might have adverse health effects.

#### *Electrochemical oxidation*

Kitazono et al. (2012) reported significant degradation of oxytetracyclines in milk by electrochemical oxidation using physically adsorbed oxidant on the surface of the anode (Ti/IrO<sub>2</sub> or Ti/PbO<sub>2</sub>) and indirect oxidation in electrogenerated NaCl or hypochlorite electrolytes in the milk. The concentration of oxytetracycline was reduced in fat-free milk from 100 mg/L to 4.2 mg/L during 6 h electrochemical treatment; this efficiency dropped to 83% in raw milk. This physicochemical technology may apply to some extent to other biorefractory antimicrobial substances that need to be degraded from milk although the electrochemical oxidation has so far only been demonstrated in the laboratory scale.

#### *Heat treatment*

With regard to the stability of antimicrobials in milk after thermal treatments, the persistence of antimicrobials after various heat treatment procedures has been examined in many studies over the course of the last 20 years revealing many of these substances to be relatively stable to heat treatment of milk. Hsieh et al. (2011) demonstrated that the heat stability of the antimicrobials used is dependent on the chemical nature of the substance. The heat stability of 14 veterinary antimicrobials

was studied under a short-term heating scenario (15 min at 100°C or 121°C) ranking with decreasing stability the antimicrobial families as follows: sulfonamides, lincomycin, colistin, tetracyclines and  $\beta$ -lactams. Moreover, the structural degradation of the drugs was in good agreement with the reduction in antimicrobial activity suggesting that degradation also diminished antimicrobial activity.

- Zorraquino et al. (2008) and Roca et al. (2011) treating nine  $\beta$ -lactams at five different time–temperature combinations (40–140°C) found that moderate heating to 60°C for 30 min and ultra-high temperature (UHT) treatment to 140°C for 10 s only caused slight decreases in antimicrobial activity, while classic sterilisation conditions (120°C) for 20 min showed a considerable inactivation of penicillins (65%) and cephalosporins (90%). Horton et al. (2015) showed that cefquinome added at a concentration of 2  $\mu$ g/mL in milk samples of 10 g was stable at 4°C and 10°C for the duration of the experiment (144 h); at 37°C and 50°C cefquinome gradually degraded, with half-lives of 65.1 and 30.9 h, respectively.
- Five quinolones in milk were investigated in the range 80–100°C, finding half-lives ranging from 102 to 456 min at the highest temperature (Roca et al., 2010). This study also showed that ciprofloxacin and norfloxacin were the most sensitive with 12% degradation at 120°C for 20 min, while enrofloxacin, flumequine and oxolinic acid were more resistant to degradation, being degraded by only 5% or less. Heating UHT milk to 140°C for 4 s, had basically no effect on degradation of all the five quinolones.
- Roca et al. (2013) also demonstrated that sulfonamides are very stable during treatments of 63°C; 30 min and 72°C; 15 s as well as during UHT sterilisation (140°C; 4 s). Therefore, the high thermostability of sulfonamides demonstrates that the heat treatments used in the dairy industry are likely to be insufficient to completely inactivate sulfonamide residues in milk.

### 3.6.2.3. Summarising remarks regarding removal of antimicrobials in milk

- Different families of antimicrobial substances are characterised by different stabilities.
- Most of the options have focused on reduction of  $\beta$ -lactams (penicillins or cephalosporins) which are the most degradable and frequently used antimicrobials in milking cows, and the highest priorities. Fewer significant interventions are demonstrated in the literature against the other antimicrobials (sulfonamides, tetracyclines, aminoglycosides, macrolides and fluoroquinolones).
- $\beta$ -Lactams (penicillins and cephalosporins) are efficiently degraded by direct incubation with specific  $\beta$ -lactamases (penicillinases, cephalosporinases). For practical application, the spectrum of activity of the commercially available enzymes and the regulatory aspects have to be further considered. This treatment could easily be feasible at the farm level.
- Fermentation can efficiently reduce penicillin and cefquinome as was reported in two studies. These treatments could certainly be feasible at the farm level but the main drawbacks are an increase in microbial load and the lack of knowledge of the mechanism of action.
- Combination of ultrafiltration and permeate washes and electrochemical oxidation are not easily applicable at the farm level but have the potential to reduce a broader spectrum of antimicrobials. These treatments are less easily transferable to farms unless there is a technological design that renders it suitable in farming practice.
- Increasing the pH in the milk to 10 has the potential to efficiently reduce the concentration of certain antimicrobials (at least cefquinome) as was shown in one study. The method is easy to apply on the farm level but the effectiveness for a broader range of antimicrobials the suitability of this milk to be fed to calves needs further consideration.
- Antimicrobial families are ranked with decreasing stability to heat as follows: sulfonamides, lincomycin, colistin, tetracyclines and  $\beta$ -lactams (cephalosporins and penicillins). Even the most thermally sensitive antimicrobial family ( $\beta$ -lactams) are still very heat stable (e.g. stable at 140°C/10 s and 60°C/30 min) making heat treatments unsuitable at the farm level for removing all antimicrobial residues from milk.

### 3.6.3. Options for the elimination of resistant bacteria in milk

Apart from the mitigation of antimicrobials in the waste milk and colostrum, it could also be considered desirable to eliminate antimicrobial-resistant bacteria.

There are some potential methods that could be used for the removal of bacteria from colostrum and milk with preservation of the vital immunoglobulins: heat treatment, microfiltration, centrifugation and curdling (see Table 19). All these methods have the potential to be used at the farm level or to be centralised at a location where technological treatment of colostrum for the farmers can occur. As an

example, in Belgium, frozen colostrum surplus is collected from dairy farms, transported to a central location, where the colostrum and its immunoglobulin quality is checked. High-quality colostrum (with an IgG concentration above 50 g/L) is then sterilised by irradiation. Farmers can purchase this sterile colostrum in a frozen or freeze-dried state. The methods differ in the complexity of processes and the demand for and availability of technical equipment.

#### *Heat treatment of colostrum*

Heat treatment of inoculated colostrum (contaminated with pathogens such as *Mycoplasma bovis*, *Listeria monocytogenes*, *E. coli* spp., *Salmonella* or MAP during 30 min at 60°C resulted in destruction of the vegetative pathogens tested except MAP. For the latter microorganism, 1 h heat treatment at 60°C was needed (McMartin et al., 2006). For lactococci, the same reduction was observed and Enterobacteriaceae and lactobacilli were reduced to a non-detectable level (Trujillo et al., 2007). Donahue et al. (2012) heated colostrum during 1 h at 60°C on a commercial batch pasteuriser and found 2–2.25 log reductions in bacterial plate count.

Likewise, incubation at 60°C for 2 h of milk containing high counts of bacteria, including ESBL-producing *E. coli*, was shown to kill most bacteria in two duplicate experiments (Horton et al., 2015).

Heat treatment can be performed with no significant effect on the immunoglobulins. Three studies investigated the effect of feeding heat-treated colostrum to calves. Two of these studies found no significant difference in the IgG blood values of the calves (Teixeira et al., 2013; Kryzer et al., 2015), in contrast to Godden et al. (2003) who found significantly higher serum IgG concentrations in the blood of calves fed unpasteurised colostrum when fed 2 L at first feeding. The contrast between the two results is likely to be related to the time and temperature used in the different studies (high temperature, short-time pasteurisation, 72°C – 15 min; treatment at 60°C – 60 min; and batch pasteurisation, 63°C – 30 min, respectively). McMartin et al. (2006) showed that treatment at 60°C for up to 120 min had no effect on IgG concentration in colostrum, while at 63°C a substantial decrease was observed. Therefore, strict adherence to an upper limit of temperature is required when heat-treating colostrum, but this may be less crucial when heating lactational whole milk.

Malmuthuge et al. (2015) explored the effectiveness of feeding heat-treated (60°C, 60 min) colostrum to calves on the bacterial colonisation of the gut. The heat treatment of colostrum drastically reduced numbers of *E. coli* in the small intestine of calves at 12 h in comparison with calves fed fresh colostrum. Moreover, they found 3.5 times more *Bifidobacterium* in the small intestine of calves when feeding heat-treated colostrum compared to untreated colostrum. The analysis of Godden et al. (2012) also showed that calves fed heat-treated colostrum were at lower risk for illness because of the significant reduction in total coliform count.

Heat treatment by means of pasteurisation is an established method of decontaminating colostrum and other milk from bacteria and could easily be established on farm.

#### *Heat treatment of raw milk*

Pasteurisation is routinely used in the dairy industry for killing vegetative bacteria in raw milk. Conditions for pasteurisation and related efficiencies for many bacteria, which are frequently present in raw milk are available. A thorough review on this knowledge is available (Kessler, 1996).

Incubation at 60°C for 2 h of milk containing high counts of bacteria, including ESBL-producing *E. coli*, was shown to kill all/most of the bacteria in two duplicate experiments (Horton et al., 2015).

#### *Microfiltration of raw milk*

Microfiltration may be used to remove bacteria from milk. Milk is first de creamed (to prevent obstruction of the filter) and afterwards microfiltered at a temperature between 35 and 55°C through a ceramic membrane with a cut-off of about 1.4 µm (Champagne et al., 1994; Fernández García et al., 2012). Although the technique is suitable for reducing the bacterial load in milk, total elimination is not achieved (Champagne et al., 1994). While this approach seems feasible for marketed milk, it would currently be difficult to perform on farms.

#### *Centrifugation of raw milk and colostrum*

Centrifugation of moderately heated raw milk or colostrum is an interesting method as different semi-industrial centrifuges exist (e.g. as decreamers in dairy industry). A bactofugation procedure was able to remove 95.3% of the bacteria (Kosikowski and Fox, 1968) and 98% of *Clostridium tyrobutyricum* with one bactofugation and 99.5% with two bactofugation (Sant'Ana, 2014). When this principle is



applied for colostrum, some pathogenic bacteria will be removed. For example, in a Belgian study, more than 1.5 log reduction was observed for MAP after centrifuging colostrum (Verheghe et al., 2015).

### **3.6.3.1. Summarising remarks regarding the removal of antimicrobial-resistant bacteria in milk**

- Heat treatment at suitable temperature/time combinations will kill the vegetative bacteria (including antimicrobial-resistant strains) in the colostrum without altering substantially the immunoglobulin content.
- Heat treatment at suitable temperature/time combinations will kill the vegetative bacteria (including antimicrobial-resistant strains) in raw milk but will not eliminate antimicrobial residues present in the milk.
- Microfiltration and centrifugation are less effective compared to heat treatment for eliminating bacteria from raw milk and are not easily applicable at the farm level.

**Table 18:** Options to reduce antimicrobial residues from milk and colostrum

Measure to reduce antimicrobial residues	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
Incubation with $\beta$ -lactamases to reduce $\beta$ -lactam antimicrobials	<ul style="list-style-type: none"> <li>• Korycka-Dahl et al. (1985)</li> <li>• Horton et al. (2015)</li> <li>• Used in the past in dairy industry to produce fermented products from contaminated milk inhibiting growth of lactic acid bacteria</li> <li>• Used to some extent in FI</li> </ul>	<ul style="list-style-type: none"> <li>• None</li> </ul>	<ul style="list-style-type: none"> <li>• Effective</li> <li>• Easy to apply</li> <li>• Commercially available</li> <li>• <math>\beta</math>-Lactam antimicrobials are most frequently used in dairy cow therapy</li> <li>• Relative short incubation time and effective at moderate temperatures</li> </ul>	<ul style="list-style-type: none"> <li>• May need regulatory clarification<sup>(a)</sup></li> <li>• Spectrum of activity of each type of <math>\beta</math>-lactamases has to be investigated for the purpose of inactivation of all used <math>\beta</math>-lactam antimicrobials</li> <li>• Do not cover other families of antimicrobials than <math>\beta</math>-lactams</li> </ul>
Heat treatment or conditioned medium from bacteria producing cefotaximase to reduce cefquinome (4th gen. cephalosporin)	<ul style="list-style-type: none"> <li>• Horton et al. (2015) (only one study, only cefquinome)</li> </ul>	<ul style="list-style-type: none"> <li>• None</li> </ul>	<ul style="list-style-type: none"> <li>• Effective</li> <li>• Relatively short incubation time and effective at moderate temperatures (10–50°C depending on method)</li> <li>• Targeting one of the highest priority CIA</li> </ul>	<ul style="list-style-type: none"> <li>• May need regulatory clarification for conditioned medium<sup>(a)</sup></li> <li>• Not available as ready-to-use commercial product for conditioned medium</li> <li>• Only applied for cefquinome in this study</li> </ul>
Fermentation to reduce penicillin-G and cefquinome	<ul style="list-style-type: none"> <li>• Keys et al. (1979) (penicillin-G)</li> <li>• Horton et al. (2015) (cefquinome)</li> </ul>	<ul style="list-style-type: none"> <li>• None</li> </ul>	<ul style="list-style-type: none"> <li>• Can be effective</li> <li>• Can use starter cultures or rely on endogenous bacteria in waste milk. For the latter success would depend on type of bacteria present</li> </ul>	<ul style="list-style-type: none"> <li>• May need regulatory clarification<sup>(a)</sup></li> <li>• Milk needs to be heated to 37°C for a prolonged time (not effective at lower temperature)</li> <li>• Fermentation would increase bacterial count of the raw milk which may require pasteurisation before feeding to calves</li> <li>• Because mechanism of activity is not known it is uncertain which antimicrobials are destroyed in each fermentation</li> <li>• pH of the milk may be acidic which may compromise calves feeding possibilities</li> <li>• Does not cover other families of antimicrobials than <math>\beta</math>-lactams</li> </ul>

Measure to reduce antimicrobial residues	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
Combination of ultrafiltration and permeate washes to reduce penicillin	<ul style="list-style-type: none"> <li>Kosikowski and Jimenez-Flores (1985)</li> </ul>	<ul style="list-style-type: none"> <li>None</li> </ul>	<ul style="list-style-type: none"> <li>Effective</li> <li>Reconstituted milk is not altered significantly in relation to raw milk</li> <li>Has the potential to also be applicable to other antimicrobials than <math>\beta</math>-lactams</li> </ul>	<ul style="list-style-type: none"> <li>Very complicated method to apply at farm level</li> </ul>
pH increase to pH 10 to reduce cefquinome	<ul style="list-style-type: none"> <li>Horton et al. (2015) (only 1 study, only cefquinome)</li> </ul>	<ul style="list-style-type: none"> <li>None</li> </ul>	<ul style="list-style-type: none"> <li>Effective</li> <li>Easy to apply and cheap</li> </ul>	<ul style="list-style-type: none"> <li>Milk unlikely to be palatable to calves</li> <li>Might have adverse health effects (adjustment to pH 6–7 may be needed)</li> <li>Lack of knowledge if this measure may be effective for a broader spectrum of antimicrobials</li> </ul>
Electrochemical oxidation to remove tetracyclines	<ul style="list-style-type: none"> <li>Kitazono et al. (2012) (only one lab scale study)</li> </ul>	<ul style="list-style-type: none"> <li>None</li> </ul>	<ul style="list-style-type: none"> <li>Experience exists for waste water treatment</li> </ul>	<ul style="list-style-type: none"> <li>Special equipment necessary</li> <li>Electrolyte (NaCl) has to be added to the milk</li> <li>Lack of knowledge if this measure may be effective for a broader spectrum of antimicrobials</li> <li>Milk fat reduces effectivity</li> </ul>
Heat treatments to remove several antimicrobials	<ul style="list-style-type: none"> <li>Well documented in scientific literature</li> </ul>	<ul style="list-style-type: none"> <li>Several studies showing that most antimicrobials are very heat stable</li> </ul>	<ul style="list-style-type: none"> <li>None</li> </ul>	<ul style="list-style-type: none"> <li>Not effective</li> <li><math>\beta</math>-lactams need sterilisation conditions (120°C for 20 min) to be degraded</li> <li>Destroy any potential beneficial bacteria</li> </ul>

CIA: critically important antimicrobial for human medicine, e.g. 3rd–4th generation cephalosporins and fluoroquinolones, according to the WHO definition (Collignon et al., 2016; WHO, 2016). See Appendix A, FI: Finland.

(a): It is likely that this use would fall under the definition of technological feed additives according to Regulation 1831/2003 which would trigger a premarketing authorisation based on a risk assessment.

**Table 19:** Options to reduce antimicrobial-resistant bacteria from milk and colostrum

Measure to reduce antimicrobial-resistant bacteria	Evidence in support of the measure	Evidence against the measure	Advantages	Disadvantages
Pasteurisation of colostrum	<ul style="list-style-type: none"> <li>• Heating conditions are well documented in literature</li> <li>• Effects on immunoglobulins and on calf health are well documented in literature</li> </ul>	<ul style="list-style-type: none"> <li>• Some contradictory reports on the effect on immunoglobulin content in blood of calves fed pasteurised colostrum</li> </ul>	<ul style="list-style-type: none"> <li>• Effective to kill resistant and other potentially pathogenic bacteria</li> <li>• Can be performed at farm level (batch pasteurisation conditions may be applied)</li> <li>• Pasteurised colostrum can be fed to calves</li> </ul>	<ul style="list-style-type: none"> <li>• Not effective against antimicrobial residues</li> <li>• Destroy any potential beneficial bacteria</li> </ul>
Pasteurisation of raw milk	<ul style="list-style-type: none"> <li>• Evidence is substantial because of dairy practice and scientific publications</li> </ul>	<ul style="list-style-type: none"> <li>• None</li> </ul>	<ul style="list-style-type: none"> <li>• Effective to kill resistant and other potentially pathogenic bacteria</li> <li>• Can be performed at farm level (batch pasteurisation may be applied)</li> <li>• Pasteurised milk can be fed to calves</li> </ul>	<ul style="list-style-type: none"> <li>• Not effective against antimicrobial residues</li> <li>• Destroy any potential beneficial bacteria</li> </ul>
Microfiltration of raw milk	<ul style="list-style-type: none"> <li>• Several scientific publications and practice in dairy industry</li> </ul>	<ul style="list-style-type: none"> <li>• None</li> </ul>	<ul style="list-style-type: none"> <li>• Milk content is not substantially changed to be fed to calves</li> </ul>	<ul style="list-style-type: none"> <li>• Knowledge on actual efficiencies in raw milk is incomplete</li> <li>• Incomplete elimination of bacteria</li> <li>• Technically demanding</li> <li>• Complicated to perform at the farm level</li> </ul>
Centrifugation of raw milk and colostrum	<ul style="list-style-type: none"> <li>• Raw milk: several scientific publications and practice in dairy industry</li> <li>• Colostrum: Verheghe et al. (2015)</li> </ul>	<ul style="list-style-type: none"> <li>• Less effective than pasteurisation</li> </ul>	<ul style="list-style-type: none"> <li>• Milk and colostrum content are not substantially changed</li> <li>• The immunoglobulins in the colostrum are not altered substantially</li> </ul>	<ul style="list-style-type: none"> <li>• Complicated to perform at farm level (heating and centrifugation are needed)</li> </ul>

### 3.7. Risks to the calf from antimicrobial exposure

Although not strictly within the remit of this Opinion, which focusses on the development of AMR from feeding antimicrobial-contaminated colostrum or milk, the WG discussed some other factors that have a bearing on whether waste milk containing antimicrobials should be fed to calves.

Intestinal health is enhanced by a high-diversity intestinal bacterial community, while low diversity is associated with poor health and disease (Mao et al., 2012; Yeoman and White, 2014). Antimicrobials decrease the diversity of the gut microbiota. Higher diversity appears to afford resilience to colonisation by pathogens. Furthermore, Lallès (2012) presented evidence that antimicrobial exposure in early life, even *in utero*, predisposes farm animals to poorer health and productivity in later life due to interference with the development of the immune system. Lallès (2012) described studies in humans, rodents and pigs which show changes that occur in tissue metabolism in later life that are caused by early antimicrobial exposure and are deleterious to health. This may be mediated by interfering with bacterial programming of gut tissues in the neonate. In man, early alterations in gut microbial colonisation, for example, by perinatal antimicrobial treatment, are suspected to be responsible for increased disease in later life (Bedford Russell and Murch, 2006). Van Vleck Pereira et al. (2016), in their review of the effects of antimicrobial exposure in human infants, concluded that the consumption of milk containing drug residues was identified as a risk factor in the subsequent development of asthma, allergy, and obesity in later life. This was interpreted as stemming from changes in the microbiota caused by antimicrobials in the developing infant. Maternal administration of antimicrobials in rat dams increased gut permeability and systemic inflammation in offspring (Fåk et al., 2008). In a swine model of perinatal disturbance of bacterial colonisation by oral antimicrobial administration to sows around farrowing, transient short-term (day 14) and long-term (month 6) alterations in paracellular permeability occurred (Boudry et al., 2011) and reductions in gene expression in offspring born to antimicrobial-treated mothers were observed by Mroz et al. (2011). Conversely, the activity of dipeptidyl peptidase IV (DPP-IV) was doubled in the jejunum of the offspring. High-intestinal DPP-IV activity was shown to specifically reduce glucose tolerance and circulating insulin through incretin breakdown and generation of deleterious dipeptides in mice (Waget et al., 2011).

Thus, although there is no direct evidence in calves, it is reasonable to extrapolate from non-ruminants to preruminants and therefore to conclude that it is likely that exposure of very young calves to antimicrobials may compromise their health and welfare in later life.

## 4. Conclusions

### 4.1. Answer to Term of reference 1: 'Assess the risk for the development of AMR due to feeding on farm of calves with colostrum potentially containing residues of antibiotics'

When the interval from the dry-off treatment until calving is as long as or longer than the minimum specified in the Summary of Product Characteristics of the antimicrobial product, faecal shedding of antimicrobial-resistant bacteria will not increase when calves are fed colostrum from treated cows.

When cows calve earlier than the minimum withdrawal period specified in the Summary of Product Characteristics for the antimicrobial product, the levels of antimicrobial in the colostrum are higher, and therefore, there is an increased probability of shedding of antimicrobial-resistant bacteria by calves receiving the colostrum. However, the available evidence is insufficient to quantify this increase, and no effect was observed in the single observational study dealing with this subject.

These conclusions are based on the information specified in the Summary of Product Characteristics, and limited observational data on shedding of antimicrobial-resistant bacteria in calves and on two studies on the antimicrobial residue concentrations in field samples of colostrum.

The context and main evidence for these conclusions are summarised below:

- Intramammary treatment of dairy cows for prevention and/or treatment of udder infections during the dry period is common in the EU and in some MS, the majority of cows are treated.
- For treatment during the dry period mainly penicillins, alone or in combination with aminoglycosides, and 1st–2nd generation cephalosporins are used. The 3rd- and 4th-generation cephalosporins are also used and in some MSs these cephalosporins are very commonly used.



- Feeding calves with colostrum from dairy cows treated with antimicrobials at the beginning of the dry period is a common practice in the majority of dairy farms in the EU.
- Feeding calves colostrum from cows treated with penicillins and aminoglycosides at the beginning of the dry period did not increase the faecal shedding of antimicrobial-resistant *E. coli*. The observational study considered does not allow conclusions to be drawn on the effect of feeding calves colostrum from cows treated at drying-off with antimicrobials other than penicillins and aminoglycosides.
- Results from scientific literature on the time needed between treatment and parturition indicated that the antimicrobial residue levels in colostrum samples are expected to be low. Only two studies presented results on the antimicrobial residue levels in field colostrum samples showing a low number of samples with levels above the MRLs or detection limit.
- The MRL assessment reports and scientific literature show that antimicrobial residue levels decrease with the length of the dry period.
- With a dry period length as long as or longer than the minimum specified in the Summary of Product Characteristics of the antimicrobial product, the antimicrobial residue levels in the colostrum are judged to be low.

#### **4.2. Answer to Term of reference 2: 'Assess the risk for the development of AMR due to feeding on farm of calves with milk of cows treated during lactation with an antibiotic and milked during the withdrawal period'**

Milk from cows receiving antimicrobial treatment during lactation contains substantial levels of antimicrobial residues during the treatment and withdrawal period. Consumption of such milk will lead to increased probability of faecal shedding of antimicrobial-resistant bacteria by calves. This conclusion is based on observational and experimental studies.

The presence of antimicrobial-resistant bacteria in milk is judged to be less important compared to the presence of antimicrobial residues in waste milk. This conclusion is based on a single study which also showed that pasteurisation of waste milk did not decrease the level of faecal shedding of antimicrobial-resistant *E. coli* in calves. The context and main evidence for these conclusions are summarised below:

- Antimicrobial treatment of dairy cows during lactation is common in the EU MSs and the main reason is infections of the udder.
- For treatment, penicillins and 1st–2nd generation cephalosporins are mainly used. Often these antimicrobials are given in combination with aminoglycosides but penicillins and 1st–2nd generation cephalosporins are also used as a single therapy. In several MSs, 3rd- and 4th-generation cephalosporins are used and in some MSs these cephalosporins are very commonly used.
- Feeding calves with milk from dairy cows treated with antimicrobials during lactation and milked during the withdrawal period is a common practice, occurring in the majority of dairy farms in Europe.
- The most pronounced effect on faecal shedding of antimicrobial-resistant bacteria was observed in calves of 2–3 weeks of age and in several studies a statistically significant reduction was seen for calves at an age of 6–7 weeks. This coincides with the observation that also in calves fed with antimicrobial-free milk, faecal shedding of resistant *E. coli* was higher in younger calves compared to older calves and dairy cows.
- Of the available experimental studies, only one reported an antimicrobial concentration at which there was no increase in faecal shedding of antimicrobial-resistant bacteria. Above this threshold concentration, there was no indication that the effect was dose-dependent. An observational study showed a comparable effect on the faecal shedding of resistant *E. coli* when feeding calves waste milk only during the withdrawal period, and when feeding calves' milk obtained during both the treatment and the withdrawal period.

#### 4.3. Answer to Term of reference 3: 'Propose possible options to mitigate the risk for the development of AMR derived from such practices if relevant'

There are three principal approaches for reducing the risk for development of AMR derived from feeding waste milk or colostrum containing antimicrobial residues to calves.

- 1) Measures in feeding management when feeding calves colostrum and milk potentially containing residues of antimicrobials.

For ToR 1, it was concluded that increased faecal shedding of antimicrobial-resistant bacteria if calves receive colostrum from cows treated with antimicrobials is not expected, provided that the interval from treatment at dry-off to calving is as long as the minimum specified in the Summary of Product Characteristics for the antimicrobial product. When cows calve earlier than the minimum withdrawal period specified in the Summary of Product Characteristics for the antimicrobial product, the levels of antimicrobial in the colostrum are higher, and therefore, there is an increased probability of faecal shedding of antimicrobial-resistant bacteria. This increased probability could be avoided by not feeding colostrum from such cows (see assessment of options in Table 16).

For ToR 2, it was concluded that milk from cows receiving antimicrobial treatment during lactation contains substantial residues during the treatment and withdrawal period and that consumption of such milk will lead to increased faecal shedding of antimicrobial-resistant bacteria by calves. A range of possible options exist for restricting the feeding of such milk to calves, which could be targeting the highest priority critically important antimicrobials (WHO, 2016). These options have varying advantages and disadvantages. A blanket ban on feeding such milk would remove the risk. Options involving partial restriction (e.g. restricting the use of milk harvested only during treatment, as practised in some MSs) would be expected to partially reduce the risk at population level, but there are limited or no data to quantify this (see Table 17).

- 2) Taking measures to destroy antimicrobial residues before feeding.

Most of the options proposed to mitigate the presence of antimicrobials in raw milk or colostrum focus on reduction of  $\beta$ -lactams (penicillins or cephalosporins) which are the most degradable and most frequently used antimicrobials in dairy cows. Fewer significant actions are demonstrated in the literature against the other antimicrobials used in dairy cows.

For  $\beta$ -lactam antimicrobials, the use of  $\beta$ -lactamases has been studied.  $\beta$ -Lactam antimicrobials can be degraded to a level below the detection limit if the specific  $\beta$ -lactamases are used. This is of importance as  $\beta$ -lactams are the most frequently used antimicrobials in dairy cows. Authorisation may be required if calves are to be fed milk treated with  $\beta$ -lactamases or fermented with  $\beta$ -lactamase-producing microorganisms. A few options (see below) have also been proposed using physicochemical approaches including heat, chemical changes in pH, combination of ultrafiltration and permeation washes, and electrochemical oxidation.

These options have varying advantages and disadvantages and are unlikely to be fully effective (see Table 18).

- Fermentation could efficiently reduce penicillin and cefquinome as was reported in two studies. The main drawbacks are an increase in microbial load and the lack of knowledge of the mechanism of action.
- Combination of ultrafiltration and permeate washes and electrochemical oxidation are not easily applicable on the farm level but have the potential to reduce a broader spectrum of antimicrobials.
- Increasing the pH in the milk to 10 has the potential to efficiently reduce the concentration of certain antimicrobials (at least cefquinome) as was shown in one study. The effectiveness for a broader range of antimicrobials and the suitability of this milk to be fed to calves needs further consideration.

- 3) Measures to eliminate antimicrobial-resistant bacteria from colostrum and waste milk.

For ToR 2, the presence of antimicrobial-resistant bacteria in waste milk and feeding to calves is likely to have an influence in the gut flora of the recipient animal. The relative contribution of this source of antimicrobial-resistant bacteria cannot be quantified with existing data.

Options to mitigate the presence of antimicrobial-resistant bacteria in raw milk or colostrum are based on thermal inactivation. Heat treatment at suitable temperature/time combinations will kill the vegetative bacteria (including antimicrobial-resistant ones) but not eliminate antimicrobial residues. In addition, some technical processes have been proposed for eliminating antimicrobial-resistant bacteria from raw milk, such as microfiltration and centrifugation. Again, all of these options have varying advantages and disadvantages (see Table 19).

## 5. Recommendations

- 1) The feeding to calves of colostrum and milk containing residues of antimicrobials that could select for antimicrobial-resistant bacteria should be avoided, particularly those selecting for resistance to highest priority CIAs.
- 2) The contribution of resistant bacteria to the environment from faeces of calves fed milk containing antimicrobials should be compared to the contribution that would arise from other methods of disposing of milk that contains both antimicrobials and resistant bacteria.
- 3) Besides antimicrobial-resistant bacteria in calf faeces, attention should be paid to the presence of antimicrobials in calf faeces, which could also contribute to the development of AMR in the farm environment.
- 4) To perform further studies regarding:
  - a) The concentration of antimicrobial residues in field colostrum and milk samples, and the thresholds at which selection for antimicrobial-resistant bacteria occurs in calves.
  - b) The effectiveness of different mitigation options.

## Documentation provided to EFSA

- 1) Gonggrijp M, Scherpenzeel C, Kappert C, Heuvelink A, Holtstege M, Nijenhuis E, Tijs S, Keurentjes J, Lam T and Velthuis A, 2015. Resistentieontwikkeling bij jonge kalveren (in Dutch). Report. Research number OND1358162 – provided by Dr. Theo Lamm (GD Animal Health, 7400 AA Deventer, the Netherlands).

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## Glossary

### Acquired resistance

A bacterial strain can acquire resistance by mutation, by the uptake of exogenous genes by horizontal transfer from other bacterial strains or by the activation/triggering of a genetic cascade,

thereby inducing the expression of resistance mechanisms. Genes encoding enzymes that can modify the structure of an antimicrobial are commonly located on transferable genetic elements (i.e. plasmids, transposons, integrons) that may usually carry more than one resistance gene. Acquisition of resistance by mutation usually arises spontaneously due to point mutations that result, for instance, in changes in an antimicrobial target – e.g. chromosomal changes that result in resistance to quinolones and fluoroquinolones (Adapted from ECDC/EFSA/EMA/SCENIHR, 2009).

## AmpC $\beta$ -lactamases

Intrinsic cephalosporinases found on the chromosomal DNA of many Gram-negative bacteria, including many members of the Enterobacteriaceae (but, notably, not in *Klebsiella* or *Salmonella*), and opportunistic pathogens such as *Pseudomonas* and *Acinetobacter*. These enzymes confer resistance to penicillins, 2nd- and 3rd-generation cephalosporins including  $\beta$ -lactam/inhibitor combinations, cefamycins (cefoxitin), but usually not to 4th-generation cephalosporins (cefepime, cefquinome) and carbapenems. It is a serious concern that a growing number of AmpC enzymes have 'escaped' on to plasmids. These are the so called 'acquired' or 'plasmidic' AmpCs. (EFSA BIOHAZ Panel, 2011).

## Antibiotic

A substance produced by a microorganism, or a chemically produced derivative thereof, that selectively destroys or inhibits the growth of other microorganisms (ECDC/EFSA/EMA/SCENIHR, 2009).

## Antimicrobial

An active substance of synthetic or natural origin which destroys bacteria, suppresses their growth or their ability to reproduce in animals or humans, excluding antivirals and antiparasitics (ECDC/EFSA/EMA/SCENIHR, 2009).

## Antimicrobial resistance (AMR)

The ability of microorganisms to survive or even to grow in the presence of a given concentration of an antimicrobial that is usually sufficient to inhibit or kill microorganisms of the same species. (ECDC/EFSA/EMA/SCENIHR, 2009).

## Calf

A calf is a young bovine which is not reproductively active. There is a gradual transition from a new born animal, dependent on milk, to an animal with many adult characteristics. In this report, calf is used to describe animals of up to 8 months of age (EFSA AHAW Panel, 2009).

## Clinical resistance

The degree of resistance to a particular antimicrobial that results in therapeutic failure in treating an infection with that specific antimicrobial, even if the bacterium is exposed to maximum levels of this antimicrobial. The **Minimum Inhibitory Concentration** (MIC, see definition below) of an antimicrobial for a bacterium isolated from clinical samples, in relation to assumed tissue concentrations in the infected patient, is used for guidance purposes. A bacterial isolate is categorised as clinically resistant when the obtained MIC of the antimicrobial is associated with a high likelihood of therapeutic failure of treatment with that antimicrobial. **Clinical breakpoints** are intended for use in everyday clinical laboratory work to advice on therapy in the patient and may vary between countries and over time. They are defined by scientific committees (e.g. CLSI, EUCAST) and on the basis of knowledge of drug pharmacology of antimicrobials and clinical efficacy (Adapted from ECDC/EFSA/EMA/SCENIHR, 2009).

## Co-resistance and co-selection

Genes conferring AMR are frequently contained in larger genetic elements such as integrons, transposons or plasmids, and as such may be 'linked' to other, unrelated resistance genes. In such cases, multiple resistance genes may be transferred in a single event. When two or more different resistance genes are physically linked, this is termed 'co-resistance'. Consequently, selection for one

resistance attribute will also select for the other resistance gene(s), termed co-selection (ECDC/EFSA/EMA/SCENIHR, 2009).

### Critically Important Antimicrobial for human medicine (CIA)

Those antimicrobials which meet following criteria: Criterion 1: *The antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people* and, Criterion 2: *The antimicrobial class is used to treat infections in people caused by either: (1) bacteria that may be transmitted to humans from nonhuman sources, or (2) bacteria that may acquire resistance genes from nonhuman sources.* (Collignon et al., 2016; WHO, 2016).

### Colostrum

Secretion of the mammalian mammary gland around parturition. Compared to mature milk, colostrum contains more proteins, especially immunoglobulins, required by the newborn to acquire passive immunity.

### Dairy cow

A dairy cow is a cow that is kept for producing milk for human consumption (as opposed to beef or suckler cows).

### Dam

Mature female bovine animal.

### Dry period

Prior to parturition, dairy cows are commonly not milked for several weeks. The procedure of not milking a cow anymore to induce involution is called the 'dry-off' of the cow. **Intramammary treatments** (see below) with long-acting antimicrobials after the last milking are termed dry cow treatments. During the dry period, the mammary gland undergoes the process of involution and renewal. At the end of the dry period, colostrum is being produced by the mammary gland.

### Extended-spectrum beta-lactamases (ESBLs)

ESBLs are plasmid-encoded enzymes in Enterobacteriaceae, frequently found in *Escherichia coli* and *Klebsiella pneumoniae*, but also present in other members of this bacterial family. ESBLs confer resistance to a variety of  $\beta$ -lactam antimicrobials, including penicillins, 2nd-, 3rd- and 4th-generation cephalosporins and monobactams (e.g. aztreonam), but usually not the carbapenems or the cephamycins (e.g. ceftiofuran) (EFSA BIOHAZ Panel, 2011).

### Feed

Any substance or product, including additives, whether processed, partially processed or unprocessed, intended to be used for oral feeding to animals.<sup>7</sup>

### Heifer

A young cow from 8 months until her first parturition (EFSA AHAW Panel, 2009).

### Inherent (intrinsic) resistance

An inherent trait of certain bacterial species. For example, the target of the antimicrobial agent may be absent in that species, the cell wall may have poor permeability for certain types of molecules or the bacterial species may inherently produce enzymes that destroy the antimicrobial agent. These bacteria are clinically resistant, but should more accurately be referred to as 'insensitive' (ECDC/EFSA/EMA/SCENIHR, 2009).

<sup>7</sup> Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1.

## Intramammary treatment

Application of antimicrobials or other drugs by local infusion into the udder through the teat canal, usually using an applicator designed for the purpose, i.e. an intramammary.

## Intramuscular injection

Application of antimicrobials or other drugs in muscular tissue by injection through the skin. From the intramuscular depot the drug is absorbed and distributed systemically by the bloodstream.

## Intravenous injection

Application of antimicrobials or other drugs directly in the bloodstream by injection through the skin into a blood vessel. The drug is immediately distributed systemically by the bloodstream.

## Limit of Quantification (LOQ)

The LOQ is the smallest concentration that can be reliably measured by an analytical procedure.

## Maximum Residue Limit (MRL)

The MRL is the maximum concentration of an individual residue accepted by European Union in a food product obtained from an animal that has received a veterinary medicine or that has been exposed to a biocidal product for use in animal husbandry. MRL are generally derived from toxicologically or microbiologically based **Acceptable Daily Intake** (ADI<sub>tox</sub> or ADI<sub>mic</sub>). The ADI is the estimate of residue, expressed in terms of micrograms or milligrams per kilogram of bodyweight that can be ingested daily over a lifetime without any appreciable health risk.

## Microbiological/epidemiological resistance

The ability of bacteria of a specific species to survive in the presence of antimicrobial concentrations at which they cannot normally survive. The values of MIC or Inhibition zone diameters used for this categorisation are termed '**epidemiological cut-off values**' (ECO<sub>FFS</sub>). The ECO<sub>FF</sub> separates isolates of a bacterial species without acquired resistance (wild type) from isolates which have acquired resistance (non wild type). The use of epidemiological cut-off values provides an appropriate level of sensitivity when measuring AMR development in bacteria of concern in both human and veterinary medicine but is not always indicative of the probability of clinical success (see [Clinical resistance](#)) (adapted from ECDC/EFSA/EMA/SCENIHR, 2009).

## Milk

Udder secretion of cows during lactation.

## Milk replacer

Compound feed administered after dilution in a given quantity of liquid for feeding young animals as a complement to, or substitute for, post-colostral milk or for feeding young animals such as calves, lambs or kids intended for slaughter.<sup>8</sup>

## Minimum inhibitory concentration (MIC)

Lowest concentration of an antimicrobial that will inhibit *in vitro* the visible growth of a microorganism after overnight incubation (Andrew, 2001). It is expressed in µg/mL (or mg/L).

## Parenteral treatment

Application of antimicrobials or other drugs intended for systemic action by routes other than the digestive tract or topical application, e.g. by injection or infusion.

<sup>8</sup> Regulation (EC) No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC. OJ L 229, 1.9.2009, p. 1.

## Post-colostral milk

See [Transition milk](#).

## Qualitative risk assessment

A risk assessment based on data which, while forming an inadequate basis for numerical risk estimations, nonetheless, when conditioned by prior expert knowledge and identification of attendant uncertainties permits risk ranking or separation into descriptive categories of risk (Codex Alimentarius Commission, 1999).

## Quantitative risk assessment

A risk assessment that provides numerical expressions of risk and indication of the attendant uncertainties (Codex Alimentarius Commission, 1999).

## Resistome

A collection of all the antimicrobial resistance genes and their precursors in pathogenic and non-pathogenic bacteria (Wright, 2007).

## Somatic cell counts

Somatic cell count (SCC) is the number of somatic cells found in a millilitre of milk. Somatic cells are mostly cells of the immune system (80% in uninfected quarters, 99% in mastitic quarters). These somatic cells are part of the natural defense mechanism and include lymphocytes, macrophages, polymorphonuclear cells and some epithelial cells. Somatic cells are therefore a reflection of the inflammatory response to an intramammary infection or another trigger of the immune system. (Schukken et al., 2003) The somatic cell count is used to define milk from cows from mastitis and a threshold value has been defined for raw milk put on the market in Reg. No. 853/2004/EC.

## Steers

Male young bovine animals, castrated before puberty and kept for meat production.

## Screening tests for detection of antimicrobial residues in milk

Screening tests for detection of antimicrobial residues in milk are based on various detection concepts: microbiological inhibitory assays; immune-enzymatic reactions (ELISA kits); immunochemical reactions (IACs); biosensing technologies (BIAs, Evidence). The results are generally either qualitative results with no formal identification of a specific antimicrobial or qualitative with a first knowledge on the family of antimicrobial in question or even for some of them with a semiquantitative approach. The Delvotest-P is a microbiological inhibitory assay using the inhibition of *Bacillus stearothermophilus* (as *Geobacillus stearothermophilus*) mostly sensitive (at  $\mu\text{g/L}$  trace level) to the  $\beta$ -lactams (penicillins and most cephalosporins) but also to several other antimicrobials from other classes.

## Systemic treatment

Any treatment given to animals that will lead to spread of the drug throughout the body.

## Subcutaneous injection

Application of antimicrobials or other drugs in the subcutaneous space by injection through the skin. From the subcutaneous depot the drug is absorbed and distributed systemically by the bloodstream.

## Transition milk

Udder secretion of the cow after the first milking. Transition milk is not colostrum anymore but not yet marketable milk. While definitions of the duration of the transition vary for this Opinion the milk of the first 5 days after calving is termed transition milk or **post-colostral milk**.



## Uncertainty

General term referring to all types of limitations in available knowledge that affect the range and probability of possible answers to an assessment question. Available knowledge refers here to the knowledge (evidence, data, etc.) available to assessors at the time the assessment is conducted and within the time and resources agreed for the assessment (EFSA Revised Draft for Internal Testing of the 'Guidance on Uncertainty in EFSA Scientific Assessment', <http://www.efsa.europa.eu/sites/default/files/160321DraftGDUncertaintyInScientificAssessment.pdf>).

## Veal calves

Veal calves are raised for producing veal. They are typically fed liquid feed (milk or milk replacer) throughout their lifespan.

## Waste milk

Milk produced on farm that cannot be marketed for human consumption (see Section 1.4.1).

## Weaning

In dairy farming, calves are often separated from their dams soon after birth and are not allowed to suckle the cow. Instead they are fed with milk or milk replacer via buckets or other feeding devices. Although separated from the dam, calves are considered as unweaned as long as they are fed milk and the term weaning is used to refer to the process of removing milk from the calf's diet (EFSA AHAW Panel, 2009).

## Withdrawal period definition

As described in the guidance for the determination of the withdrawal period for milk (EMEA/CVMP/473/98-Final) the following definition shall apply: 'the withdrawal period is defined as the interval between the last administration of veterinary medicinal product to the animal under normal conditions of use and the production of foodstuff from such animals to ensure that such foodstuffs do not contain residues in quantities in excess of the maximum residue limits laid down. Withdrawal period: Period necessary between the last administration of the veterinary medicinal product to animals under normal conditions of use and the production of foodstuffs from such animals, in order to ensure that such foodstuffs do not contain residues in quantities in excess of the maximum limits laid down in application of Regulation (EEC) No 2377/90'. The European harmonised method for withdrawal period determination in milk is the Time To Safe Concentration (TTSC), which calculates a tolerance limit on the number of milking per animal. This tolerance limit is the time necessary for residue concentration in the milk of most animals to reach the safe concentration (**MRL**, see above).

## Abbreviations

AM	antimicrobial
AMP	ampicillin
AMR	antimicrobial resistance
AMX	amoxicillin
AHAW	EFSA Panel on Animal Health and Welfare
APHA	UK Animal and Plant Health Agency
BIOHAZ	EFSA Panel on Biological Hazards
BSAC	British Society of Antimicrobial Chemotherapy
CFL	cefalonium
CFQ	cefquinome
CFU	colony-forming units
CI	confidence interval
CIA	critically important antimicrobial
CLSI	Clinical and Laboratory Standards Institute
CLX	cloxacillin
CTX-M	cephalosporinase-type cefotaximase
CVMP	Committee for Medicinal Products for Veterinary Use

DCA	desfuroylceftiofur acetamide
DADD	defined animal daily dosage
DPP-IV	dipeptidyl peptidase IV
DR	drug residue
ECOFF	epidemiological cut-off value
EMA	European Medicines Agency
ESBL	extended-spectrum beta-lactamases
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
GIT	gastrointestinal tract
HAP	cefapirin
HPLC	high-performance liquid chromatography
HPLC–MS/MS	high-performance liquid chromatography –tandem mass spectrometry
IAC	immunoaffinity chromatography
Ig G	immunoglobulin G
LEX	cefalexin
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MAP	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>
MIC	minimum inhibitory concentration
MPC	mutation prevention concentration
MRL	maximum residue limit
MS	Member State
MSW	mutant selection window
NCCLS	National Committee for Clinical Laboratory Standards
NR	no drug residue
PCR	polymerase chain reaction
PEN-G	penicillin G
PD	pharmacodynamic
PK	pharmacokinetic
SCC	somatic cell count
ToR	Term of reference
TTSC	time to safe concentration
UHT	ultrahigh temperature
WG	Working Group
WHO	World Health Organization

## Appendix A – Antimicrobials classes used in human medicine

**Table A.1:** Listing and categorisation of antimicrobials classes used in human medicine (examples of veterinary use only drugs are also listed at the end of each category)

Antimicrobials classes	Antimicrobials compounds
<b>Highest priority critically important antimicrobials</b>	
Quinolones	e.g. <i>ciprofloxacin, flumequine, gatifloxacin, levofloxacin, moxifloxacin, nalidixic acid, norfloxacin, danofloxacin (V*), enrofloxacin (V*), marbofloxacin (V*)</i>
3rd- and 4th-generation cephalosporins	e.g. <i>cefepime, cefoperazone, cefotaxime, cefpodoxime, ceftazidime, ceftriaxone, ceftiofur (V*), cefovecin (V*), cefquinome (V*)</i>
Macrolides and ketolides	e.g. <i>azithromycin, clarithromycin, erythromycin, spiramycin, gamithromycin (V*), tildipirosin (V*), tilmicosin (V*), tulathromycin (V*), tylosin (V*)</i>
Glycopeptides	e.g. <i>teicoplanin, telavancin, vancomycin, avoparcin (V*)</i>
<b>Critically important antimicrobials</b>	
Aminoglycosides	e.g. <i>amikacin, neomycin, kanamycin, streptomycin, gentamicin, apramycin (V*)</i>
Carbapenems	e.g. <i>imipenem, meropenem, ertapenem, panipenem</i>
Penicillins	e.g. <i>amoxicillin, ampicillin, meticillin, penicillin G (=benzylpenicillin), penicillin V (=phenoxymethylpenicillin), penethamate hydriodide (V*)</i>
Polymyxins	e.g. <i>colistin, polymyxin B</i>
Phosponic acid derivatives	e.g. <i>fosfomicin</i>
Glycylcyclines	e.g. <i>tigecycline</i>
Lipopeptides	e.g. <i>daptomycin</i>
Monobactams	e.g. <i>aztreonam, carumonam</i>
Oxazolidinones	e.g. <i>linezolid</i>
Ansamycins	e.g. <i>rifampicin, rifaximin, rifapentine, rifamycin</i>
Drugs used solely to treat tuberculosis or other mycobacterial diseases	e.g. <i>capreomycin, pyrazinamide</i>
<b>Highly important antimicrobials</b>	
Amidinopenicillins	e.g. <i>mecillinam, pivmecillinam</i>
Amphenicols	e.g. <i>chloramphenicol, thiamphenicol, florfenicol (V*)</i>
1st- and 2nd- generation cephalosporins and cephamycins	e.g. <i>cefaclor, cefalexin, cefalotin, cefapirin, ceftazolin, cefoxitin, cefuroxime, cefalonium (V*)</i>
Lincosamides	e.g. <i>clindamycin, lincomycin, pirlimycin (V*)</i>
Penicillins (antistaphylococcal)	e.g. <i>cloxacillin, dicloxacillin, flucloxacillin, oxacillin, nafcillin</i>
Pleuromutilins	e.g. <i>retapamulin, tiamulin (V*), valnemulin (V*)</i>
Pseudomonic acids	e.g. <i>mupirocin</i>
Riminofenazines	e.g. <i>clofazimine</i>
Steroids antibacterials	e.g. <i>fusidic acid</i>
Streptogramins	e.g. <i>quinupristin/dalfopristin, pristinamycin, virginiamycin (V*)</i>
Sulfonamides	e.g. <i>sulfadiazine, sulfadimethoxine, sulfamethoxazole, trimethoprim, formsulfathiazole (V*), phthalylsulfathiazole (V*)</i>
Sulfones	e.g. <i>dapsone, aldesulfone</i>
Tetracyclines	e.g. <i>chlortetracycline, doxycycline, minocycline, oxytetracycline, tetracycline</i>

Antimicrobials classes	Antimicrobials compounds
<b>Important antimicrobials</b>	
Aminocyclitols	e.g. <i>spectinomycin</i>
Cyclic polypeptides	e.g. <i>bacitracin</i>
Nitrofurantoin	e.g. <i>furazolidone, nitrofurantoin, furaltadone (V*)</i>
Nitroimidazoles	e.g. <i>metronidazole, tinidazole, ornidazole</i>

Table adapted from WHO 4th revision 2013 (WHO, 2016).

(V\*): Antimicrobial compound authorised only for veterinary medicine only.

## Appendix B – Letter from the European Commission to the MSs (Ref. Ares (2014)3179090 – 26/09/2014)

EUROPEAN COMMISSION

HEALTH AND CONSUMERS DIRECTORATE GENERAL

Veterinary and International affairs

Director

### Subject: FEEDING OF CALVES WITH MILK CONTAINING RESIDUES OF ANTIBIOTICS

Dear Chief Veterinary Officer,

I refer to the discussions at the Standing Committee on the Food Chain and Animal Health, section animal nutrition, on 20 March 2014 in which the practice of feeding calves with milk containing residues of antibiotics was on the agenda.

As regards the legal framework it was clarified that:

- Regulation (EC) No 767/2009 on the placing on the market and use of feed<sup>1</sup> requires that feed must be safe and harmonises the conditions for the placing on the market and the use of feed, in order to ensure a high level of feed safety and thus a high level of protection of public health.
- Withdrawal periods are established to define from which point of time the animal product is fit for human consumption. In reverse, milk produced within the withdrawal period is not safe for human consumption.
- Directive 2002/32 on undesirable substances in animal feed<sup>2</sup> establishes limits for coccidiostats and histomonostats in feed for non-target species, but not for antibiotics.
- Regulation (EC) No 1069/2009<sup>3</sup> on animal by-products stipulates:
  - that raw milk, colostrum and products derived therefrom which are obtained, kept, disposed of or used on the farm of origin do not fall under the provisions of this Regulation (Art 2(2)(e)).
  - animal by-products derived from animals which have been submitted to illegal treatment as defined in Article 1 (2)(d) of Directive 96/22/EC or Article 2(b) of Directive 96/23/EC, are categorised as Category 1 materials (prohibited for placing on the market for animal feeding) (Art 8(c)).
  - animal by-products containing residues of authorised substances or contaminants exceeding the permitted levels as referred to in Article 15(3) of Directive 96/23/EC, are categorised as Category 2 materials (prohibited for placing on the market for animal feeding) (Art 9(c)).
- Feeding of calves with milk containing residues of antibiotics on farm is not harmonised at EU level and is subject to national rules (Art. 2(2e)).

During the meeting it was observed that two cases should be differentiated:

- Feeding calves with milk from cows treated during lactation within the withdrawal period;
- Feeding of calves with colostrum or milk from fresh milking cows treated with antibiotics during their dry period.

The potential risk for development of Antimicrobial Resistance due to the practice of feeding calves with milk that might contain residues of antibiotics has not been assessed so far by EFSA.

In order to collect information for a request to EFSA on a risk assessment and subsequently to evaluate the need of harmonisation management measures at EU level, I would appreciate if you could answer the questions in the Annex to this letter.

Please feel free to contact stakeholders in your country in order to gather those data.

We would appreciate to have your **replies** by email to SANCO G1 **SANCOCONSULT-G1@ec.europa.eu** by **30 November 2014**.

For further questions you can contact directly Wolfgang Trunk (tel.: 0032 2 2986375, email: wolfgang.trunk@ec.europa.eu)

cc:

Enclosure: Questions concerning the feeding of calves with milk containing residues of antibiotics

<sup>1</sup> OJ L 229, 1.9.2009, p. 1

<sup>2</sup> OJ L 140, 30.5.2002, p. 10

<sup>3</sup> OJ L 300, 14.11.2009, p. 1



## **Annex – Questions concerning the feeding of calves with milk containing residues of antibiotics**

- 1) Country concerned:
- 2) In your country, are there any (national or regional) rules, legislation or guidelines on practice of feeding calves on farm with milk that might contain antibiotic residues?
  - (a) If yes, please provide the reference and a description of the rules/legislation/guidelines.
  - (b) Provide also the result of the (official) controls on the implementation of these rules/legislation/guidelines and the body in charge of these controls.
- 3) Please provide information about:
  - (c) The percentage of herds in your country that receive regularly an antibiotic treatment (prophylactic treatment) at the beginning of or during the dry period.
  - (d) The percentage of herds in your country that are screened (diagnostic) before receiving a treatment at the beginning of or during the dry period.
  - (e) The percentage of cows that are treated with an antibiotic during lactation (systemic or intramammary antimicrobial treatment).
  - (f) The percentage of farms in your country that feed the milk from the treated cows to their calves?
  - (g) If the farm is not feeding their calves with milk from treated cows, what is the destination of the milk in these farms? Please specify the other ways of disposal of that milk in practice and if possible percentage of farms applying them.
- 4) Would you support an EU initiative for a harmonised approach on this issue?

## Appendix C – Antimicrobials used in dairy cows (Section 3.2)

Table C.1: Antimicrobials used in dairy cows

<u>Country</u>	Penicillins (benzylpenicillin, aminopenicillins, $\beta$ -lactamase stable penicillins)	1–2 gen Cephalosporins	3–4 gen Cephalosporin	Aminoglycosides	Amphenicols	Macrolides/Lincosamides	Fluoroquinolones	Tetracyclines	Aminocoumarins (Novobiocin)	Other antimicrobials	Penicillins and aminoglycosides	Penicillins and polypeptides	Penicillins and aminoglycosides and novobiocin	Amoxicillin and clavulanic acid	1–2 gen cephalosporins and aminoglycosides	Aminoglycosides and macrolides	Sulfonamides and trimethoprim	Aminoglycosides, polypeptides and tetracyclines	Other combinations	Reference	Comment
<b>Injectables</b>																					
<b>Lactation and dry period</b>																					
BE	27.3	0.1	36.4	0.1	–	14.9	8.7	4.6	–	–	4.2	–	–	0.5	–	1.7	–	0.9	Stevens et al. (2016)	Proportion (%) of total daily dosages in 57 dairy herds, 2012–2013	
CZ	29.0	0.6	33.4	10.0	–	5.0	5.0	4.0	–	8.0	–	–	–	–	–	5.0	–	–	EC Questionnaire 2015 – see Appendix B	Proportion (%) of sales data and questionnaire to veterinarians on use in cattle, 2012	
DK	76.0	–	0.3	2.5	–	1.2	–	11.3	–	0.1	–	–	–	–	–	8.6	–	–	DANMAP (2014)	Proportion (%) of total amount active compound used in cows and bulls, 2014	
NL	28.0	–	–	–	4.0	8.0	–	22.7	–	–	–	–	–	–	–	21.3	–	4.0	SDa (2015)	Proportion (%) of total daily dosages in dairy herds, 2014	
SE	84.2	–	0.2	–	–	3.3	6.9	6.9	0.4	–	–	–	–	–	–	5.0	–	–	Växa Sverige (2014)	Proportion (%) of total number of treatments of dairy cow affiliated to Växa Sverige, 2013	

<u>Country</u>	Penicillins (benzylpenicillin, aminopenicillins, $\beta$ -lactamase stable penicillins)	1–2 gen Cephalosporins	3–4 gen Cephalosporin	Aminoglycosides	Amphenicols	Macrolides/Lincosamides	Fluoroquinolones	Tetracyclines	Aminocoumarins (Novobiocin)	Other antimicrobials	Penicillins and aminoglycosides	Penicillins and polypeptides	Penicillins and aminoglycosides and novobiocin	Amoxicillin and clavulanic acid and aminoglycosides	1–2 gen cephalosporins and aminoglycosides	Aminoglycosides and macrolides	Sulfonamides and trimethoprim	Aminoglycosides, polypeptides and tetracyclines	Other combinations	Reference	Comment
UK (England and Wales)	6.2	13.9	5.9	0.1	–	27.4	5.5	6.9	–	–	20.7	–	–	9.4	–	–	3.9	–	–	Brunton et al. (2012)	Most frequently used antimicrobial (%), 557 dairy farms in England and Wales, 2010/11
<b>Intramammaries</b>																					
<b>Lactation and dry period</b>																					
DE	69.7	3.0	4.9	20.8	–	1.5	–	–	–	0.1	–	–	–	–	–	–	–	–	–	Hauck et al. (2014)	Proportion (%) of the amount of antimicrobials in intramammaries sold 2012
DK	59.8	20.9	3.9	–	–	–	–	–	–	–	16.6	–	–	–	–	–	–	–	–	DANMAP (2014)	Proportion (%) of Defined Animal Daily Dosages (DADD), 2014
<b>Lactation</b>																					
BE	5.1	14.9	31.0	–	–	0.5	–	–	–	–	11.4	–	–	–	–	–	–	–	1.3	Stevens et al. (2016)	Proportion (%) of total daily dosages for mastitis in 57 dairy herds, 2012–2013
IE	36.0	17.4	2.0	33.1	–	0.8	–	–	10.1	0.7	–	–	–	–	–	–	–	–	–	More et al. (2012)	Proportion (%) of the amount of antimicrobials sold, 2010
NL	52.2	–	2.0	2.8	–	1.9	–	–	–	–	2.5	–	–	–	–	–	–	–	–	Kuipers et al. (2016)	Proportion (%) of total daily dosages for mastitis in 94 dairy herds – 95% intramammaries, 2102

<u>Country</u>	Penicillins (benzylpenicillin, aminopenicillins, $\beta$ -lactamase stable penicillins)	1–2 gen Cephalosporins	3–4 gen Cephalosporin	Aminoglycosides	Amphenicols	Macrolides/Lincosamides	Fluoroquinolones	Tetracyclines	Aminocoumarins (Novobiocin)	Other antimicrobials	Penicillins and aminoglycosides	Penicillins and polypeptides	Penicillins and aminoglycosides and novobiocin	Amoxicillin and clavulanic acid	1–2 gen cephalosporins and aminoglycosides	Aminoglycosides and macrolides	Sulfonamides and trimethoprim	Aminoglycosides, polypeptides and tetracyclines	Other combinations	Reference	Comment
NL	80.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	19.9	SDa (2015)	Proportion (%) of total daily dosages in dairy herds, 2014
FR	19.8	8.9	15.2	1.0	–	0.1	–	–	–	–	2.8	4.0	–	11.4	–	–	–	36.8	–	ANSES ANMV (2015)	Data from the French National Antimicrobial sales Monitoring Programme, 2104
UK (England and Wales) Lactating	1.4	1.4	29.5	–	–	–	–	–	–	–	7.7	–	37.0	6.5	–	0.4	–	–	–	Brunton et al. (2012)	Most frequently used antimicrobials (%), 557 dairy farms in England and Wales, 2010/11
UK	25.7	8.1	7.0	43.4	–	0.6	–	–	14.3	–	0.6	–	–	–	–	0.3	–	–	–	UK-VARSS (2015)	Proportion of the amount of antimicrobials active ingredient sold, 2014
<b>Dry period</b>																					
BE	30.8	16.1	48.3	–	–	–	–	–	–	6.2	0.1	–	–	–	–	–	–	–	–	Stevens et al. (2016)	Proportion (%) of total daily dosages in 57 dairy herds, 2012–2013
FR	22.5	42.3	14.9	–	–	–	–	–	–	6.7	11.4	–	–	–	2.2	–	–	–	–	ANSES ANMV (2015)	Data from the French National Antimicrobial sales Monitoring Programme, 2014

<u>Country</u>	Penicillins (benzylpenicillin, aminopenicillins, $\beta$ -lactamase stable penicillins)	1–2 gen Cephalosporins	3–4 gen Cephalosporin	Aminoglycosides	Amphenicols	Macrolides/Lincosamides	Fluoroquinolones	Tetracyclines	Aminocoumarins (Novobiocin)	Other antimicrobials	Penicillins and aminoglycosides	Penicillins and polypeptides	Penicillins and aminoglycosides and novobiocin	Amoxicillin and clavulanic acid	1–2 gen cephalosporins and aminoglycosides	Aminoglycosides and macrolides	Sulfonamides and trimethoprim	Aminoglycosides, polypeptides and tetracyclines	Other combinations	Reference	Comment
IE	70.4	25.5	0.7	3.3	–	–	–	–	0.1	–	–	–	–	–	–	–	–	–	–	More et al. (2012)	Proportion (%) of the amount of antimicrobials sold, 2010
NL	43.9	–	–	–	–	–	–	–	–	56.1	–	–	–	–	–	–	–	–	–	Kuipers et al. (2016)	Proportion (%) of total daily dosages, 94 dairy herds, 2012
NL	37.3	–	–	–	–	1.7	–	–	–	–	–	–	–	–	–	–	–	61.0	–	SDa (2015)	Proportion (%) of total daily dosages in dairy herds, 2014
SE	–	–	–	–	–	–	–	–	–	100	–	–	–	–	–	–	–	–	–	Swedres-Svarm (2015)	Proportion (%) of the amount of antimicrobials sold, 2015
UK (England and Wales)	23.8	43.2	16.4	–	–	–	–	–	–	16.6	–	–	–	–	–	–	–	–	–	Brunton et al. (2012)	Most frequently used antimicrobial (%), 557 dairy farms in England and Wales, 2010/11
UK	62.1	24.3	–	3.3	–	–	–	–	–	10.3	–	–	–	–	–	–	–	–	–	UK-VARSS (2015)	Proportion of the amount of antimicrobials sold, 2014

–: no data.



## Appendix D – Answers received from Dr. Theo Lamm (GD Animal Health, 7400 AA Deventer, The Netherlands) regarding the report 'Resistentieontwikkeling bij jonge kalveren' from Gonggrijp et al (see below)

At your request we give you, to the best of our knowledge, answers to a number of questions related to the 'Scientific Opinion on the risk for the development of Antimicrobial Resistance (AMR) due to feeding of calves with milk containing residues of antibiotics' of the EFSA, as asked by Dr Lieve Herman. These answers are based on a report we published March this year, entitled 'Resistentieontwikkeling bij jonge kalveren' (Resistance development in young calves), by Maaïke Gonggrijp, Christian Scherpenzeel, Carlijn Kappert, Annet Heuvelink, Manon Holtstege, Ellen Nijenhuis, Sabine Tijs, Judith Keurentjes, Theo Lam en Annet Velthuis. This report was made available for you, but is written in Dutch. We currently are preparing a scientific publication 'ESBL-/AmpC-producing *Escherichia coli* in feces from young calves in relation to the presence of antimicrobial residues of dry cow antibiotics in colostrum'.

- 1) Can you provide us information on the antimicrobial content of colostrum samples after dry cow therapy?
- 2) Can you provide us information on the influence of the use of a teat sealer together with the antimicrobial injection on the antimicrobial content later on in the colostrum? Can you also document results on how the length of the dry period influence the amount of antimicrobial-positive colostrum samples?
- 3) Can you document the results obtained from cows treated with cloxacilline and benzylpenicilline during dry therapy in relation to the antimicrobials consumed and the excretion in the colostrum and the first bucket of transition milk?
- 4) Can you document on the shedding of antimicrobial-resistant *E. coli* and antimicrobial residues after feeding calves colostrum from cows treated during dry therapy?

**Can you provide us information on the antimicrobial content of colostrum samples after dry cow therapy?**

Colostrum samples from 118 cows from 114 farms were analyzed by a microbiological screening method followed by LC-MS confirmation for the presence of antimicrobial residues. Eighty-eight (75%) of the cows were intramammary treated with a dry cow therapeutic treatment with a  $\beta$ -lactam antibiotic and 30 (25%) with a combination of an aminoglycoside and a  $\beta$ -lactam antibiotic. The study was carried out in the Netherlands in 2013. In Table 1, the concentrations of the antibiotic residues found in the colostrum samples are summarized. 67% of the colostrum samples did not exceed the MRL concentration of any antimicrobial applied, 29% of the samples contained cloxacillin at a concentration above the MRL with a median concentration of 86.5  $\mu\text{g}/\text{kg}$  and a mean of 229.8  $\mu\text{g}/\text{kg}$ , 3% exceeded the MRL concentration of ampicillin and 1% of penicillin. These were all samples collected during the withdrawal period for human consumption of the antibiotic treatment of the cow.

**Table 1:** Concentrations (in  $\mu\text{g}/\text{kg}$ ) of the antimicrobial residues in 118 colostrum samples

Antimicrobial residue	Number of samples	Concentration ( $\mu\text{g}/\text{kg}$ ), median (mean)	MRL ( $\mu\text{g}/\text{kg}$ )	Samples above MRL (% of total)
Cloxacillin	48	86.5 (229.8)	30	34 (29%)
Neomycin	25	250 (297.6)	1500	0 (0%)
Ampicillin	8	3.9 (25.5)	4	3 (3%)
Penicillin	4	2.7 (26.8)	4	1 (1%)
Cefalexin	1	5 (5)	100	0 (0%)
Streptomycin	1	118 (118)	200	0 (0%)

**Can you provide us information on the influence of the use of a teat sealer together with the antimicrobial injection on the antimicrobial content later on in the colostrum? Can you also document results on how the length of the dry period influence the amount of antimicrobial-positive colostrum samples?**

In 23 of the 118 cows (19%) an internal teat sealer was used together with the intramammary antimicrobial treatment at drying off. In 11 of these cows (48%) antimicrobials were detected in the colostrum while this was the case in 67% of the colostrum samples from cows not treated with an internal teat sealer.

As expected, a longer dry period decreased the amount of samples in which antimicrobial residues were found (Table 2).

**Table 2:** Dry period length as related to the percentage of colostrum samples in which antimicrobial residues were found

Length of dry period	Number of samples	Percentage positive (%)
Shorter than 44 days	32	72%
Between 44 and 62 days	59	64%
Longer than 62 days	27	52%

**Can you document the results obtained from cows treated with cloxacillin and benzylpenicillin during dry therapy in relation to the antimicrobials consumed and the excretion in the colostrum and the first bucket of transition milk?**

In order to study the length of excretion of antimicrobial residues in milk and the amount of residues calves consumed, colostrum samples were collected from 28 cows on eight farms; 22 were treated with cloxacillin (500/600 mg) and 6 with a combination of neomycin (500 mg) and benzylpenicillin (1314 mg). None of these cows was treated with an internal teat sealer. Two of these cows did not excrete detectable antimicrobial residues at all during the first three days after calving.

The total amount of cloxacillin in the first 300 ml colostrum of 22 cows had a median of 0.005 mg (mean 0.017 mg, range 0–0.079 mg) and in the first bucket a median of 0.25 mg (mean 0.91 mg, range 0–8.03 mg) for the cows treated with cloxacillin.

The total amount of neomycin in the first 300 ml colostrum of 6 cows had a median of 0.05 mg (mean 0.08 mg, range 0–0.18 mg) and in the first bucket a median of 0.68 (mean 1.05 mg, range 0.14–3.57 mg).

Based on these results, it is concluded that non-feeding the first 300 ml of colostrum cannot be considered as a plausible control method to reduce the calves' uptake of antimicrobial residues from colostrum which result from dry cow therapy.

Assuming a calf consumed a total of 12 liters of colostrum during the first two days, it was estimated based on the results of 22 cows, that calves from cows treated with cloxacillin (500/600 mg) at drying off consumed during those two days a total amount of cloxacillin with a median of 0.34 mg cloxacillin (mean 1.16 mg, range 0–6.64 mg).

Based on the results of 6 cows treated with a combination of neomycin (500 mg) and benzylpenicillin (1314 mg), and assuming a consumption of 12 liters of colostrum during the first two days, it was estimated that calves from cows treated with neomycin at drying off consumed during the first two days a total amount of neomycin with a median of 1.14 mg (mean 2.02 mg, range 0–4.33 mg).

**Can you document on the shedding of antimicrobial-resistant *E. coli* and antimicrobial residues after feeding calves colostrum from cows treated during dry therapy?**

A study was performed on 10 conventional dairy herds in which 87 cow-calf couples were studied. Of these, 20 cows were dried off with 500 mg cloxacillin (4 quarters), 38 with 600 mg cloxacillin (4 quarters), and 29 were control cows that were not dried off with antibiotics. Calves were only fed colostrum from their own mother (cow-calf couples).

Colostrum samples were collected from the first bucket, and from the 2<sup>nd</sup>-5<sup>th</sup> milking (pooled). Fecal samples (swabs) were collected from the cows at the day of birth of the calf (day 1) and from the calves at day 1, 7, and 14.

Colostrum samples were tested for the presence of antimicrobial residues (microbiological screening and confirmation by LC-MS), ESBL/AmpC-producing *E. coli* (growth in the presence of 1 mg/L cefotaxime and confirmation by a combination disk diffusion test), total aerobic count (sheep blood agar) and coliform count (MacConkey agar). Fecal samples were tested for the presence of ESBL/AmpC-producing *E. coli*. Fecal samples collected from the calves on day 7 were also tested for the presence of antimicrobial residues.

In 60% (95% CI 47-73%) of the colostrum samples from cows treated with cloxacillin at drying off, cloxacillin residues were detected. The median concentration in the first bucket of colostrum the calves were drinking from was 148 µg/kg (mean 218 µg/kg), while it was 67 µg/kg (mean 94 µg/kg) in the second until the fifth milking. There was no difference between residue levels in the colostrum from the cows with a dry therapy of 500 or 600 mg cloxacillin. In the colostrum sample of one cow in the control group 6.3 µg/kg of amoxicillin was found, and in the colostrum of one cow dried off with cloxacillin, 2.0 µg/kg of benzylpenicillin was found.

In none of the fecal samples collected from 84 calves of cows treated with cloxacillin at drying off, cloxacillin residues were found. Fecal samples from two calves contained low concentrations of benzylpenicillin.

Culture results from colostrum revealed that two out of 173 samples (2%, 95% BI: 0-8%) tested positive for ESBL/AmpC-producing *E. coli*, both pool samples from the 2<sup>nd</sup>-5<sup>th</sup> milking, from cows with a dry therapy of cloxacillin. Most samples (>95%) had a total aerobic count of  $\geq 10^5$  cfu/mL, with approximately 50% being coliforms.

At day 1, from 10 of 86 fecal samples (12%; 95% CI 6-20%) ESBL/AmpC-producing *E. coli* were isolated. At day 7, from 32 of 84 (38%; 95% CI 28-49%) samples and at day 14 from 26 of 74 (35%; 95% CI 24-47%) samples. At day 7 and 14 significantly more positive samples were collected than at day 1.

No significant association was found between the dry cow treatment of the cows with cloxacillin (and the presence of antimicrobial residues in the colostrum) and the number of ESBL/AmpC-positive calf fecal samples or the amount of ESBL/AmpC-producing *E. coli* (cfu/g) excreted in the positive calf fecal samples. This was according to expectation, because cloxacillin is not selecting for ESBL/AmpC-producing *E. coli*.

No significant association was found between the total aerobic count of colostrum and the presence of ESBL/AmpC-producing *E. coli* in calf feces. In calves in which no ESBL/AmpC-producing *E. coli* was found in their feces, as compared to ESBL/AmpC-positive calves, a significantly higher total coliform count was found in the colostrum. This could be explained by the fact that a high number of other coliform bacteria is limiting the colonisation by ESBL/AmpC-producing *E. coli*.

In conclusion, in this study we found no effect on consumption of colostrum of cows that were treated with cloxacillin containing dry cow antibiotics, on the fecal excretion of ESBL/AmpC-producing *E. coli* by their calves.

## Appendix E – Methods and Criteria for susceptibility testing applied by the different studies considered in Sections 3.3 and 3.4

**Table E.1:** Methods and criteria used for antimicrobial susceptibility testing in referenced publications (Sections 3.3 and 3.4)

Reference	Year published	Testing performed and breakpoints determined using
Aust et al.	2013	CLSI, 2008 (M31–A3)
Beneragama et al.	2013	Plate count method, 50 mg/L cefazolin
Bengtsson et al.	2009	CLSI (M31-A2, formerly NCCLS, 2002)
Berge et al.	2006	CLSI (M31-A2, formerly NCCLS, 2002)
Botrel et al.	2010	French Society Microbiology (CA-SFM, 2007a,b)
BVL	2015	CLSI, 2013a (M100-S23)
Dolejska et al.	2008	Literák et al., 2007; CLSI (M31-A2, formerly NCCLS, 2002)
Duse et al.	2015	CLSI, 2013b (VET01-A4)
Duse et al.	2016	MICs by CLSI method, EUCAST ECOFFs used
Kalmus et al.	2011	CLSI (M31-A2, formerly NCCLS, 2002; M31-A3, CLSI, 2008)
Oliver et al.	2012	Not stated in paper, data from many different sources
Pereira et al.	2014	CLSI, 2008 (M31-A3)
Persson et al.	2011	CLSI, 2007 (M100-S17)
Randall et al.	2012	MICs by BSAC method, EUCAST ECOFFs used
Randall et al.	2014	MICs by BSAC method, EUCAST ECOFFs used
Roesch et al.	2006	CLSI (M31-A2, formerly NCCLS, 2002; M7-A6, formerly NCCLS, 2003)
Thomas et al.	2015	CLSI (Vet01-A4, CLSI, 2013b; Vet01-S2, CLSI 2013c; M100-S series)

BSAC: British Society of Antimicrobial Chemotherapy; CLSI: Clinical and Laboratory Standards Institute; ECOFF: Epidemiological Cut-Off value (EUCAST) to denote isolates as sensitive or resistant; EUCAST: European Committee on Antimicrobial Susceptibility Testing; NCCLS: National Committee for Clinical Laboratory Standards.