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The risk of development of antimicrobial resistance with the use of coccidiostats in poultry diets

Opinion of the Panel on Animal Feed of the Norwegian Scientific Committee for Food Safety

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Assessed and approved

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Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Summary

Key words: VKM, Risk assessment, Norwegian Scientific Committee for Food Safety, Norwegian Food Safety Authority, coccidiostats, antimicrobials, resistance, poultry

Background

Antimicrobials revolutionized human as well as animal medicine in the 20th century by providing effective treatment of diseases caused by pathogenic microorganisms. However, microorganisms have the ability to develop antimicrobial resistant strains. This occurs when microorganisms mutate or when resistance genes are exchanged between them. The use of antimicrobial drugs accelerates the emergence of drug-resistant strains. A priority is to safeguard the efficacy of antimicrobial drugs we depend on for treatment of infectious diseases in humans. Use of antimicrobials in food animals can create a source of antimicrobial resistant bacteria that can spread to humans both by direct contact and through the food supply.

Coccidiosis is an intestinal disease in animals caused by unicellular parasites called coccidia. As most of the damage of this infection is done by the time signs of the disease are widespread, preventive measures are preferred. Coccidiostats are animal feed additives used to prevent coccidiosis by inhibiting or killing coccidia. There are two major groups of coccidiostats; ionophores and non-ionophores, the latter also referred to as "non-ionophore coccidiostats" (but also called chemicals). One main difference between these groups is that ionophores also inhibit or kill some bacterial species, whereas non-ionophore coccidiostats do not. Consequently, some bacterial infections may also be controlled by ionophore coccidiostats, e.g. the poultry disease necrotic enteritis caused by the bacterium *Clostridium perfringens* (*C. perfringens*).

Eleven different coccidiostats have been authorised for use in the EU, both ionophores and non-ionophore coccidiostats. Norway has been exempted from the EEA Agreement in this field and has approved only five; all ionophores. The two ionophore coccidiostats currently used in Norway are narasin for broilers and monensin for turkeys.

Resistance to coccidiostats in coccidia and bacteria

Development of resistance in coccidia to all eleven coccidiostats has been described in the scientific literature, but the prevalence of resistance is unknown. Cross-resistance between various ionophore coccidiostats has also been shown, i.e. development of resistance to one ionophore may also render the coccidia resistant to another ionophore. Various rotation and shuttle programmes with exchange between ionophores and non-ionophore coccidiostats are believed to prevent or delay development of resistance in coccidia. In Norway, such

programmes will have little effect as long as only ionophores and not non-ionophore coccidiostats are approved for use.

Development of resistance against ionophores has also been observed in bacteria. In the Norwegian surveillance programme NORM-VET during the years 2002 - 2013, between 50 - 80 % of the tested flocks had narasin resistant faecal enterococci, which are bacteria that are part of the normal intestinal microbiota. However, the pathogenic bacterium *C. perfringens* has not been shown to be resistant against any ionophore. Cross-resistance in bacteria to more than one ionophore has been observed. In addition, a limited amount of data may indicate an association between narasin and resistance to the antibacterials bacitracin and vancomycin. As these are antibacterials used for treatment in humans, more research should be performed to validate these results. Non-ionophore coccidiostats, which do not have antibacterial effect, are not approved in Norway. If such coccidiostats were approved in Norway, coccidiostats with negligible probability of inducing resistance in bacteria would be available.

Human exposure to resistant bacteria and coccidiostats

Humans may theoretically be exposed to coccidiostat resistant bacteria from poultry in a number of ways, e.g. by handling live animals and their manure, through slaughtering and processing, and by preparation and consumption of poultry meat. Furthermore, bacteria of the human normal microbiota, which cover all skin and mucosal surfaces, might develop resistance if they are exposed to coccidiostats.

In this assessment, the probabilities of exposure are classified as: Negligible (extremely low), Low (possible, but not likely), Medium (likely), High (almost certain) and Not assessable.

The Panel has estimated the following probabilities of human exposure:

- Handling manure from coccidiostat fed poultry without sufficient risk-reducing measures entails a high probability of exposure to both resistant bacteria and coccidiostats. Without proper protection, the probability of exposure to coccidiostats is also high when handling coccidiostat premixes and feeds containing coccidiostats without proper protection measures. Various treatments, e.g. composting, of the manure may reduce the probability.
- The probability of exposure to resistant bacteria is medium for workers handling carcasses and raw meat on a daily basis if risk-reducing measures are not applied, whereas the probability of exposure to coccidiostats is negligible.
- For consumers, the probability of exposure to coccidiostats is negligible. The probability for exposure to resistant bacteria is also negligible in heat treated food since heat treatment kills the bacteria. The probability of exposure to coccidiostat resistant bacteria

is low to medium if handling raw meat without proper hygienic procedures, because raw meat may harbour resistant bacteria.

Risk-reducing measures will lower the probabilities.

However, little is known concerning the consequences of human exposure to coccidiostat resistant bacteria or to coccidiostats. There is little information in scientific literature indicating whether such bacteria in fact will colonize the human body, either transitionally or permanently. Furthermore, there is no information on the probability of exchange of resistance genes from transferred bacteria to bacteria of the human natural microbiota or to pathogens. Likewise, the Panel has no information on the level of exposure, e.g. the amount of coccidiostats and their metabolites, or the time period, necessary for the various bacteria to give rise to resistant variants. As coccidiostats are not used to treat infectious diseases in humans, concern of resistance is related to possible cross- or co-resistance with antibacterials considered important in human medicine. Such resistance has so far not been confirmed.

Use of therapeutic antibacterials for poultry

If the ionophore coccidiostats used in Norway are replaced by one or more non-ionophore coccidiostat with no antibacterial effect and no other changes are done, the coccidiostats used will no longer inhibit the bacterium *Clostridium perfringens*, which is the cause of necrotic enteritis. Over time this will likely to lead to a need for intermittent or continuous use of higher levels of therapeutic antibacterials due to increased incidence of this disease in poultry production. The magnitude of the increase is difficult to predict.

Alternatives to in-feed antimicrobials

Eradication from the birds' environment of coccidia causing coccidiosis is difficult to achieve because the coccidia form oocysts that survive outside the host and resist commonly used disinfectants.

Vaccination with non-pathogenic vaccines is now used increasingly in commercial Norwegian broiler farms, instead of in-feed coccidiostats. So far coccidiosis has not been reported as a problem in this transition process to broiler rearing without in-feed coccidiostats in Norway.

Non-antimicrobial feed additives with purported health-promoting benefits, i.e. acid-based products, probiotics, prebiotics, synbiotics, yeast-based products, plant-derived products, combinations of these, and other products have been developed and used in feed. These products have been tested for efficacy against coccidia with conflicting, non-consistent or non-convincing results. The majority of these products appear to target the bacterial microbiota rather than coccidia. The Panel has not assessed possible effects of other types of management changes.

Sammendrag på norsk

Antimikrobielle midler har revolusjonert medisinen i det 20. århundre ved å gi effektiv behandling mot infeksjonssykdommer. Mikroorganismer har imidlertid evnen til å utvikle resistens mot slike stoffer. Bruk av antimikrobielle midler bidrar til utvikling av resistente stammer. Det er viktig å forsøke å hindre resistens mot antimikrobielle stoffer som vi er avhengige av for behandling av smittsomme sykdommer hos mennesker. Bruk av antimikrobielle midler til matproduserende dyr kan skape en kilde til resistente bakterier som kan spres til mennesker både ved direkte kontakt og gjennom mat.

Koksidiose er en tarmsykdom hos dyr forårsaket av encellede parasitter som kalles koksidier. Ettersom det meste av skaden har skjedd før man rekker å oppdage infeksjonen, foretrekkes preventive tiltak. Koksidiostatika er tilsetningsstoffer i dyrefôr som brukes til å forebygge koksidiose ved å hemme eller drepe koksidier. Det er to store grupper av koksidiostatika; ionoforer og ikke-ionoforer, sistnevnte blir også referert til som «kjemiske koksidiostatika». En hovedforskjell mellom disse gruppene er at ionoforer også hemmer eller dreper enkelte bakteriearter. Det gjør ikke kjemiske koksidiostatika. Følgelig kan noen bakterielle infeksjoner også bli kontrollert av ionofore koksidiostatika, f.eks fjørfesykdommen nekrotiserende enteritt som forårsakes av bakterien *Clostridium perfringens* (*C. perfringens*).

Elleve ulike koksidiostatika har blitt godkjent for bruk i EU, både ionoforer og kjemiske koksidiostatika. Norge har blitt unntatt fra EØS-avtalen på dette feltet og har godkjent bare fem; alle ionoforer. De to ionofore koksidiostatika som brukes i Norge i dag er narasin for slaktekylling og monensin for kalkuner.

Resistens mot koksidiostatika i koksidier og bakterier

I den vitenskapelige litteraturen er det beskrevet resistens mot alle elleve koksidiostatika hos koksidier, men prevalensen av resistens er ukjent. Kryssresistens mellom ulike ionofore koksidiostatika er også vist, det vil si utvikling av resistens mot en ionofor kan også gi resistens mot en annen ionofor. Forskjellige såkalte «rotasjons-» og «skyttel-» programmer med veksling mellom ionoforer og kjemiske koksidiostatika er antatt å forhindre eller forsinke utvikling av resistens hos koksidier. I Norge vil slike programmer ha liten effekt ettersom bare ionoforer og ikke kjemiske koksidiostatika er godkjent for bruk.

Det er observert at bakterier også kan utvikle av resistens mot ionoforer. I det norske overvåkingsprogrammet NORM-VET ble det i årene 2002 – 2013 funnet at mellom 50 og 80 % av de testede slaktekylling- og kalkunflokkene hadde narasinresistente enterokokker, dvs bakterier som er en del av den normale tarmfloraen. Det har imidlertid ikke vært rapportert at sykdomsfremkallende *C. perfringens* som har vært resistente mot noen ionofor. Kryssresistens mellom ionoforer er observert hos bakterier, på samme måte som hos koksidier. I

tillegg kan en begrenset mengde data tyde på at det hos bakterier kan være en sammenheng mellom narasin og bacitracinresistens, og mellom narasin og vancomycinresistens. Ettersom bacitracin og vancomycin brukes til behandling av mennesker, bør det forskes mer for å bekrefte eller avkrefte disse resultatene. Kjemiske koksidiostatika som ikke har antibakteriell effekt har neglisjerbar risiko for å indusere resistens i bakterier. Kjemiske koksidiostatika er imidlertid ikke godkjent i Norge.

Human eksponering for resistente bakterier og koksidiostatika

Mennesker kan teoretisk sett bli utsatt for koksidiostatika-resistente bakterier fra fjørfe på en rekke måter, f.eks ved håndtering av levende dyr og gjødsel, ved slaktning og prosessering, og ved bearbeiding og inntak av fjørfekjøtt. Bakterier i normalefloraen hos mennesker, som dekker alle hud- og slimhinneoverflater, kan teoretisk utvikle resistens hvis de blir utsatt for koksidiostatika.

Sannsynlighet for eksponering klassifiseres på følgende måte: Neglisjerbar (ekstremt lav), Lav (lite sannsynlig, men mulig), Middels høy (sannsynlig), Høy (nesten sikker), Ikke klassifiserbar (ikke mulig å anslå nivå på sannsynligheten).

Faggruppen har konkludert med følgende sannsynligheter for at mennesker kan bli eksponert:

- Håndtering av gjødsel fra fjørfe gitt før tilsatt koksidiostatika uten tilstrekkelige risikoreduserende tiltak innebærer en høy sannsynlighet for eksponering for både resistente bakterier og for koksidiostatika. Behandling av gjødsel, f.eks. kompostering kan redusere sannsynligheten. Uten tilstrekkelig beskyttelse, er sannsynligheten for eksponering for koksidiostatika også høy ved håndtering av koksidiostatika-holdig premiks- blandinger for tilsetning til fôr.
- Sannsynligheten for eksponering for resistente bakterier er middels høy for arbeidere som tilnærmet daglig håndterer slakt og rått kjøtt uten risikoreduserende tiltak, mens sannsynligheten for eksponering for koksidiostatika er neglisjerbar.
- For forbrukerne, er sannsynligheten for eksponering for koksidiostatika neglisjerbar. Sannsynligheten for eksponering for resistente bakterier er også ubetydelig i varmebehandlet mat, men lav til middels høy hvis man håndterer rått kjøtt uten tilstrekkelige hygieniske rutiner.

Risikoreduserende tiltak vil redusere sannsynligheten for at mennesker utsettes for koksidiostatika og resistente bakterier.

Det finnes lite kunnskap om konsekvenser av eksponering av mennesker for resistente bakterier og for koksidiostatika. Det er lite informasjon i vitenskapelig litteratur om hvorvidt bakterier som er resistente mot koksidiostatika vil slå seg ned, enten kortvarig eller permanent, hos mennesker. Videre er det ingen informasjon om sannsynligheten for

overføring av resistensgener fra fjørfebakteriene verken til bakterier i menneskers naturlige bakterieflora eller til sykdomsfremkallende bakterier. Likeledes har faggruppen ingen informasjon om graden av eksponering, for eksempel mengden av koksidiostatika og deres nedbrytningsprodukter eller tidsperioden, som er nødvendig for at bakterier hos mennesker skal utvikle resistente varianter. Koksidiostatika brukes ikke ved behandling av smittsomme sykdommer hos mennesker. En eventuell risiko er derfor spesielt knyttet til om det er en sammenheng mellom koksidiostatika og resistens hos bakterier mot antibakterielle midler som er viktige i humanmedisin. Slik sammenheng har så langt ikke blitt bekreftet.

Bruk av terapeutiske antibakterielle midler hos fjørfe

Dersom ionofore koksidiostatika som brukes i Norge blir erstattet av et eller flere kjemiske koksidiostatika uten antibakteriell virkning, og ingen andre endringer gjennomføres, vil de koksidiostatika som brukes ikke lenger hemme bakterien *Clostridium perfringens* som kan gi nekrotiserende enteritt. Dette vil trolig over tid føre til et behov for intermitterende eller kontinuerlig bruk av høyere nivåer av terapeutisk antibiotika på grunn av økt forekomst av nekrotiserende enteritt. Hvor mye høyere forbruket av terapeutiske antimikrobielle midler kan bli er vanskelig å forutsi.

Alternativer til koksidiostatika i føret

Utrydding av koksidier i fuglenes miljø er ønskelig, men det er vanskelig fordi koksidiene danner oocyster som overlever utenfor verten og som f.eks. motstår vanlig brukte desinfeksjonsmidler.

Vaksinasjon med ikke-patogene vaksiner i stedet for koksidiostatika i føret brukes i økende grad i kommersielle norske slaktekyllig-besetninger. Så langt har ikke koksidiøse blitt rapportert å være et problem.

Ikke-antimikrobielle førtilsetninger med påståtte helsebringende fordeler, dvs. syrebaserte produkter, probiotika, prebiotika, synbiotika, gjærbaserte produkter, plantebaserte produkter, kombinasjoner av disse, og andre produkter har blitt utviklet og markedsføres. Disse produktene er testet for effekt mot koksidier med motstridende, ikke-konsistente eller ikke-overbevisende resultater. De fleste av disse produktene synes å være rettet mot bakteriefloraen i stedet for mot koksidier.

Faggruppen har ikke vurdert mulige effekter av andre former for driftsendringer.

Abbreviations and glossary

Abbreviations

AGP	Antibiotic growth promoters
DANMAP	The Danish Programme for surveillance of antimicrobial consumption and resistance in bacteria from animals, food and humans http://www.danmap.org/
EFSA	European Food Safety Authority, http://www.efsa.europa.eu/
EUCAST	The European Committee on Antimicrobial Susceptibility Testing, http://www.eucast.org/
FAO	Food and Agriculture Organization of the United Nations
FDA	U.S. Food and Drug Administration, http://www.fda.gov/
FINRES-vet	The Finnish antimicrobial resistance monitoring programme, http://www.evira.fi
LOD	Limit of Detection
LOQ	Limit of Quantification
MIC	Minimum Inhibitory Concentration, the lowest concentration of a given agent that inhibits growth of a microorganism under standard laboratory conditions. MIC data can provide information about the activity of antimicrobials
MRL	Maximum Residue Limit, the legal maximum concentration of a residue, resulting from the registered use of an agricultural or veterinary chemical
NFSA	Norwegian Food Safety Authority, http://mattilsynet.no/
NIPH	Norwegian Institute of Public Health Institute, http://www.fhi.no/
NORM-VET	The Norwegian monitoring programme on antimicrobial resistance in bacteria from food, feed and animals http://www.vetinst.no/eng/Publications/NORM-NORM-VET-Report
NVI	Norwegian Veterinary Institute, http://www.vetinst.no
OIE	The World Organization for Animal Health, http://www.oie.int/
PCR	Polymerase Chain Reaction
P95-exposure	The estimated exposure at the 95-percentile
SWARM	The Swedish Veterinary Antimicrobial Resistance Monitoring programme http://www.sva.se/en/antibiotics-/svarm-resistance-monitoring
TTGE	Temporal temperature gradient gel electrophoresis.
VMPs	Veterinary medicinal products
VKM	Norwegian Scientific Committee for Food Safety, http://www.vkm.no/
WHO	World Health Organization, http://www.who.int/en/

Glossary

Acquired resistance: is resistance to a particular antimicrobial agent, to which the microorganism previously was susceptible. The change is the result of genetic alteration in a microorganism due to mutation(s), the acquisition of foreign genetic material or a combination of both mechanisms.

Antibacterials: A general term for the drugs (antibiotics), chemicals, or other substances that either kill or inhibit the growth of bacteria.

Antibiotics: Traditionally; natural organic compounds produced by microorganisms, acting already in low concentration against other microorganisms. Today "antibiotics" comprises also synthetic and semisynthetic compounds with similar effects.

Antimicrobials: A general term for the drugs (antibiotics), chemicals, or other substances that either kill or inhibit the growth of microbes. The concept of antimicrobials applies to antibiotics, disinfectants, preservatives, sanitizing agents and biocidal products in general.

Antimicrobial resistance is defined as by Davison et al. (2000); a property of bacteria that confers the capacity to inactivate or exclude antibiotics, or a mechanism that blocks the inhibitory or killing effects of antibiotics.

1. The ability of a microorganism to withstand an antibiotic.
2. A relative term which provides an interpretation of the clinical significance of concentrations of an antimicrobial that inhibits the growth of an organism or kill it in laboratory systems (*in vitro*).
3. Either microbiological resistance, where resistant organisms are those that possess any kind of resistance mechanism or resistance gene, or clinical resistance, where a bacterium is classified as susceptible or resistant depending on whether an infection with that bacterium responds to therapy or not.

Coccidia: Microscopic, spore-forming, single-celled, eukaryote parasites of the subclass Coccidiasina. Unless otherwise noted, the term "coccidia" in this assessment is used to describe coccidia of the genus *Eimeria* which can infect poultry.

Coccidiostats: Agents added to animal feed (as for poultry) that serves to retard the life cycle or reduce the population of pathogenic coccidia to the point that disease is minimized and the host develops immunity.

Conjugation: Transfer of genetic material between different bacterial cells by direct cell-to-cell contact.

Co-resistance: Occur when the genes specifying different resistant phenotypes are located together on a mobile genetic element (like plasmid, transposon, integron).

Cross-resistance: Resistance occurring when the same or similar mechanism(s) of resistance applies to different antimicrobials.

Cut-off values: Microbiological cut-off values are defined for the purpose of distinguishing resistant from susceptible strains. Epidemiological cut-off values are used for surveillance, whereas clinical cut-off values are used to predict the effect of an antimicrobial in a clinical setting. More explanation in NORM-VET report 2014.

Eukaryotes: Any organism having as its fundamental structural unit a cell type that contains specialized organelles in the cytoplasm, a membrane-bound nucleus enclosing genetic material organized into chromosomes, and an elaborate system of division by mitosis or meiosis, characteristic of all life forms except bacteria, blue-green algae, and other primitive microorganisms. Coccidia are eukaryotes.

Genetic transmission of antimicrobial resistance: Between microbes is both 'vertical' (new generations inherit resistance genes) and 'horizontal' (bacteria share or exchange sections of genetic material with other bacteria, including bacteria of other species). Environmental spread of antimicrobial resistance takes place as the microbes move from place to place; via animals, persons, food and feed, water and wind, airplanes and cars, etc.

Gram-negative bacteria: Most bacterial species can be differentiated into two large groups (Gram-positive and Gram-negative) based on the physical properties of their cell walls by bacteriological laboratory technique called Gram staining (developed by Hans Christian Gram in 1884).

Intrinsic resistance: The inherent or innate ability of a microbial species to resist a particular antimicrobial agent. Intrinsic resistance occurs in organisms that have not been susceptible to that particular antimicrobial agent.

***In vitro*:** In an artificial environment, such as a test tube; not inside a living organism (Latin for "in glass").

***In vivo*:** Being or occurring within a living organism or in a natural setting.

Microbiota: Collective term for microflora (i.e., any type of microorganisms) that may be found within a given environment.

Minimum Inhibitory Concentration (MIC): The lowest concentration of a given agent that inhibits growth of a microorganism under standard laboratory conditions. MIC data can provide information about the activity of antimicrobials.

Pathogen: An agent that is capable of causing disease. Bacteria and coccidia may be pathogens.

Pathogenic: Capable of causing disease.

Percentile: A common used term for visualising the low, medium and high occurrences of a measurement (e.g. acrylamide intake) by splitting the whole distribution into one hundred equal parts. The 95-percentile is the value (or score) below which 95 percent of the observations may be found.

Prebiotics: Non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and / or activity of a limited number of bacterial species already resident in the colon, and thus attempt to improve host health.

Probiotics: Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host.

Prokaryot: Any organism in which the genetic material is in a single DNA chain that is not enclosed in a nucleus. Bacteria are prokaryotes.

Rotation programme: A programme where the coccidiostats used are changed at regular intervals.

Shuttle programme: Two or more coccidiostats are used during the grow-out of a poultry flock, e.g. one for starter and others for grower and finisher.

Susceptibility: Describes the degree to which a target microorganism is affected by an antimicrobial agent.

Synbiotic: Refers to nutritional supplements combining a mixture of probiotics and prebiotics in a form of synergism. The concept of synbiotics was proposed to “characterise some colonic foods with interesting nutritional properties that make these compounds candidates for classification as health-enhancing functional ingredients” (Gibson and Roberfroid, 1995).

Therapeutic antibiotics: Antimicrobials used to treat clinical diseases caused by microbes, as opposed to antimicrobials used for prevention.

Background as provided by the Norwegian Food Safety Authority

Coccidiostats are authorised for use as an additive in feed for chickens and turkeys. Eleven different coccidiostats have been authorised for use in the EU. Norway has been exempted from the EEA Agreement in this field and has approved only five. The reason is that these five were already in use in Norway when the EEA Agreement was signed in 1994. In the light of the extent of the production of poultry for slaughter at the time and Norway's restrictive approach to feed antibacterials and coccidiostats, exemption was granted from approval of the other coccidiostats that are authorised in the EU.

Norwegian Food Safety Authority has discussed with the feed industry, relevant organisations and institutions to establish whether it would be appropriate to ask for the exemption for coccidiostats under the EEA Agreement to be repealed. The same enquiry was repeated to the Norwegian Ministry of Agriculture and Food in 2011. This would simplify our regulations and unify all the regulations concerning limit values for coccidiostats contents in feed and limits for permitted residual values in food. The feedback from the industry was largely that there was unlikely to be a professional basis for more/other coccidiostats than those that are currently permitted, but that it would be completely safe to use all those that had been authorised by the EU. Nevertheless, the Ministry of Agriculture and Food did not see the need for an amendment of the EEA Agreement on this matter.

The EU intended to ban coccidiostats as a feed additive with effect from 2012. Trials were conducted in a number of European countries to identify alternative measures to reduce or prevent coccidiosis in poultry. The conclusion was that neither vaccination nor other measures tested could replace the use of coccidiostats in feed. Coccidiostats as a preventive measure to manage coccidiosis in commercial poultry farming are necessary for reasons of both animal health and animal welfare. The proposal to ban coccidiostats as a feed additive was therefore put on ice. Authorised coccidiostats are currently being evaluated by the EFSA for re-authorisation as and when the current authorisations expire. With regard to the coccidiostats approved in Norway, we will comply with the EU regulations in full.

Recently concern has been raised that the use of coccidiostats in feed could result in the development of bacteria with antimicrobial resistance in both humans and animals. It is therefore necessary to evaluate whether, and potentially how, the use of coccidiostats in feed for poultry can contribute to an increased occurrence of bacteria with antimicrobial resistance. It would also be appropriate to evaluate whether there are differences between the various coccidiostats and the status of the use of those approved in Norway compared

with the others that have been authorised for use in the EU with regard to the development of potential antimicrobial resistance.

The development of antimicrobial resistance is an increasing problem. Given the suggestions that the widespread use of coccidiostats as a feed additive for poultry might be a contributing factor to this development, it would be desirable to evaluate the 11 EU-approved preparations with regard to potential development of antimicrobial resistance.

Narasin is the active ingredient most commonly used in Norway and dominates broiler chicken production. In addition to acting as a coccidiostat, narasin has also been found to have an antimicrobial effect on gram-positive bacteria including enterococci, staphylococci and *C. perfringens*. The latter may cause necrotic enteritis in chicken, and narasin in feed has a preventative effect. Gram-negative bacteria such as *E. coli* are resistant to narasin. For such reasons the use of narasin should be assessed separately.

Terms of reference as provided by the Norwegian Food Safety Authority

1. To what extent can the 11 EU-authorized coccidiostats induce resistance and/or cross-resistance in bacteria?
2. To what extent can the 11 EU-authorized coccidiostats induce resistance in coccidia?
3. Are there advantages or disadvantages associated with the development of resistance in bacteria under the current practice in Norway with only five coccidiostats available compared to the 11 EU authorized coccidiostats?
4. Are there advantages or disadvantages associated with the development of resistance in coccidia under the current practice in Norway with only five coccidiostats available compared to the 11 EU authorized coccidiostats?
5. What are the risks of antibacterial resistance being developed in and/or transferred to people (workers) handling coccidiostat preparations, feed, poultry, poultry meat or manure from poultry production using coccidiostat feed additives? If so, what risk-reducing measures are available?
6. What are the risks of antibacterial resistance being developed in and/or transferred to people (consumers) handling and eating meat from poultry production using coccidiostat feed additives?
7. What are the risks of an increase in the therapeutic use of antimicrobials in poultry production under current production practices if coccidiostats with antibacterial effects are replaced by coccidiostats without such effects?
8. Do alternative measures exist that can be employed to reduce the risk of coccidiosis in broiler chickens as effectively as coccidiostats?

Terms of Reference in Norwegian

1. I hvilken grad har de 11 EU-godkjente koksidiostatika evne til å kunne fremme resistens og/eller kryssresistens hos bakterier?
2. I hvilken grad har de 11 EU-godkjente koksidiostatika evne til å fremme resistens hos koksidier?
3. Er det fordeler eller ulemper for utvikling av resistens hos bakterier med dagens praksis i Norge, der det er betydelig færre koksidiostatika (bare 5 preparater) å velge blant, sammenlignet med EU der 11 koksidiostatika er godkjent?
4. Er det fordeler eller ulemper for utvikling av resistens hos koksidier med dagens praksis i Norge, der det er betydelig færre koksidiostatika (bare 5 preparater) å velge blant, sammenlignet med EU der 11 koksidiostatika er godkjent?
5. Er det risiko for utvikling og/eller overføring av antibakteriell resistens hos mennesker som håndterer koksidiostatika-preparater, fôr, fjørfe, fjørfekjøtt eller gjødsel som følge av at fôr tilsatt koksidiostatika er brukt i fjørfeproduksjonen? I så fall hvilke risikoreduserende tiltak finnes?
6. Er det risiko for utvikling hos og/eller overføring av antibakteriell resistens til mennesker som håndterer og spiser fjørfekjøtt som følge av at fôr tilsatt koksidiostatika er brukt i fjørfeproduksjonen?
7. Er det risiko for økning i terapeutisk bruk av antibiotika i fjørfeproduksjonen, dersom en tilsetter koksidiostatika uten antibakteriell effekt i fôret?
8. Hvilke tiltak er aktuelle for å redusere risikoen for koksidiøse i slaktekyllingproduksjonen, slik at bruk av koksidiostatika blir unødvendig?

Assessment

1 Introduction

1.1 Why is antimicrobial resistance a concern?

An antimicrobial agent is a compound that can destroy or inhibit the growth of microorganisms. Both antibacterials and coccidiostats are antimicrobials, intended for use primarily against bacteria and coccidia, respectively.

Antimicrobials revolutionized human as well as animal medicine in the 20th century by providing effective treatment of diseases caused by pathogenic microorganisms. However, microorganisms have the ability to develop antimicrobial resistant strains. The evolution of such strains is a natural phenomenon. This occurs when microorganisms replicate themselves erroneously causing mutations or when resistance traits are exchanged between them. The use of antimicrobial drugs accelerates the emergence of drug-resistant strains (<http://www.who.int/mediacentre/factsheets/fs194/en/>).

Use of antimicrobials in food animals can create a source of antimicrobial resistant bacteria that can spread to humans by contact with such bacteria, either directly from the animals or through the food supply (Figure 1.1-1). Emergence of resistance in non-pathogenic bacteria does not pose an immediate threat to humans and animals. However, they may transfer their resistance genes to pathogenic bacteria and thereby reduce the possibilities of treating and managing infectious diseases. It is therefore important to keep the prevalence of resistant strains, pathogenic and non-pathogenic, as low as possible.

WHO has classified antimicrobials according to their importance for human medicine (http://www.who.int/foodsafety/areas_work/antimicrobial-resistance/cia/en/). Antimicrobials classified as “critically important” should be reserved for treatment of severe infections in humans. A priority is therefore to safeguard the efficacy of antimicrobial drugs we depend on for treatment of infectious diseases in humans. Improved management of the use of antimicrobials in food animals on a world-wide basis, particularly reducing those critically important for human medicine, is recognized as an important step towards preserving the benefits of antimicrobials for people.

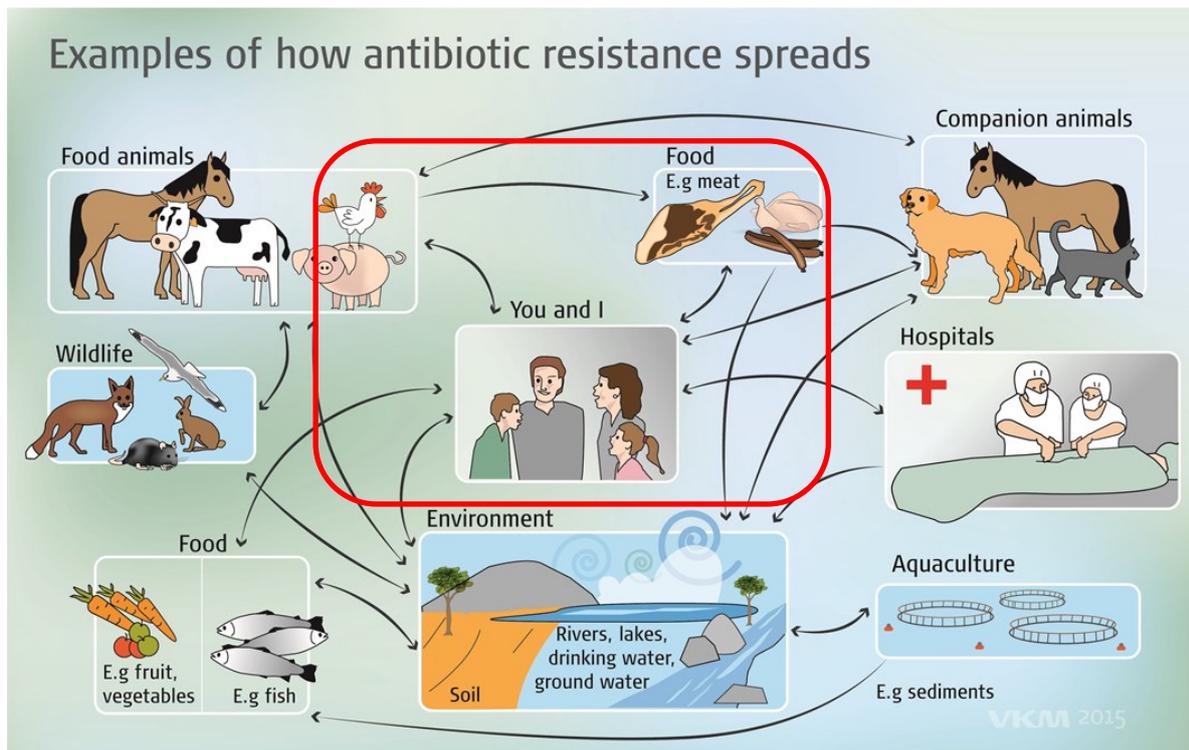


Figure 1.1-1 Spread of resistant bacteria between different compartments in the society. The present assessment includes humans, poultry and poultry meat.

1.2 Definition, development and spread of antimicrobial resistance

Resistance against antimicrobials can be either intrinsic or acquired. Intrinsic resistance is the innate ability to resist activity of a particular antimicrobial agent in an organism that has never been susceptible to that particular drug. Acquired resistance can be defined as the capacity of a species or strain of microorganism to survive exposure to drug formerly effective against it, due to genetic mutation and selection for and accumulation of genes conferring protection from the agent (<http://www.merriam-webster.com/medical/resistance>). In this assessment the expression "resistance" refers to acquired resistance, unless specifically stated otherwise.

The difference between "resistant" and "non-resistant" is not always clearly defined, and the terms "reduced susceptibility" or "reduced sensitivity" can therefore sometimes be scientifically more correct than "resistant". However in this assessment, only the terms "resistance" and "resistant" will be used to ease readability.

When microorganisms are exposed to an antimicrobial, any cells with random mutations in the DNA rendering them resistant to this antimicrobial will have a proliferative advantage

(Figure 1.2-1). Consequently, large numbers of this strain may rapidly arise. In the case of bacteria, the new resistant strain may also spread the resistance genes by horizontal gene transfer to other strains, from the same species but also from other bacterial species. Of particular concern is the development and spread of antibacterial resistance in pathogenic bacteria, and especially when they become resistant to multiple antibacterials.

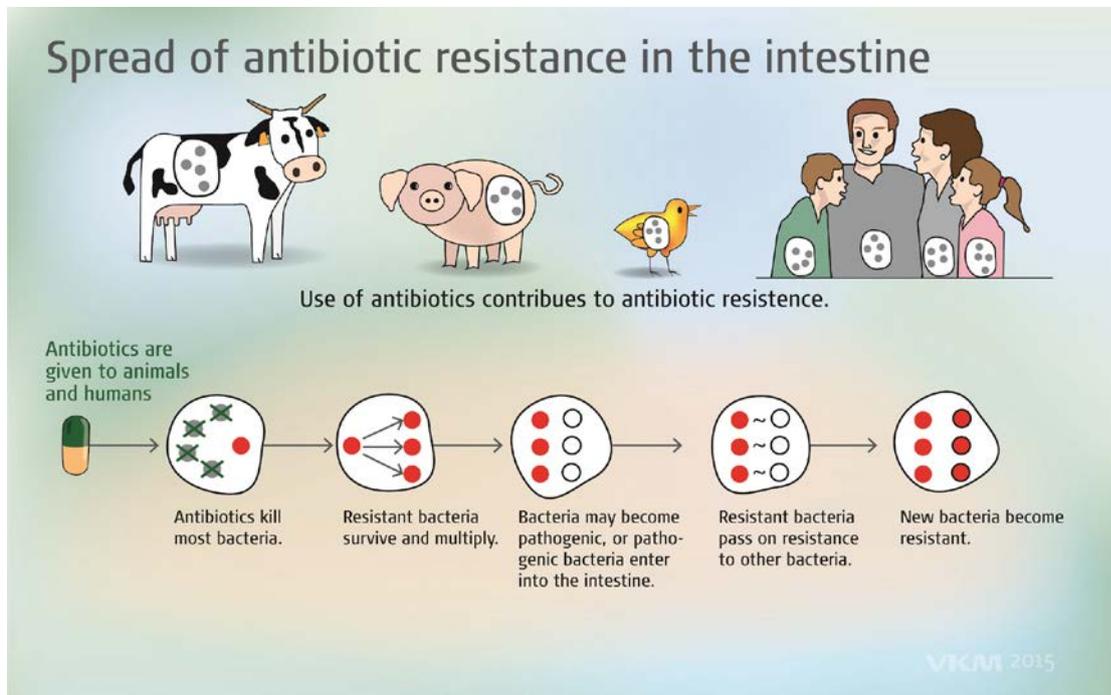


Figure 1.2-1 Development and spread of antibacterial resistance in the intestine of both humans and animals.

Exposure to one antimicrobial may also render the microorganism resistant to other antimicrobials through cross-resistance and co-resistance. Cross-resistance occurs when the bacteria can use the same resistance mechanism against several antibacterials. Co-resistance can occur when mechanisms encoding resistance are genetically linked. Bacterial resistance genes are frequently contained in larger, often transferable, genetic elements, and as such may be 'linked' to other, unrelated resistance genes. In such cases, multiple resistance genes may be transferred in a single event, meaning that selection for one resistance gene will also select for the other resistance gene(s).

Co-resistance between antibacterials and disinfectants has also been observed, e.g. for quaternary ammonium compounds (qac) and sulphonamide in Gram-negative bacteria (Sidhu et al., 2002). Furthermore, use of zinc and copper in animal feed has been linked to development of antibacterial resistance in bacteria. Resistance to zinc is often linked with resistance to methicillin in staphylococci, and resistance to is often associated with resistance to antibacterial drugs like macrolides and glycopeptides (e.g. vancomycin) (Yazdankhah et

al., 2014). The cross- and co-resistance between coccidiostats and disinfectant agents as well as metals with antibacterial properties have not been evaluated in this assessment.

Experiments suggest that the use of antibacterial agents may also indirectly be associated with development of resistance through disturbances in the ecologic balance of the intestine, as indicated in Norwegian studies on horses, dogs and calves (Gronvold et al., 2010a; Gronvold et al., 2010b; Gronvold et al., 2011). Furthermore, in an overview article by Rice (2013) the author suggests that the spread of glycopeptide resistance in enterococci is promoted by the administration of non-glycopeptide.

1.3 General information on coccidia

Coccidia (Coccidiasina) constitute a subclass of microscopic, spore-forming, single-celled, eukaryote parasites with a complex structure and life cycle. They are obligate intracellular parasites, meaning that they must reproduce within a host cell. Almost all livestock can be affected by different types of coccidia. Poultry are infected by coccidia of the genus *Eimeria*. These are generally host-specific, and the different species parasitize specific parts of the intestinal tract. There are seven different *Eimeria* species that infect chicken – *E. acervulina*, *E. brunette*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella* – and six that infect turkey – *E. adenoides*, *E. dispersa*, *E. gallopavonis*, *E. innocua*, *E. meleagridis*, *E. meleagrimitis*, and *E. subrotunda*. There are large variations in pathogenicity of the different *Eimeria* species.

Unless otherwise noted, the term “coccidia” in this assessment is used to describe coccidia of the genus *Eimeria* which can infect poultry.

The life cycle of *Eimeria* in poultry (Figure 1.3-1) takes place partly outside and partly inside the host, in which the latter is where both asexual and sexual stages of reproduction occur. It begins when active oocysts are picked up by the bird and swallowed. An oocyst is a capsule with a thick wall protecting the parasite eggs. Each oocyst has four sporocysts in it, and each sporocyst contains two sporozoites. In the digestive tract, the eight sporozoites are released from the oocyst, and they move into the epithelial cells lining the digestive tract where they develop into trophozoites. Within the host cells, the trophozoites undergo asexual reproduction to produce merozoites, which when released from the damaged epithelial cell can in turn penetrate other healthy epithelial cells causing further tissue damage. There may be several generations of asexual multiplication. However, this stage is self-limiting and eventually stops. A sexual stage then occurs at which the merozoites in host cells differentiate into either male (microgamonts) or female (macrogamonts) forms. The microgamonts divide to form microgametes, which fertilize the macrogamonts leading to the development of oocysts which are shed in the faeces.

In the environment outside the host, fresh oocysts are not infective until they have sporulated. Under optimal conditions (20 – 30 °C with adequate moisture and oxygen), this requires 1–2 days. Once sporulated, the oocyst remains infective for months if protected from very hot, dry, or freezing conditions. Chickens pick them up by pecking on the ground or litter used for bedding in the house.

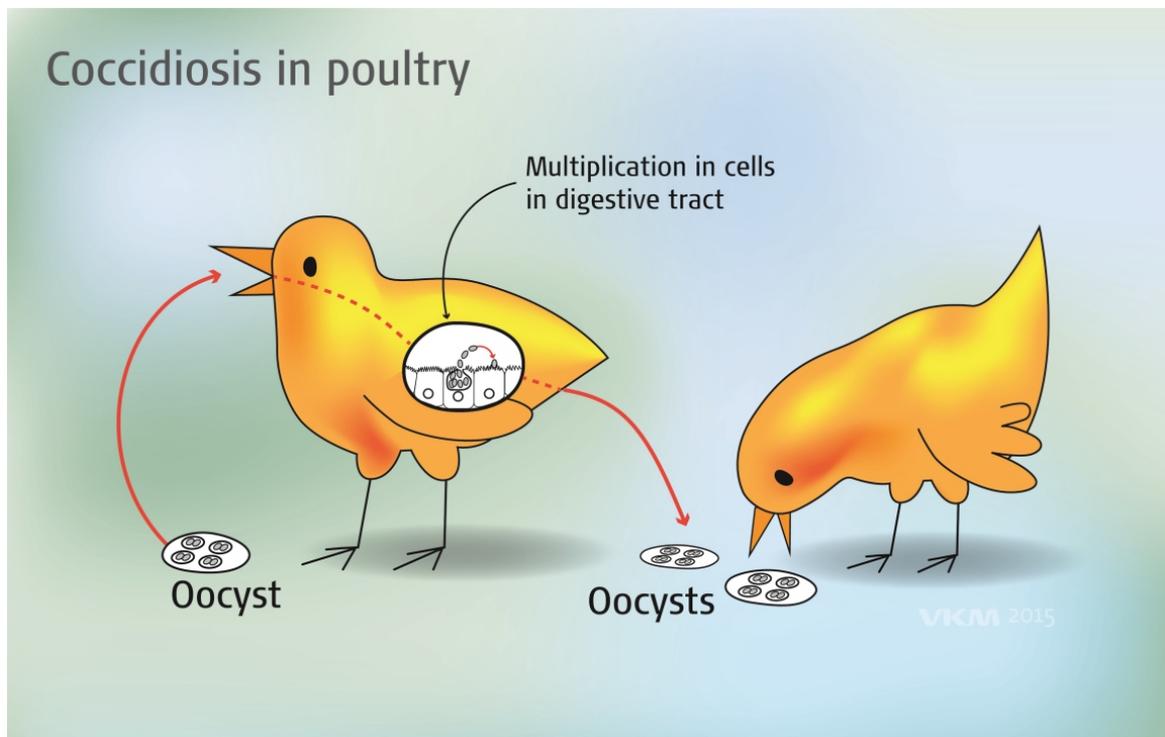


Figure 1.3-1 The life cycle of coccidia (*Eimeria*) in poultry. For more explanation, see text in chapter 1.3.

1.4 Coccidiosis in poultry

Coccidiosis is a term used for intestinal health problems caused by several species of protozoan parasites of the genus *Eimeria*. Damage to the host is caused by the reproduction of the parasite in the intestinal epithelial cells. Pathogenicity is influenced by host genetics, nutritional factors, concurrent diseases, age of the host, and species of the coccidium (http://www.merckmanuals.com/vet/poultry/coccidiosis/overview_of_coccidiosis_in_poultry.html).

In chicken, signs of coccidiosis range from decreased growth rate to a high percentage of visibly sick birds with severe diarrhoea, and high mortality. Feed and water consumption are depressed. *Eimeria necatrix* and *Eimeria tenella* are the most pathogenic in chickens. Mild infections by *Eimeria* species, which would otherwise be classified as subclinical, may cause depigmentation and can potentially lead to secondary infection, particularly by *Clostridium* spp. Infection (for more details see section 1.6).

Common signs of coccidiosis in infected turkey flocks include reduced feed consumption, rapid weight loss, droopiness, ruffled feathers, and severe diarrhoea. Wet droppings with mucus are common. Clinical infections are seldom seen in poults >8 wk old. Morbidity and mortality may be high.

Most of the damage is done by the time signs of coccidiosis are widespread in the flock. This is the rationale behind the preventive medication (in-feed coccidiostats) that is common practice in conventional broiler and turkey rearing.

1.5 General information on bacteria

Bacteria are microscopic, single-celled organisms. They are prokaryotes, meaning that the DNA is not enclosed in a nuclear membrane, but resides in a nuclear region of the cell. Bacterial cells multiply by cell division, which may occur as often as approximately every twenty minutes under optimal conditions.

Bacteria may well be the most successful life form on Earth when it comes to survival. Bacterial ancestors appeared approximately 3.5 billion years ago. Today there are estimated to be approximately 5×10^{30} bacteria on Earth (Whitman et al., 1998), forming a biomass which exceeds that of all plants and animals and appearing in almost any man-made or natural environment, including in soil, water and the atmosphere, as well as on and inside living organisms.

The key to such success is the ability of bacteria to undergo rapid genetic adaptation to changing environments. This is mediated by mutations in the DNA and by horizontal gene transfer, which is defined as the exchange and stable integration of genetic material

between different strains or species (Doolittle, 1999). Various mechanisms for antimicrobial resistance are demonstrated in Figure 1.5-1, and mechanisms for gene transfer are described in Figure 1.5-2.

The microbiota associated with humans and animals represent a complex assemblage of microorganisms covering all three domains of life (Bacteria, Archaea and Eukarya) (Ley et al., 2008). All body sites are colonized, with the lower gastrointestinal tract being the most densely populated. In number of cells, the microbiota generally outnumbers the host by a factor of 10. Therefore, the gut microbiota can be considered an organ in itself (O'Hara and Shanahan, 2006).

Most bacterial species can be differentiated into two large groups – Gram-positive and Gram-negative – based on the physical properties of their cell walls by a bacteriological laboratory technique called Gram staining developed by Hans Christian Gram in 1884. Due to the thicker cell wall of the Gram-negative bacteria, these are in general more inherently resistant to antibacterials than the Gram-positive bacteria. Most pathogenic bacteria are Gram-negative.

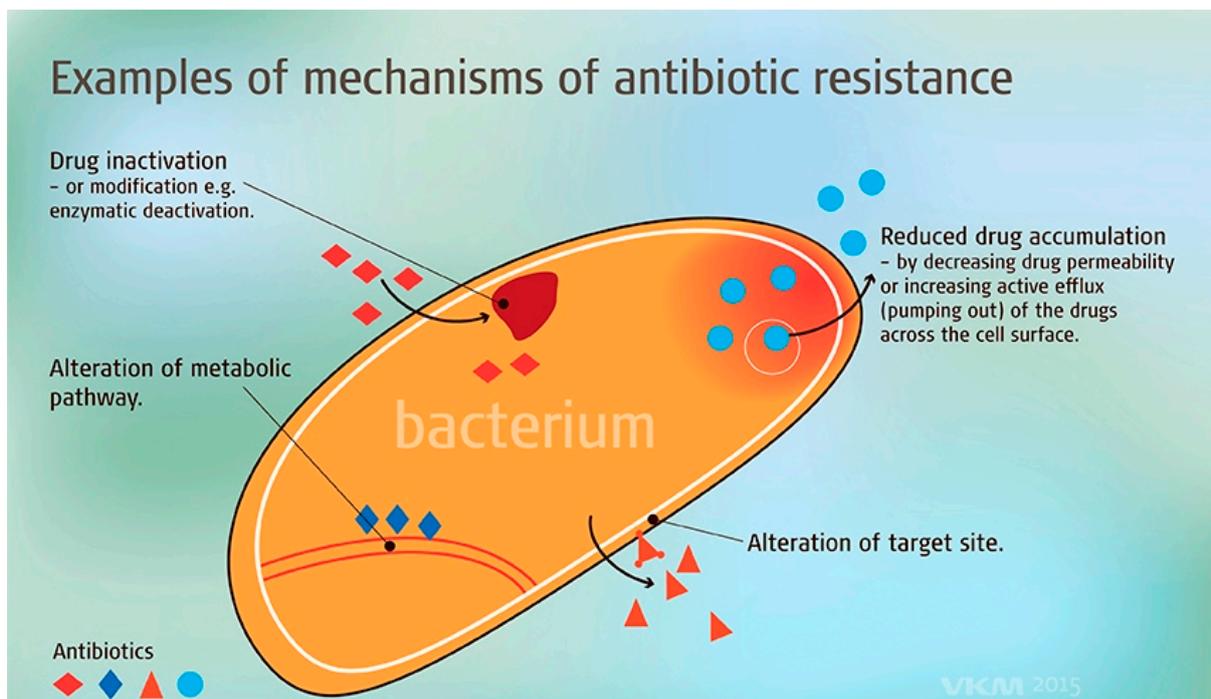


Figure 1.5-1 Examples of mechanisms used by bacteria to resist antibacterials.

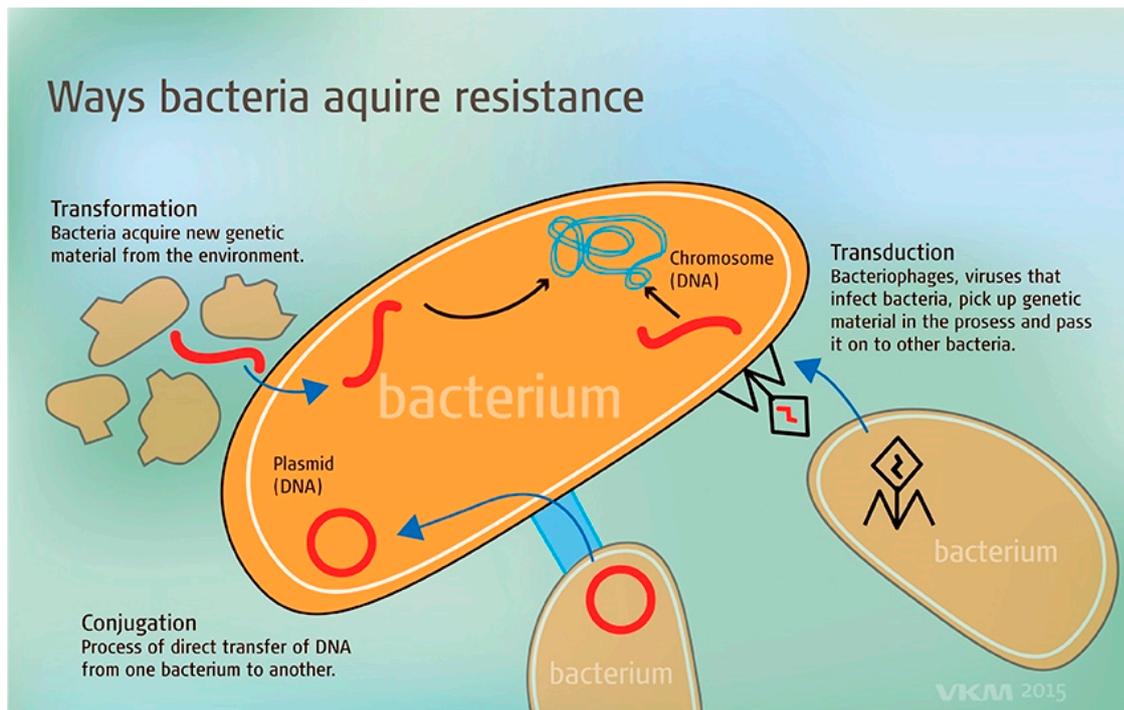


Figure 1.5-2 Main mechanisms used to transfer resistance genes between bacteria.

1.6 Relevant bacterial infections in poultry

Necrotic enteritis is caused by the intestinal bacterium *Clostridium perfringens* (*C. perfringens*). This bacterium is commonly found in caecal contents, but will under certain conditions start proliferating and producing toxins in the small intestine where damage is inflicted. Coccidial infection is considered the most important predisposing factor for necrotic enteritis. The interplay between coccidia and *C. perfringens* is therefore very important. As for coccidiosis, most of the damage is done by the time the clinical signs are widespread. The clinical form of necrotic enteritis causes diarrhoea and decreased appetite or anorexia, as well as signs associated with lethargy, such as ruffled feathers, relative immobility, and depression. Mortality rates can be up to 50%. In subclinical forms of necrotic enteritis there is no marked increase or peak in mortality and no clinical signs are present. Impaired production performance (growth and feed utilisation) is a main consequence of both subclinical and clinical forms of coccidiosis and necrotic enteritis, as are poor quality of the litter bedding and adverse environmental conditions to the detriment of the birds' welfare. Treatment requires the use of antibacterials, specifically penicillin, which is of importance to human medicine and its use should therefore be kept to a minimum in order to maintain the efficacy. Hence, prevention of both diseases is considered a better course of action than treatment. The Norwegian broiler industry has experienced three necrotic enteritis epidemics in the early 1970's, mid-80's and mid-90's. The disease has been under control since the mid-90's when narasin was introduced as an in-feed coccidiostat. Narasin is an ionophorous

coccidiostat which also exerts effect against Gram-positive bacteria including *C. perfringens*, the causative agent of necrotic enteritis.

Gizzard erosion and ulceration syndrome (GEU) has been identified as a health problem in chickens since the early 1930's. The gizzard mucosa becomes inflamed. Several causative factors have been suggested by Gjevre et al. (2013), including nutritional deficiencies, a toxin (gizzerosine) found in fish meal, and infection by *C. perfringens* and certain strains of Fowl adenovirus 1 (FAdV-1). All of these factors may play a part in the pathogenesis, but recent research suggests that FAdV-1 is an important primary pathogen (Grafl et al., 2012; Ono et al., 2003). The severity of GEU has been shown to be associated with intestinal counts of *C. perfringens* (Novoa-Garrido et al., 2006). GEU may therefore be a predisposing factor for necrotic enteritis. The ionophorous coccidiostat narasin does not prevent the emergence of GEU, but does reduce the severity of the gizzard lesions (Kaldhusdal et al., 2012). The majority of GEU cases are subclinical and impair production performance (Grafl et al., 2012), like most cases of coccidiosis and necrotic enteritis.

Enterococcus hirae is an intestinal bacterium that has been associated with growth depression, septicemia, right-sided endocarditis, focal brain necrosis (encephalomalacia) and osteomyelitis in broilers. In many cases encephalomalacia causes locomotion problems and mortality towards the end of the first week after hatch, whereas the endocarditis is more typically found later on in the grow-out period. *E. hirae* shows biochemical characteristics that are between *Enterococcus faecium* and *Enterococcus durans* (Thayer and Waltman, 2013). Clinical disease associated with *E. hirae* has been detected in Norwegian broilers offered in-feed narasin. This disease emerged as a problem in Norwegian broilers around year 2000, but does not appear to have been of major significance during recent years.

1.7 Antimicrobial resistance testing

1.7.1 Bacteria resistance testing

The degree of antibacterial resistance is commonly measured as Minimum Inhibitory Concentration (MIC), i.e. the lowest concentration of a given agent that inhibits growth of a defined number of the microorganisms under standard laboratory conditions.

To distinguish resistant from susceptible strains, microbiological cut-off values (also called break point values) are defined. So-called epidemiological cut-off values are used for surveillance, whereas clinical cut-off values are used to predict the effect of an antimicrobial in a clinical setting. In the present assessment, only epidemiological cut-off values are used. These are the MIC- values for each antibacterial agent that distinguishes wild-type populations of bacteria from those with acquired or selected resistance mechanisms. For more information on cut-off values; see (NORM/NORM-VET, 2014). Depending on the test

system, a cut-off value is given as either a concentration (in mg/L or µg/ml) or a zone diameter (in mm). Cut-off values for an antibacterial agent may differ between different bacterial species. The European Committee on Antimicrobial Susceptibility Testing - EUCAST gives recommendations for cut-off values to be used.

Surveillance of antibacterial resistance in animals and animal products include testing of both pathogenic bacteria, as well as commonly occurring bacteria that normally are non-pathogenic. The Gram positive bacterial species *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) are commonly used intestinal bacteria to monitor antibacterial resistance in animals, including poultry.

1.7.2 Coccidia resistance testing

Coccidiostat resistance of coccidia field isolates is studied by the use of either *in vivo* or *in vitro* assays. *In vivo* anticoccidial sensitivity testing (AST) is a well-known technique to assess resistance of a certain coccidial isolate to different coccidiostats (Chapman, 1998; McDougald and Reid, 2003; Naciri et al., 2003; Peek and Landman, 2003). The assay requires the diagnostic slaughtering of a large number of chickens in order to determine the efficacy of the coccidiostats. Although a valid method for a certain isolate, this technique is not routinely used. The main reasons are the long duration and very high cost associated with the complicated, *in vivo* character of the test. The short period of testing (around six days) without allowing the initially naive birds to recover from an artificially high infective dose makes interpretation of the results complicated. However, in a review paper devoted to coccidiostat resistance in fowl coccidia, Abbas et al. (2011) cited the definition of resistance in the broad terms as given by the World Health Organization (1965): the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject. Several authors have also described *in vitro* culture systems for studying invasion and development of *Eimeria tenella* in the presence of ionophore drugs and other compounds. These assays are based on counting intracellular sporozoites after fixation and staining of the *E. tenella*-infected cell monolayer. More modern methods of quantification are currently being studied, e.g. the use of quantitative PCR.

1.8 Norwegian chicken and turkey production

In Norway poultry meat production consist mainly of chicken (broiler) and turkey and a relatively small production of ducks. Geese and other species are of marginal importance in this assessment. The commercial poultry production has developed extensively in Norway during the last years. The chicken meat production has increased from 69 375 tonnes in 2009 to 91 931 tonnes in 2013, the turkey production is stabilised at approximately 10 000 tonnes in the same period while the duck production increased from 355 tonnes to 593

tonnes (Animalia, 2014). Since anti-coccidials are not used in duck feed, this production is not mentioned further in this document.

The amount of organic poultry meat production of 83 tonnes for chicken and 130 tonnes for turkey is only 0.21% of the total poultry meat production (Animalia, 2014). Organic poultry meat production is done without the use of coccidiostats.

The amount of poultry production without the use of coccidiostats has until 2015 been limited to the production of selected broiler brands in a few farms. Compensatory measures in these flocks has been limited to reduced animal density in early life, feeding that reduces energy intake and restricts growth, enrichment of the environment etc. Vaccination against coccidiosis has been used to some degree.

Organic broilers have been produced according to EU legislation that includes restrictions according to age, density, feed and environment. Organic broilers are vaccinated against coccidiosis.

The need for in-feed coccidiostats in poultry feed has been under investigation by the Norwegian poultry industry for several years. Different trials with different remedies have been performed, but no measures have been suggested that can compete with coccidiostats with regard to feed efficacy, animal health or –welfare (Kaldhusdal, 2006).

In 2015 there has been a general commercial demand for broilers that are raised without the use of coccidiostats. High quality standard of management practices and feed combined with general good health status and vaccination against coccidiosis seems to be factors that now allows for Norwegian broiler production without the routine use of in-feed coccidiostats. (Atle Løvland, Nortura, personal communication).

A study based on data from 2000 to 2004 produced data suggesting that at least 25 % of Norwegian broiler flocks were coccidia negative. However, the data suggested an increasing trend in the prevalence of infected flocks (Haug et al., 2008).

Import to and export from Norway of poultry products varies with the market situation in Norway. Currently, there is no import but some export.

1.9 The role of in-feed coccidiostats in broiler rearing

A specialised broiler production first evolved in the USA, beginning with the rearing of single purpose meat type chickens in the 1920's and developing further with separate hatcheries, feed-mills, farms and processing plants during the 1930's and 1940's (http://www.poultryegginstitute.org/educationprograms/PandEP_Curriculum/Documents/PDFs/Lesson2/HistoryofPoultryProductionver3Pres.pdf). Intestinal disease, usually ascribed to

coccidiosis, was identified as an important problem from the start of the industry. Initially the problem was controlled through treatment of outbreaks of clinical disease, mainly by means of sulphonamides. Gradually the concept of preventive medication emerged with the realization that most of the damage is done by the time signs of intestinal disease is widespread in a flock. Preventive medication was implemented through routine incorporation of coccidiostats in broiler feeds. This routine was firmly established in 1948, when sulfaquinoxaline was introduced commercially as a poultry coccidiostat (Campbell, 2008). Sulfaquinoxaline was initially developed and tested for use in human medicine, but proved too toxic for humans.

The sulphonamides were introduced into human medicine in the 1930s. New types of antimicrobials were developed during the 1940's. These new substances were mostly produced by a microorganism and were antagonistic to the growth of other microorganisms in high dilution. The term 'antibiotic' was coined for these substances. Some of these new drugs were tested for efficacy against diseases in animals. During such experiments it was found that some substances (e.g. chlortetracycline produced by *Streptomyces aureofaciens*); (Castanon, 2007) could enhance the growth and feed efficiency of chickens, and in 1951 the United States Food and Drug Administration approved the use of antibacterials as animal additives without veterinary prescription. Similar approvals were given in European countries during the 1950' and 1960's. This group of substances was named 'antibiotic growth promoters (AGP)'.

Whereas the coccidiostats were approved as a preventative medication against a specific disease problem such as intestinal coccidiosis, the use of AGPs was not based on efficacy against a specific disease, but rather that these substances improved production performance. It is, however, clear that researchers observed mitigating effect of AGPs on diarrhoea, and even suspected that the positive effects of these substances might be associated with their suppressive effect on the intestinal bacterium *C. perfringens* (Bakke et al., 1954). These observations preceded the detection of the important intestinal disease necrotic enteritis, which was described in its clinical form in 1961 (Parish, 1961) and its subclinical form in 1992 (Kaldhusdal and Hofshagen, 1992).

The introduction of preventive in-feed medication during the late 1940s and early 1950s can be seen as an essential technological component in the development of the new broiler industry. It has been claimed that the anticoccidial drug sulfaquinoxaline 'played an important part in the demotion of roast chicken from vaunted Sunday-dinner status to an unexpected position on the everyday menu of the Western world' (Campbell, 2008). The AGPs were likely to play a similar part in this process, at least during the first decades of the industry's history.

Both AGPs and coccidiostats are antimicrobial agents, but they are grouped separately according to the microorganisms they act primarily against. In some cases a compound

belonging to one of these categories may also exert an effect against several types of microbes. The ionophorous coccidiostats such as narasin is an example of a group of compounds with efficacy against coccidia as well as bacteria.

The Norwegian broiler industry was established during the 1960's and 1970's. Whereas tetracyclines as antibacterial growth promoters were included in Norwegian broiler feed already during the 1960's, coccidiostats (amprolchloride) were not introduced until 1972. From 1972 both coccidiostats and antibacterial growth promoters were used routinely in Norwegian broiler feeds until 1995, when the broiler industry implemented a voluntary abolishment of the use of antibacterial growth promoters (Grave et al., 2004).

The emergence of bacterial strains resistant to antibacterials was of concern to researchers already during the 1950 and 1960s (Anonymous, 1968; Bakke et al., 1954) published a report recommending that antibacterials used for disease treatment in human and veterinary medicine should not be used as growth promoters. Specifically, it was recommended that the use of penicillins, tetracyclines, tylosin, and sulfonamides as growth promoters be discontinued. This policy was implemented in Norway in 1972.

However, in the 1990's it was discovered that the antibacterial (avoparcin) used only for growth promotion in animals could confer cross-resistance to vancomycin (Grave et al., 2004), an antibacterial on the World Health Organization's List of Essential Medicines (http://www.who.int/medicines/publications/essentialmedicines/18th_EML_Final_web_8Jul13.pdf , <http://en.wikipedia.org/wiki/Vancomycin>, http://en.wikipedia.org/wiki/WHO_Model_List_of_Essential_Medicines). On this background avoparcin was banned in Norway from May 31, 1995. The avoparcin case demonstrated that avoiding the use of antibacterials that are used for treatment of diseases in humans or animals is not enough to prevent induction of antibacterial resistance that can endanger animal or human health. It is also crucial that substances used in animal feeds cannot confer cross-resistance to antibacterials of therapeutic importance.

Avoparcin had been introduced in 1987, following an epidemic of necrotic enteritis in the Norwegian broiler population. The epidemic disappeared following the introduction of in-feed avoparcin in 1987, but necrotic enteritis reappeared as a severe disease problem shortly after the avoparcin ban in 1995. As a response to this development, the ionophorous coccidiostat narasin was approved for use in broiler feeds as from November 1995. Narasin was introduced because of the beneficial effect of this substance against *C. perfringens* and necrotic enteritis, as evidenced by experience from Sweden, where AGPs had been banned and narasin was used as the only in-feed medication as from 1990. The introduction of narasin in the broiler feed in Norway was followed by a gradual decline in prevalence of necrotic enteritis during 1996-98 (Lovland and Kaldhusdal, 2001), and the prevalence has remained low since then.

In 2003, the European Union banned the use of antibacterials as growth promoters (Regulation (EC) No 1831/2003), which was implemented from 2006. In Article 11 of this regulation, the European Union states that the use of coccidiostats as feed additives should be evaluated by December 2012. However, in 2008, the European Commission submitted a report on the use of these substances as feed additives and existing alternatives to the Council and the European Parliament (http://www.ipex.eu/ipex/cms/home/Documents/dossier_COM20080233). In this report, the European Commission recommended maintaining the current legislation and allows the use of coccidiostats, including ionophores, as feed additives because of the lack of alternatives and to preserve the economic viability of the poultry industry. Therefore, the use of in-feed coccidiostats is still approved.

1.10 The role of in-feed coccidiostats in turkey rearing

Turkeys are susceptible to the same kind of intestinal diseases as broiler chickens, and antibacterial growth promoters and coccidiostats have also been added to turkey feeds. The use of antibacterial growth promoters was abolished in turkey feeds in the same way as in broiler feeds (in 1995 in Norway, in 2006 in the EU). The transitional time period following the abolishment of growth promoters was characterised by an increased prevalence of clinical and subclinical necrotic enteritis. As mentioned above, these problems disappeared in the Norwegian broiler industry shortly after the introduction of the in-feed coccidiostat narasin. However, this compound cannot be used in turkey feeds, because narasin (and salinomycin) is too toxic to turkeys (Markiewicz et al., 2014). Necrotic enteritis has remained a challenge to the Norwegian turkey industry. However, the causes of persisting problems with necrotic enteritis in turkeys are unknown. Monensin is used most extensively as an in-feed coccidiostat for Norwegian turkeys. Practical experience from Sweden and Norway suggests that narasin efficiently prevents necrotic enteritis in broilers. Monensin (Watkins et al., 1997) and lasalocid (Lanckriet et al., 2010), however, appear to have slightly higher minimum inhibitory concentration (MIC) values than narasin for *C. perfringens*, the causative agent of necrotic enteritis.

1.11 Coccidiostats included in the present assessment

The coccidiostats can be divided into two main groups; ionophores and non-ionophores, see Table 1.11-1 for overview. Ionophores are originally fermentation products of *Streptomyces* and other fungi species. Non-ionophore coccidiostats are most often chemically synthesised, and are thus sometimes referred to as "synthetic" or "chemicals". One important difference between the two groups is that most of the ionophores display antibacterial effects.

Table 1.11-1

Coccidiostats used as poultry feed additives to control coccidiosis

Compound	Active substance	Antibacterial activity	Approved in Norway
Ionophores	Narasin	Mainly active against Gram-positive bacteria	Yes
	Lasalocid sodium	Active against Gram-positive bacteria, but not against Gram-negative bacteria.	Yes
	Monensin sodium	Mainly active against Gram-positive bacteria.	Yes
	Salinomycin sodium	Active against Gram-positive bacteria, but not against Gram-negative bacteria.	Yes
	Maduramicin ammonium	Active against Gram-positive bacteria, but not against Gram-negative bacteria.	Yes
	Semduramicin sodium	Has limited antibacterial activity against Gram-negative microorganisms tested, and a minimal activity against selected Gram-positive control organisms	No
Non ionophores	Robenidine hydrochloride	No known antibacterial effect	No
	Diclazuril	No substantial antibacterial activity	No
	Decoquinatate	Most tested strains of bacteria appear resistant to the effects of decoquinatate at concentrations of > 64 mg /-1, substantially higher than the concentration of decoquinatate expected in the digestive tract	No
	Halofuginon	No known antibacterial effect	No
	Nicarbazin	No known antibacterial effect	No

The ionophores are currently only used in animals, where they are considered “critically important in poultry” by OIE, the reason being that: “Ionophores are essential for animal health because they are used to control intestinal parasitic coccidiosis (*Eimeria* spp.) where there are few or no alternatives available”

(http://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/Eng_OIE_List_antimicrobials_May2015.pdf). On the other hand, none of the ionophores are listed as “critically important”, “highly important” or “important” for use in human medicine, according to the WHO listing (http://www.who.int/foodsafety/areas_work/antimicrobial-resistance/cia/en/).

1.11.1 Narasin

According to EFSA (2004c), narasin is a polyether ionophore that exhibits both antibacterial and anticoccidial activities. It is used to prevent coccidiosis in poultry.

Narasin, as other polyether ionophores, is effective against sporozoites and early and late asexual stages of coccidia in the intestine of the chicken. The biological activity of ionophores/narasin is based on their ability to form lipid soluble and dynamically reversible complexes with cations, preferably monovalent cations such as the alkaline ions K^+ , Na^+ and Rb^+ . They function as carriers by mediating an electrically neutral exchange-diffusion type of cation transport across the membranes. The resultant changes in transmembrane ion gradients and electrical potentials produce critical effects on cellular function and metabolism of coccidia.

Narasin is mainly active against Gram-positive bacteria.

1.11.2 Lasalocid sodium

According to EFSA (2004f), lasalocid belongs to the divalent polyether ionophore family. It is a feed additive for chickens for fattening (broilers) and chickens reared for laying.

Lasalocid sodium, as for other ionophores, has different ionic affinities, binding divalent cations as well as monovalent ions, increasing their passage through biological membranes. This leads to the disruption of the normal physiological processes of cells.

Coccidial sporozoites exposed to lasalocid sodium in the intestinal lumen exhibit considerable swelling, large vacuoles, pitting and holes in the surface suggesting extreme and potentially lethal osmotic damage. Any ionophore accumulated while coccidia sporozoites are exposed in the intestinal lumen continues its disruptive effects after host cells are invaded. Consequently, lasalocid sodium can selectively destroy intracellular sporozoites while remaining relatively non-injurious to the host cell.

One study on rat, reported that lasalocid stimulated release of catecholamines from pheochromocytoma and this effect may be dependent on increasing intracellular Ca^{2+} (Perlman et al., 1980).

Lasalocid sodium is active against Gram-positive bacteria, but not against Gram-negative bacteria.

1.11.3 Monensin sodium

According to EFSA (2004e), monensin sodium is a polyether ionophore that exhibits both antibacterial and anticoccidial activities. It is a feed additive used to control coccidiosis in chickens for fattening (broilers), fattening turkeys and replacement layers.

Monensin sodium acts as an ionophore, i.e. a chemical substance that complexes monovalent cations and facilitates the transport of the bound ion through biological membranes. Coccidial sporozoites exposed to monensin sodium in the intestinal lumen exhibit considerable swelling, large vacuoles, pitting and holes in the surface suggesting extreme osmotic damage which is potentially lethal. Development of a sporozoite that successfully invades a host cell is inhibited as the ionophore continues its destructive process. Monensin sodium selectively destroys intracellular sporozoites while remaining relatively non-injurious to the host cell (Chapman, 1993). It has been shown that monensin sodium has an effect on second generation merozoites but not upon developing gametocytes.

Monensin sodium is primarily coccidiocidal in action and active against *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mivati*, *E. necatrix* and *E. tenella* of the chicken and *E. melagrimitis*, *E. pallida*, *E. dispersa* and *E. adenoeides* of the turkey.

Monensin sodium is active against Gram-positive bacteria, but not against Gram-negative bacteria.

1.11.4 Salinomycin sodium

According to EFSA (2004d), salinomycin sodium is a monocarboxylic polyether ionophore with both antibacterial and anticoccidial effects. It is a feed additive used for control of coccidiosis in chickens for fattening.

Salinomycin (SAL) is effective against sporozoites, and early and late asexual stages of coccidia in the intestine of the chicken. The biological activity of SAL is based on the ability of the ionophores to form lipid soluble, dynamically reversible complexes with mono- and divalent cations (preferably the alkali ions K^+ , Na^+ and Rb^+). SAL encloses the cation in a hollow ball, in the center of which the cation is fixed and immobilised. It functions as a carrier by mediating an electrically neutral exchange-diffusion type of cation transport across the membranes. The resultant changes in transmembrane ion gradients and electrical potentials often produce profound effects on cellular function and metabolism.

SAL-Na is active against Gram-positive bacteria, but not against Gram-negative bacteria.

1.11.5 Maduramicin

According to EFSA (2011), maduramicin is a polyether ionophore. It is intended to be used to control coccidiosis in chickens for fattening.

Maduramicin is considerably more potent as a coccidiostat than the other polyether ionophores that are used as coccidiostatic feed additives. These compounds are branch-chained, polyoxygenated carboxylic acids that act as mobile carriers of cations by rendering cations lipid-soluble, thereby enabling them to pass across membranes. This process disrupts cationic cross-membrane gradients and is responsible for their anticoccidial activity.

Maduramicin is active against Gram-positive bacteria, but not against Gram-negative bacteria.

1.11.6 Semduramicin sodium

According to SCAN (2002), semduramicin sodium is a monocarboxylic acid polyether ionophore. It is used to control coccidiosis in chickens for fattening.

These compounds are branch-chained, polyoxygenated carboxylic acids that act as mobile carriers of cations by rendering them lipid-soluble, thereby enabling them to pass across membranes. This process disrupts cationic cross-membrane gradients and is responsible for their anticoccidial activity.

Effect on bacteria: According to FDA semduramicin, an ionophorous agent, had no remarkable activity against Gram-negative microorganisms tested, and activity against selected Gram-positive control organisms was minimal (<http://www.fda.gov/downloads/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/OIADrugSummaries/ucm062337.pdf>).

1.11.7 Robenidine hydrochloride

According to EFSA (2004a), robenidine hydrochloride is a chemically synthesized substance. It is used to control coccidiosis in chickens for fattening and turkeys.

Robenidine hydrochloride activity against *Eimeria* results from dual action exerted upon different stages of the parasite as it develops in the intestinal mucosa. Initially it acts as a coccidiostat and arrests the development of the first schizont generation. Secondly, robenidine hydrochloride is coccidiocidal, killing the second generation of schizonts and possibly the merozoites.

The compound has no known antibacterial effect.

1.11.8 Diclazuril

According to EFSA (2010), diclazuril is a synthetic compound of the triazinone family. It is used to control coccidiosis in chickens for fattening.

The exact mode of action of Diclazuril is not known but it has a potent coccidicidal action on some *Eimeria* strains (*E. tenella* and *E. acervulina*) and coccidiostatic action on others. According to the anticoccidial properties of triazinones, diclazuril is active against intracellular development stages of coccidia, namely during schizogony and gametogony.

Diclazuril possesses negligible antifungal and no antibacterial activity at 100 µg/ml (Van Cutsem and Ribbens-Pavella, 1985). Diclazuril has no substantial antibacterial activity (EFSA, 2014).

1.11.9 Decoquinat

According to EFSA (2003a), decoquinat is a 4-hydroxyquinoline, manufactured chemical synthesis. It is used to control coccidiosis in chickens for fattening.

Based on data obtained from clinical studies, decoquinat is thought to act by arresting the development of sporozoites following their penetration of the gut epithelium. The degree of damage to the gut in terms of lesions is significantly reduced and oocyst output is also reduced. Decoquinat significantly inhibits mitochondrial respiration and electron transport in *Eimeria*.

Most strains of bacteria appear resistant to the effects of decoquinat at concentrations of > 64 mg l⁻¹, substantially higher than the concentration of decoquinat expected in the digestive tract.

1.11.10 Halofuginone

According to EFSA (2003b), halofuginone hydrobromide is a synthetic derivate of an alkaloid originally isolated from an Asiatic plant, *Dichroa febrifuga* Lour. It is used to control coccidiosis in chickens for fattening and turkeys.

Halofuginone hydrobromide (HBR) acts especially on the 1st generation schizonts early after sporozoite invasion. HBR has a coccidiocidal activity against *Eimeria maxima* and *Eimeria tenella*.

The compound has a cryptosporidiostatic effect on *Cryptosporidium parvum*. It is mainly active on the free stages of the parasite (sporozoite, merozoite).

The compound has no known antibacterial effect.

1.11.11 Nicarbazin

According to EFSA (2008g), nicarbazin is a non-ionophoric synthetic complex composed of an equimolar amount of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). It is used to control coccidiosis in chickens for fattening.

Nicarbazin acts primarily by inhibiting the further development of the 2nd generation and, to a lesser extent, 1st generation schizont stage of *Eimeria* spp. parasites. The anticoccidial effect of nicarbazin was shown to be mediated to a large extent by its systemic absorption.

The compound has no known antibacterial effect.

1.12 Coccidiostat in-feed control programmes

Various programmes are applied to prevent or delay development of coccidiostat resistance in coccidia (<http://www.omafra.gov.on.ca/english/livestock/poultry/facts/coccidiosis.htm>).

Continuous program means that the same coccidiostat(s) are used indefinitely, usually until a problem develops, or until a new product is introduced on the market. This type of programme is used in Norway.

Shuttle refers to the use of two or more products during the grow-out of a flock. The principle of shuttles is that the best drug is used for each phase of the grow-out i.e. most suitable coccidiostat is used for starter, while another coccidiostats are used for grower and finisher. Non-ionophore coccidiostats usually follow ionophores. This is to prevent late cycling of coccidiosis in the grow-out.

Rotation means that a conscious decision is made to change the drug(s) used at a given time in the future i.e. every four months, after two crops etc. Shuttle programs may fit into rotation programs i.e. a decision may be made to use a shuttle program (coccidiostat A and B) for the summer. For the winter program other coccidiostats may be used in a shuttle, or only one coccidiostat. The key aspect of rotations is to alternate drug chemistries i.e. non-ionophore coccidiostats follow ionophores. Use of two ionophores back to back in a rotation is unlikely to give desired results. Vaccines can also be included in a rotation programme.

Clean-up program If coccidiocidal, non-ionophores coccidiostats can be used in order to reduce the infection pressure of coccidiosis (De Iguassu, 2005), in a so-called clean-up program. To achieve this, non-ionophore coccidiostats are preferably used during a complete grow-out, a so-called full program. Some producers do not, in order to limit risk of resistance, use non-ionophore coccidiostats in full program, but switch from one non-ionophore coccidiostats to another in the same grow-out, i.e. shuttle program.

1.13 The chicken gastrointestinal (GI) tract microbiota

Intestinal health is essential for production success, and changes in this complex system may lead to poor performance, increased mortality and condemnations as well as higher medication cost. Microbiota in the intestine of an animal species has evolved together with the host. The intestinal microbiota has an enormous metabolic potential and it affects both the nutrition and health of the host (Lan et al., 2005; Rantala and Nurmi, 1973; Rinttilä and Apajalahti, 2013) and shifts in intestinal microbiota can result in a series of implications, including disease, welfare, environmental and food safety concerns (Roberts et al., 2015). The domestic chicken (*Gallus gallus domesticus*) has a unique status as 'both the model and the system' – chickens are common model organisms for human biological research and also comprise a global economically valuable protein industry. The intestinal bacterial communities play important roles, in the influence on the immune system, in nutrition and for the health of the host by inhibiting the establishment of intestinal pathogens.

Studies on the composition of the intestinal microbiota of chickens date back to 1901 (Rahner, 1901) and continued in the 1940s (Shapiro and Sarles, 1949). Comprehensive surveys that attempted to culture as many of the intestinal bacteria as possible were mostly carried out until the 60s and 70s (Barnes, 1979; Barnes and Goldberg, 1962; Barnes and Impey, 1968; Barnes et al., 1972; Fuller, 1973a; Mead and Adams, 1975; Salanitro et al., 1974), but few studies have been carried out using cultivation during the last decade (Kaldhusdal et al., 2001; Oakley et al., 2014a). Cultivation studies are technically difficult since strict anaerobic conditions have to be maintained during isolation and biochemical differentiation of the bacteria, and Gaskins et al. (2002) suggested that less than 20% of the bacterial taxa inhabiting the poultry gastrointestinal tract are recovered by cultivation.

Why do we care about the intestinal microbiota of chickens? One answer is 'healthy normal intestinal microbiota is the first line of defense for all animals to invading pathogens'. In chickens; during the first two to four days post hatched, streptococci and Enterobacteria colonize the small intestine and ceca. After the first week, lactobacilli predominate in the small intestine while mainly *Escherichia coli* and *Bacteroides* (anaerobes) colonize the ceca with lower proportion of facultative aerobe bacteria. Although other parts of the digestive tract of chickens might also be important sites of bacterial residents and for different pathogen-host microbiota interactions, the ceca have received most of the attention because the microbiota of the ceca is very diverse and caecal content may contain 10^{11} bacteria g^{-1} (Apajalahti et al., 2004; Mead, 1997). Potential human pathogens such as *Salmonella enterica* and *Campylobacter jejuni* are frequently most numerous in the ceca (Barrow et al., 1987; Doyle, 1991; Duchet-Suchaux et al., 1995). However, as earlier culturing and microscopic evaluations indicated that only a fraction of bacteria in the caecum of chicken could be grown in the laboratory, more recent studies have focused on the use of molecular methods to evaluate bacterial abundance and diversity (Amit-Romach et al., 2004;

Danzeisen et al., 2011; Gabriel et al., 2006; Gong et al., 2002b; Konsak et al., 2013; Lee and Newell, 2006; Lu et al., 2003; O'Hara and Shanahan, 2006; Oakley et al., 2014a; Oakley et al., 2014b; Oakley et al., 2013; Qu et al., 2008; Sekelja et al., 2012; Stanley et al., 2015; Stanley et al., 2013a; Stanley et al., 2013b; Stanley et al., 2014; Stanley et al., 2012; Torok et al., 2013; Torok et al., 2011a; Torok et al., 2011b; Torok et al., 2008; Van der Hoeven-Hangoor et al., 2013; Van der Wielen et al., 2002a; Yeoman et al., 2012; Zhao et al., 2013; Zhu et al., 2002; Zoetendal et al., 1998). Recent progress in technology for microbial community analysis has evolved the understanding of the chicken intestinal microbiome, and it is now a general understanding that shifts in the microbial communities occur. These shifts can result in a series of implications including disease, reduced welfare, environmental and food safety concerns.

The intestinal microbiota are classified as autochthonous or indigenous, when they are able to colonize the host's epithelial surface or are associated with the microvilli, or as allochthonous or transient (associated to digesta or are present in the lumen) (Cole and Fuller, 1984; Savage, 1977). However, few studies have evaluated both the allochthonous and autochthonous microbiota of poultry (Gong et al., 2002a; Zhu et al., 2002).

In the following part we focus on two studies that evaluated the gut microbiota of chickens (Oakley et al., 2014a; Zhu et al., 2002), and two recent review papers (Oakley et al., 2014b; Stanley et al., 2014).

Zhu et al. (2002), carried out a survey of cecal bacteria by retrieval of 16S rRNA gene sequences from DNA isolated from the cecal content and the cecal mucosa. The ribosomal gene sequences were amplified with universal primers and cloned or subjected to temporal temperature gradient gel electrophoresis (TTGE). Partial 16S rRNA gene sequences were determined from the clones and from the major bands in TTGE gels. A total of 1,656 partial 16S rRNA gene sequences were obtained and compared to sequences in the GenBank.

Comparison indicated that 243 different sequences were present in the samples (Figure 1.13-1). Overall, sequences representing 50 phylogenetic groups or subgroups of bacteria were revealed, but approximately 89% of the sequences represented just four phylogenetic groups (*Clostridium leptum*, *Sporomusa* sp., *Clostridium coccooides*, and enterics). Sequences of members of the Bacteroides group, the *Bifidobacterium infantis* subgroup, and of *Pseudomonas* sp. each accounted for less than 2% of the total gene sequences. Sequences related to the *Escherichia* sp. subgroup and from *Lactobacillus*, *Pseudomonas*, and *Bifidobacterium* spp. were generally between 98 and 100% identical to sequences already deposited in the GenBank. Sequences most closely related to those of the other bacteria were generally 97% or less identical to those in the databases and therefore might be from currently unknown species. TTGE and random cloning indicated that certain phylogenetic subgroups were common to all birds analysed, but sequence data from random cloning also

provided evidence for qualitative and quantitative differences among the cecal microbiota of individual birds reared under very similar conditions.

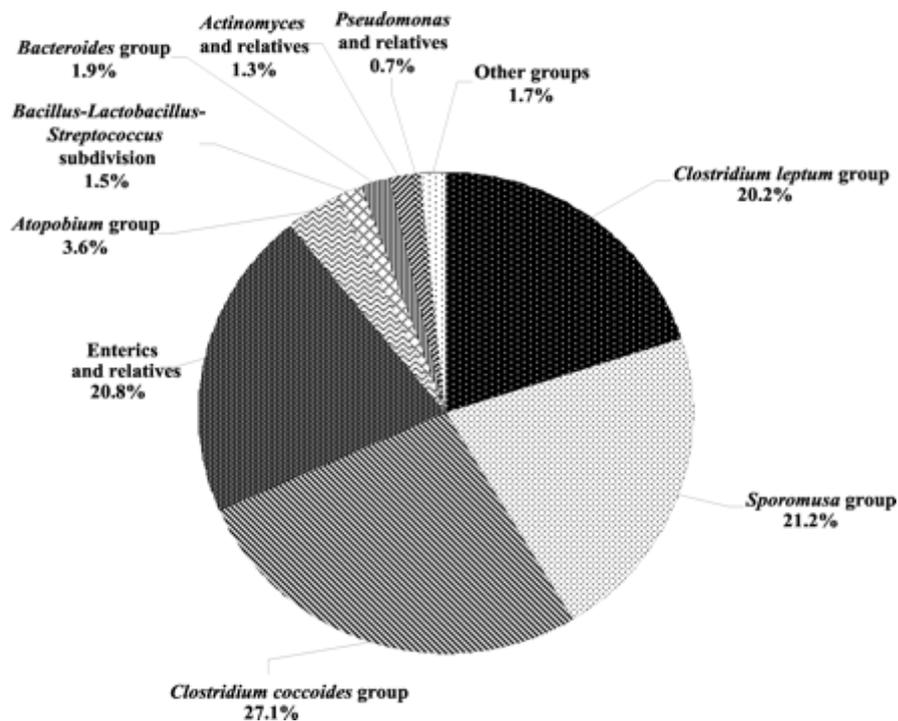


Figure 1.13-1 Percentage of the total number of sequences obtained in the study by Zhu et al. (2002) that were classified by the sequence match program of the Ribosomal Database Project as belonging to different phylogenetic groups or subdivisions. Sequences most closely related to *Eubacterium*, *Desulfovibrio*, *Clostridium propionicum*, *Xanthomonas*, *Clostridium botulinum*, *Acholeplasma-Anaeroplasmata*, *Aeromonas*, *Rhizobium-Agrobacterium*, and *C. lituseburense* groups were combined under "other groups." (Source: Zhu et al. (2002) with permission)

As a percentage of the total number of sequences obtained from each library, sequences related to those of the *Selenomonas ruminantium* subgroup predominate (Figure 1.13-2). These sequences were reported in all four libraries (cecal content; libraries 1A and 1B, and from the cecal mucosa; libraries 1C and 1D) and were therefore retrieved from bacteria in cecal content and the mucosa. Sequences representing the *Phascolarctobacterium faecium* and *Veillonella parvula* subgroups were also present in each of the libraries. Similarly, sequences representing the *Ruminococcus gnavus* subgroup of the *Clostridium coccoides* group of bacteria and the *Clostridium leptum* subgroup of the *C. leptum* group of bacteria were reported in all four libraries. Sequences assigned to most other subgroups were generally also represented in cecal content and the cecal mucosa libraries. A small number of sequences belonging to the *Butyrivibrio fibrisolvens*, *Clostridium lituseburense*, and *Prevotella buccae* subgroups were revealed only in the mucosal libraries. One sequence belonging to the *Clostridium polysaccharolyticum* and the *Lactobacillus mali* subgroups, respectively, was only reported in the cecal content libraries. Whether these findings indicate

true differences in the bacterial populations or are merely the result of chance events is not known. For example, sequences belonging to the *S. paucivorans* subgroup were reported in the cecal content and the mucosal clone libraries, but only in the libraries generated with primer pair 8FPL/1492RPL; out of 22 primers used for PCR. Similarly, sequences belonging to the *Clostridium xylanolyticum* and *Ruminococcus hansenii* subgroups were revealed only in the content and mucosal samples amplified with primer pair 515FPL/1492RPL.

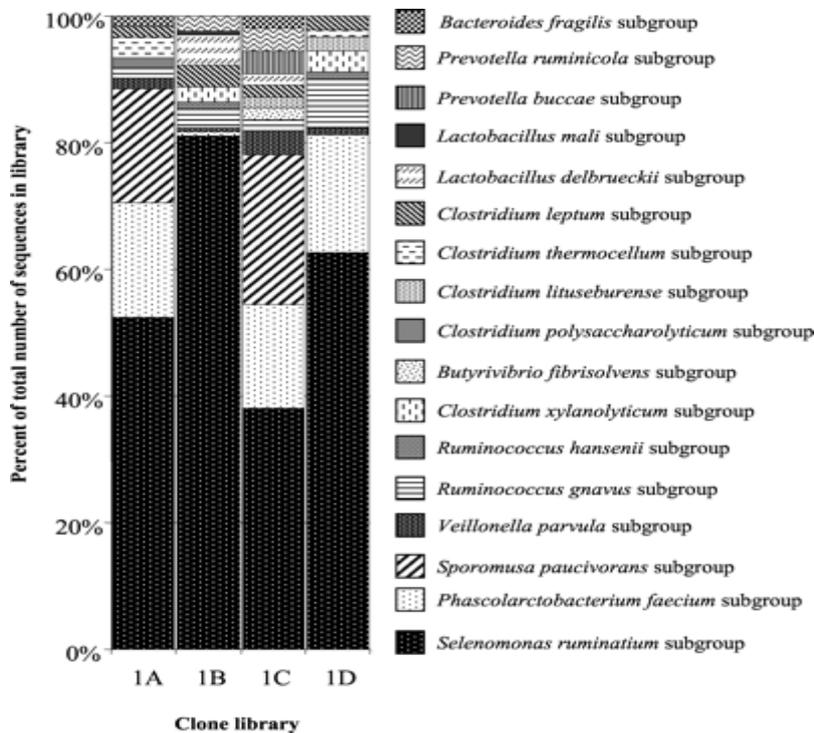


Figure 1.13-2 Percentages of sequences from clone libraries 1A to 1D most closely related to 16S rRNA gene sequences from particular phylogenetic subgroups of bacteria. The libraries were obtained from DNA extracted from cecal content (libraries 1A and 1B) and from the cecal mucosa (libraries 1C and 1D) from one 6-week-old broiler. (Source: Zhu et al. (2002) with permission).

Oakley et al. (2014a), evaluated the cecal bacterial communities at day of hatch (n = 5 birds), 7d (n = 32), 21d (n = 27), and 42d (n = 36) post-hatch using direct 454 sequencing of 16S rRNA gene amplicons from each bird in combination with cultivation-based recovery of a *Salmonella typhimurium* marker strain and quantitative-PCR targeting *C. perfringens*. By day 21 post-hatch, a single genus (*Faecalibacterium*) accounted for 23-55% of sequences (Figure 1.13-3). Sokol et al. (2008), reported that *Faecalibacterium prausnitzii* has anti-inflammatory properties and an inverse correlation with severity and recurrence of colitis in humans and murine models. Whether or not members of the genus *Faecalibacterium* have similar roles in chickens merits further investigations. By day 42, *Faecalibacterium* sequences were recovered at approximately equal proportions to *Roseburia*, a saccharolytic, butyrate-producing bacterium reported by Duncan et al. (2002). In this respect, it is worth mention

that butyrate is known to increase disease resistance (Stanley et al., 2012). At day 42 relatively abundant sequences were classified as *Lachnospiraceae incertae sedis*, and *Oscillibacter*, previously encountered in chickens (Luo et al., 2013). Some of these members are known to produce short chain fatty acids (Katano et al., 2012; Lee et al., 2012). Data revealed by Oakley et al. (2014a) are consistent with previous results identifying various members of the poultry GI microbiome (Bjerrum et al., 2005; Geier et al., 2009; Gong et al., 2008; Knarreborg et al., 2002; Oviedo-Rondón et al., 2010; Rehman et al., 2007; Sun et al., 2013a; Tillman et al., 2011; Torok et al., 2013; Torok et al., 2011a; Torok et al., 2011b). Thus, exhaustive sequencing with modern methods from a fairly large number of birds can provide important new information regarding the generic composition of the chicken cecal microbial community and how the community changes over time. Proper understanding and management of temporal modulation of the GI tract microbiome will be important for maintaining bird health and improving productivity.

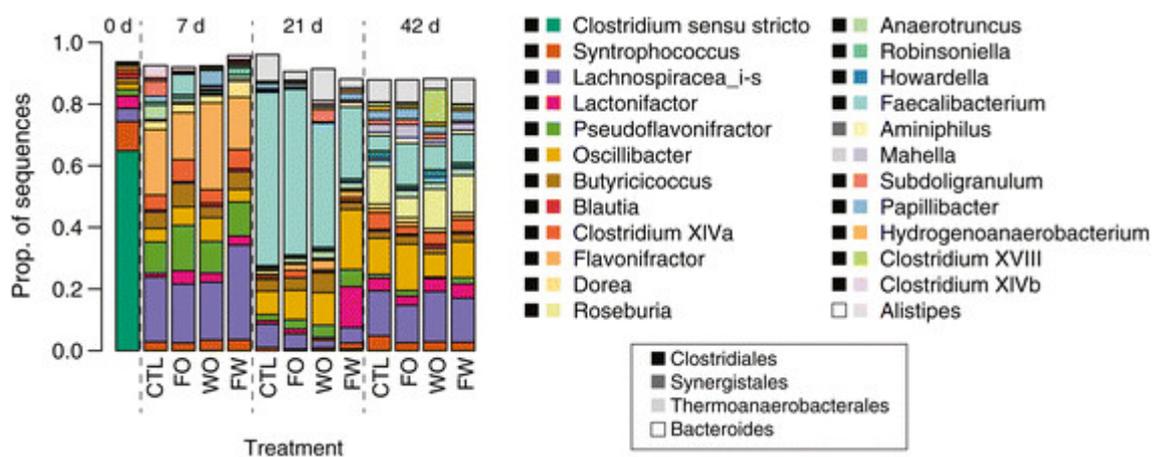


Figure 1.13-3 Relative abundance at the genus level for sequences by treatment and time with taxonomic classifications performed with the RDP classifier as described in the text. Only sequences with a total relative abundance greater than 5% are shown. For day-of-hatch birds and each subsequent time point (7d, 21d, and 42 d post-hatch), the relative proportions are shown for each treatment. Day-of-hatch birds were proportionally high in *Clostridium*. Treatment designations are Ctl, control; FO, feed-only; WO, water-only; and FW, feed and water as described in the text. (Source: Oakley et al. (2014a) with permission).

Sequencing data also demonstrated small treatment effects on taxonomic groups containing known pathogens (Figure 1.13-4, part A and B). Consistent with the cultivation data, *Salmonella* sequences decreased in relative abundance with time and were almost entirely absent by day 21 (Figure 1.13-4, part A and B). Sequences classified as *Clostridium* increased to a maximum of 0.5% at day 21, subsequently decreasing in relative abundance after 42 days of feeding (Figure 1.13-4; part A). In general, taxa considered as putative pathogens (*Campylobacter*, *Clostridium*, *Escherichia Shigella*, *Klebsiella* and *Salmonella*) were a minor component of the bacterial community (<1.5% total relative abundance).

Quantitative-PCR for the *Clostridium* clade containing the *C. perfringens* subgroup was qualitatively consistent with the sequencing data and showed a significant increase in the abundance of this group from hatching to day 21 post-hatch, followed by a significant decline by day 42 to the same levels at day 7 (Figure 1.13-4; part B).

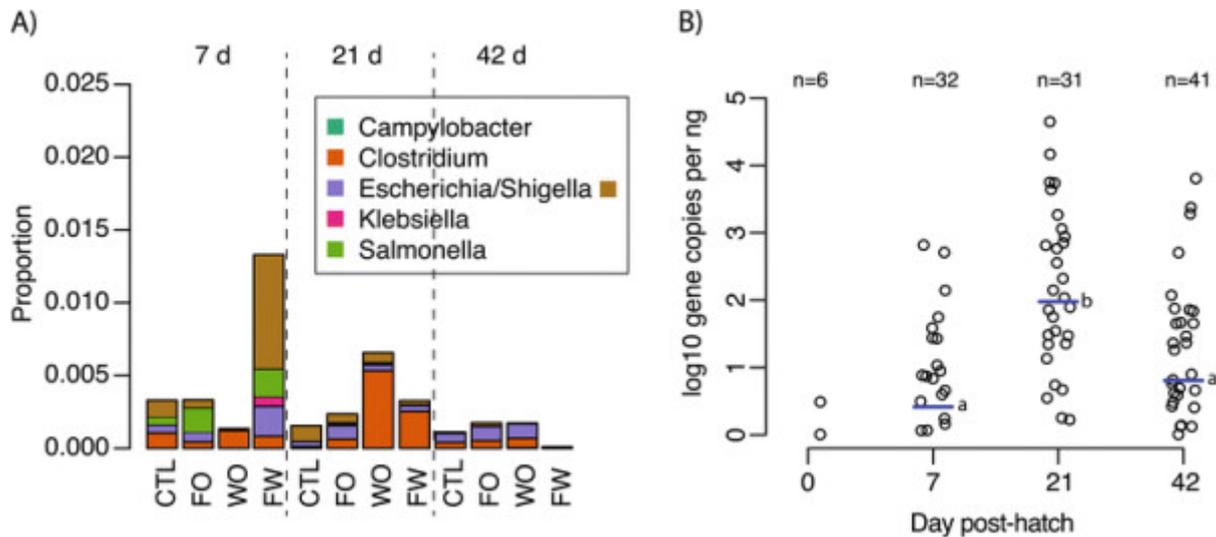


Figure 1.13-4, part A and part B Changes in relative abundance of putative pathogens by treatment and time. **A)** For each time point (7d, 21d, and 42 d post-hatch), the relative proportions are shown for each of the four treatments. Putative pathogens were defined using the intersection of independent taxonomic classifications with the RDP classifier and the Silva database as described in the methods. Sequences classified as Escherichia or Shigella by Silva are shown separately but not distinguished by RDP. Treatment designations are Ctl, control; FO, feed-only; WO, water-only; and FW, feed and water as described in the text. Note scale of Y axis. **B)** Number of gene copies of *Clostridium* as determined by quantitative-PCR for each time point. Treatments for each time point are grouped due to the non-significant effect of treatment. Quantitative loads of *Clostridium* were significantly higher at 21 d than 7d or 42 ($p < 0.0001$, one-sided t-tests). (Source: Oakley et al. (2014a) with permission).

Based on their results, the authors concluded that over the 42 days experiment, the cecal bacterial community changed significantly as measured by a variety of ecological metrics and increases in the complexity of co-occurrence networks. Management of poultry to improve animal health, nutrition, or food safety may need to consider the interactive effects of any treatments with the dramatic temporal shifts in the taxonomic composition of the cecal microbiome as described here.

Recently, two review papers have been published with regard to the gut microbiota of chickens (Oakley et al., 2014b; Stanley et al., 2014). Oakley et al. (2014b) revealed that Firmicutes, Bacteroides, and Proteobacteria are the most common phyla in the chicken ceca, with Actinobacteria accounting for the remainder (Figure 1.13-5a). At finer scales of taxonomic resolutions, the majority of sequence types can be shown to belong to various

members of the Clostridiales (Figure 1.13-5b). Although Clostridiales are known generally as important contributors to short chain fatty acid metabolism, further information of the members of this diverse group and their interactions merits evaluations.

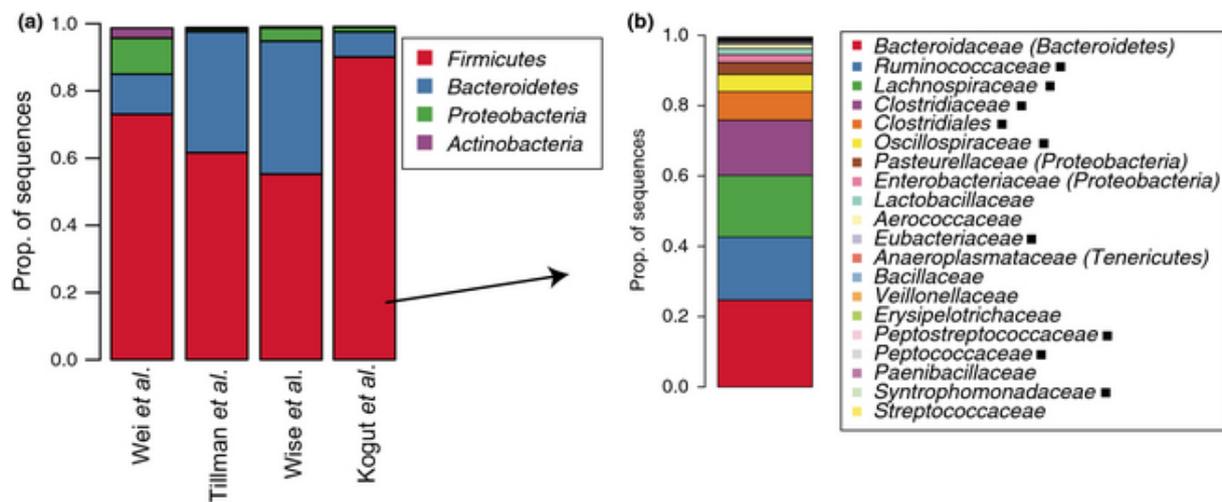


Figure 1.13-5, part a and part b Relative proportions of bacterial phyla (a) and families (b) reported in chicken ceca. Data from Wei et al. (2013) represent publicly available sequences retrieved as described. Data from Tillman et al. (2011) and Wise and Siragusa (2007) are re-analyzed from data included in the report from Oakley et al. (2013) representing 8 and 10 birds, respectively. Data from Kogut et al. are unpublished, collected, and analyzed as previously described by (Oakley et al., 2012; Oakley et al., 2013) representing 20 birds and ca. 20 000 sequencing reads. Data for each of these three flocks are from 3 weeks post hatch. Sequences from Wei et al. (2013) were additionally screened by removing sequences with ambiguous base calls, and all sequences were classified against a reference database of type strains from SILVA v115 (Pruesse et al., 2007). Many of the sequences reviewed in Wei et al. (2013) do not contain metadata regarding bird age, which can have strong effects on community composition and structure. For (b) families belong to the phylum Firmicutes unless otherwise noted; families followed by black squares belong to the Clostridiales. (Source: Oakley et al. (2014b), with permission).

Stanley et al. (2014) revealed that other parts of the digestive tract of chickens might also be important sites of bacterial residents (Table 1.13-1) and the authors cited van der Wielen (Van der Wielen et al., 2002b) suggested that the bacterial communities originating from different gut sections should be considered as separate ecosystems. For example; the crop where starch breakdown and lactate fermentation is performed, the cell densities are up to 10^9 cells g^{-1} (Rehman et al., 2007; Stanley et al., 2014) gizzard; low pH of gastric juices containing pepsin and HCl limits the total bacterial population level $<10^8$ g^{-1} , in contrast to caecum that harbor up to 10^{11} bacteria g^{-1} .

Table 1.13-1 A general overview of the most abundant bacterial residents in the gastrointestinal (GI) tract of chicken. After Stanley et al. (2014).

Section of GI tract	Dominant and abundant bacteria	References
Crop	<i>Lactobacillus</i> (dominant), Clostridiaceae, <i>Bifidobacterium</i> , Enterobacteriaceae, <i>Enterococcus</i>	(Fuller, 1973a; Rehman et al., 2007; Sekelja et al., 2012)
Gizzard	<i>Lactobacillus</i> (dominant), Clostridiaceae, <i>Enterococcus</i> , coliforms	(Fuller, 1973a; Rehman et al., 2007; Sekelja et al., 2012)
Duodenum	<i>Lactobacillus</i> (very dominant, up to 99% in some birds), <i>Streptococcus</i> , coliforms	(Gong et al., 2002a; Konsak et al., 2013; Lu et al., 2003; Salanitro et al., 1974)
Ileum	<i>Lactobacillus</i> (very dominant), <i>Streptococcus</i> , coliforms, Enterobacteriaceae, Clostridiaceae	(Gong et al., 2002a; Lu et al., 2003; Salanitro et al., 1974; Van der Hoeven-Hangoor et al., 2013)
Caecum	Unknown and uncultured bacteria, <i>Lactobacillus</i> , Bacterioides, <i>Clostridium</i> , <i>Bifidobacterium</i>	(Gong et al., 2002a; Lu et al., 2003; Stanley et al., 2013b; Torok et al., 2011a; Zhu et al., 2002)
Faeces	Variations between sampling time, <i>Lactobacillus</i> , <i>Clostridium</i> , <i>Fecalibacterium</i> , <i>Ruminococcus</i> , <i>Bacillus</i> , <i>Eubacterium</i> , <i>Fusobacterium</i>	(Sekelja et al., 2012; Zoetendal et al., 1998)

Pathogenic bacteria in the gastrointestinal (GI) tract

In newly hatched chickens, the rapid establishment of an "adult-type" intestinal microbiota including pathogenic bacteria occurs. Several studies have reported pathogenic bacteria, *Campylobacter jejuni*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Enterococcus*, *Escherichia coli*, *Yersinia enterocolitica* and *C. perfringens* in the GI tract of chicken (Table 1.13-2). In a review paper devoted to colonization factors of *C. jejuni* in the intestine of chickens, Hermans et al. (2011) revealed that *C. jejuni* have developed several survival and colonization mechanisms responsible for the bacterium highly adapted nature to the chicken host. It is generally accepted that *Campylobacter* adhesion to epithelial cells is an important step in successful colonization, and the caecum is the predominant site for colonization (Beery et al., 1988; Stern et al., 1988), but administration of selected probiotics in combination with prebiotics may reduce colonization (Arsi et al., 2015). According to Stern et al. (1988) as few as 35 cfu of *C. jejuni* can be sufficient for successful colonization in chicken. According to Brownell et al. (1970), the caecum is the main site of *Salmonella* colonization in chicken.

Fuller (1973b) reported that *E. coli* were associated preferentially to the crop epithelium. In a later study, Snoeyenbos et al. (1982) reported highest colonization ration of *E. coli* in the caecum of holoxenic chickens in contrast to Barrow et al. (1988b) revealing that *E. coli* in the caecal contents were higher than those of the caecal epithelium. The verotoxin-producing *E.*

coli O157 is not generally reported in poultry, despite being able to colonise the caecum of chickens (Schoeni and Doyle, 1994). Soerjadi-Liem et al. (1984) reported that newly hatched monoxenic chickens subcutaneously injected or orally exposed to *Y. enterocolitica*, the major colonisation of the bacterium was revealed in the crop and caecum compared to the other regions of the GI tract.

C. perfringens is a part of the "normal" intestinal microbiota of chicken, but the levels reported in the intestine vary considerably from sporadic and low numbers to 10^8 or more (Engberg et al., 2002; Ficken and Wages, 1997). Discrepancy seems to occur with regard to colonization of *C. perfringens*. (Vissiennon et al., 1994) reported *C. perfringens* in the intestinal lumen without adhesion onto the intestinal mucous membrane. Craven (2000) revealed that *C. perfringens* was recovered more frequently from the crop, proventriculus, duodenum, jejunum, ileum and caecum, but not the gizzard of chicken fed a 50% rye diet than birds fed the corn based diet. However, no conclusion could be drawn with respect to colonisation as the intestinal segments and their contents were investigated and no distinction could be made between the autochthonous (adherent) and the allochthonous bacteria. Pedersen et al. (2003) concluded that none of the three *C. perfringens* type A used in their study was able to colonise the intestine permanently as they were eliminated within nine days. In contrast to these results, Collier et al. (2003) suggested colonization of *C. perfringens* to ileal and jejunal mucosa.

Table 1.13-2 Pathogenic bacteria in the gastrointestinal (GI) tract of chicken

GI tract segment	Pathogenic bacteria	References
Caecum	<i>Campylobacter jejuni</i>	(Beery et al., 1988; Stern et al., 1988)
	<i>Campylobacter</i>	(Amit-Romach et al., 2004)
"Fecal samples"	<i>Campylobacter</i>	(Gaucher et al., 2015)
	<i>Campylobacter jejuni</i>	(Bolder et al., 1999)
Caecum	<i>Salmonella typhimurium</i>	(Brownell et al., 1969; Brownell et al., 1970); (Snoeyenbos et al., 1982)
	<i>Salmonella typhimurium</i>	(Amit-Romach et al., 2004)
"Fecal samples"	<i>Salmonella enteritidis</i>	(Bolder et al., 1999)
Crop	<i>Escherichia coli</i>	(Fuller, 1973b)
Caecum	<i>E. coli</i>	(Snoeyenbos et al., 1982); (Barrow et al., 1988a); (Amit-Romach et al., 2004)
Small intestine	<i>E. coli</i>	(Hock E., 1997)
Jejunum	<i>Enterococcus</i>	(Schokker et al., 2015)
Crop and caecum	<i>Yersinia enterocolitica</i>	(Soerjadi-Liem et al., 1984)
Small intestine	<i>Clostridium</i>	(Amit-Romach et al., 2004)
Ileum	<i>Clostridium</i>	(Lu et al., 2006)
Digesta in jejunum and ileum	<i>Clostridium perfringens</i>	(Collier et al., 2003)
Ileum and caecum	<i>Clostridium</i> -like	(Olsen et al., 2008)
Contents from ileum and caecum	<i>Clostridium</i> spp.	(Czerwiński et al., 2012)
Ileum, caecum and rectum	<i>C. perfringens</i>	(Engberg et al., 2000)
Jejunum, caecum, cloaca and feces	<i>C. perfringens</i>	(Mitsch et al., 2004)
	<i>C. perfringens</i> was not changed at day 7 but significantly reduced at day 21 in zinc bacitracin-fed chicken	(Chee et al., 2010)
"Fecal samples"	<i>C. perfringens</i>	(Bolder et al., 1999); (Knarreborg et al., 2002); (Gaucher et al., 2015)

Preventing colonisation of foodborne pathogens in the digestive tract of poultry is of high importance to evaluate as contamination of poultry products by foodborne human pathogens, such as genus *Campylobacter* and *Salmonella* is a considerable challenge for commercial producers in several countries. Readers with interest on this topic are referred to the review papers of Mead, Revollo and Ghareeb (Ghareeb et al., 2015; Mead and Adams, 1975; Revollo et al., 2006) and the research paper of Varmuzova et al. (2015).

1.14 Do coccidiostats exert a growth promoting effect?

Whereas coccidiostats are approved because of their efficacy against a specific disease condition (intestinal coccidiosis), antibiotic growth promoters (AGPs) were approved because of their ability to improve production performance, i.e. growth rate and feed efficiency. This distinction may seem to indicate that coccidiostats are used solely to prevent disease, whereas AGPs have been used only to increase profits for the farmers and the industry. However, in reality there is no clear-cut difference between these two aspects. Both groups of feed additives have prevented disease and therefore also improved production efficiency. The reason is that the diseases mitigated by these additives impair growth and feed utilization. Preventing these diseases therefore means not only improving broiler health but also increasing broiler growth and improving feed efficiency.

The historical distinction between a disease-preventing and a growth-promoting group of feed antimicrobials may in part be caused by the fact that coccidiosis was identified as a health problem before the 1940's, while necrotic enteritis was not described until 1961 (Parish, 1961).

Substances that were effective against coccidia were not effective (at least not to the same degree) against *C. perfringens*, and vice versa. There was therefore a need for two groups of feed additives, although the reason for this need was not recognized.

The intestinal microbiota of broilers is complex and dynamic. Many other microbes than *C. perfringens* and coccidia (*Eimeria* spp.) are likely to affect the function and structure of the digestive system. This complexity is probably one of the reasons that the mode of action of the AGPs still is incompletely understood.

In spite of the historical distinction between coccidiostats and AGPs, some of the used substances actually inhibit the growth of both coccidia and bacteria. The sulfonamides are an example of such substances, the polyether or ionophorous antibacterials is another. The first ionophore (monensin) was discovered in 1967 (Kevin et al., 2009), and several other ionophores, including narasin, were discovered during the following years. The ionophores show a broad spectrum of bioactivity, including antibacterial and anticoccidial activities. Their activity is characterized by a strong selectivity towards Gram-positive bacteria, as is a majority of the AGPs that have been used in broiler feeds.

In conclusion, coccidiostats exert a 'growth promoting' effect by preventing intestinal diseases that adversely affect intestinal functions (in particular nutrient digestion and absorption), and consequently also impair feed utilization and growth of the birds. 'Prevention of diseases and intestinal microbiota causing growth depression' is probably a more appropriate designation for this effect than 'growth promotion'. In any case, this effect of coccidiostats should not be confused with the growth promoting effect of hormones

administered to food-producing animals. Growth-promoting hormones were mostly used in cattle from the mid-1950s until the early 1970s, when a ban on diethylstilbestrol was imposed in the US (<http://www.fao.org/docrep/004/X6533E/X6533E01.htm>).

2 Hazard identification and characterisation

2.1 Literature

Coccidiostat resistance in bacteria

A literature search using relevant terms such as; Narasin AND bacteria, AND resistance using the Advanced Search Builder provided by PubMed (www.ncbi.nlm.nih.gov/pubmed) or Web of Science was performed.

A similar search using the same terms, but Salinomycin / Monensin / Lasolocid / Maudoramycin / Non-ionophore / Diclazuril / Decoquinat / Halofuginon / Nicrabazin / Narasin-Nicrabazin instead of narasin was also performed. The reference lists in the selected citation were scrutinized to identify additional articles or reports, overlooked by the searches. Titles and abstracts of all identified citations were screened and were excluded if they did not relate to the terms of reference. The titles of all hits were scanned, and for those that were of potential relevance, the abstracts were also scanned. Of these, for those of potential relevance, the full text was obtained and assessed whether it was of relevance to this Opinion. Original and review articles, and textbook content were included in this assessment.

A list of the articles on coccidiostatics, which fulfilled the inclusion criteria with summary of the findings and main conclusion, is presented in Appendix I, Tables AI-1 – AI-5.

Coccidiostat resistance in coccidia

Search was provided by webofknowledge.com [v.519] using key words; anticoccidial resistance. Relevant literature was checked; newest to oldest and highest to lowest citation. Papers with highest citation were carefully checked for relevant papers published in peer-reviewed open access scientific journals, and books, as well as minimally circulated investigations available as short communications, and abstract presented in books from international conferences. In addition, search using the following key words; Narasin / Lasolocid / Monensin / Salinomycin / Maudoramycin / Semduramicin / Robenidine / Diclazuril / Decoquinat / Halofuginone and Nicrabazin was also performed.

Effects of coccidiostats on intestinal microbiota

Search was provided by webofknowledge.com [v.519] using the key words; Narasin / Lasolocid / Monensin / Salinomycin / Maudoramycin / Semduramicin / Robenidine / Diclazuril

/ Decoquinat / Halofuginone and Nicrabazin in combination with the following key words; bacteria / gut bacteria / intestinal bacteria / gut microbiota and intestinal microbiota.

Transfer of resistance genes

Literature searches using the terms Horizontal gene transfer or Lateral gene transfer combined with the terms Narasin, Salinomycin, Monensin, Lasolocid, Maudoramycin, Semduramicin, Robenidine hydrochloride, Diclazuril, Decoquinat, Halofuginon, Nicarbazin or ionophores were performed on PubMed. The searches provided a total of 114 scientific papers of which only two were relevant. A Google search provided one additional, relevant paper.

Searches on PubMed using the terms Horizontal gene transfer or Lateral gene transfer combined with *Eimeria* did not provide any scientific papers. A Google search with the same terms did not provide any additional relevant information.

Searches on PubMed using the terms Horizontal gene transfer or Lateral gene transfer combined with Eukaryotic parasite or Protozoa provided more than 200 scientific papers of which three, including a review from February 2015, were selected to be the most informative.

Alternatives to in-feed antimicrobials

Searches for use of in-feed anticoccidials and therapeutic antibacterials, alternatives to in-feed antimicrobials, and use of therapeutic antibacterials if ionophorous anticoccidials are replaced by non-ionophorous anticoccidials or anticoccidial vaccines all followed the same procedure. References were searched in Web of Science and PubMed. Initial search strings were adapted to search engine and topic. The following terms were important in initial searches: (alternative OR replac*)/ (antibiotic*/ antibiotic growth promoter/coccidiostat/anticoccidial/broiler/turkey//poultry/necrotic enteritis/*C. perfringens*). The titles of all hits were scanned, and for those that were of potential relevance, the abstracts were also scanned. Of these, for those of potential relevance, the full text was obtained and assessed whether it was of relevance to this Opinion. New search terms were used if relevant key word were found during the search process. References in examined full text articles with potential relevance were scrutinized by means of new searches, and full text obtained if deemed appropriate. Original and review articles, and textbook content were included in this assessment.

2.2 Hazard identification and characterisation

2.2.1 Resistance to coccidiostats in bacteria

A list of scientific articles published in the international journals which fulfilled the inclusions criteria is found in Appendix I. Only original papers regarding the antibacterial activity of the coccidiostats and their ability to induce resistance in bacteria were included. Evaluation of data from the national antimicrobial surveillance programmes in Norway (NORM-VET), Sweden (SWARM), Denmark (DANMAP) and Finland (FINRES-vet) showed similar trends in resistance patterns of bacterial studied.

Test materials for isolation of bacterial species in all of these studies were faeces. Test materials other than faeces have been specifically indicated in the Tables in the Appendix IV.

The evaluated scientific articles gave limited information regarding antimicrobial agents used for therapeutically purposes of animals in these studies.

Definite confirmation of cross or co-resistance between the coccidiostats and other antibacterial agents in bacteria is dependent on identification of gene(s) encoding resistance. However, the Panel is not aware of any scientific literature identifying such genes against any of the ionophores in any bacterial species.

2.2.1.1 Narasin

Thirteen original articles fulfilled the criteria (see Chapter 2.1) to be included in this part of the risk assessment. Enterococci (*E. faecium* and *E. faecalis*) and *Clostridium perfringens* were the most frequently bacterial species examined for development of resistance. Data regarding development of resistance in many Gram-positive bacteria like staphylococci and including non-cultural species is lacking. Most of the studies were performed in poultry (faeces, meat).

Resistance to narasin:

Resistance against narasin in enterococci (primarily *E. faecium* and *E. faecalis*) has been reported in several scientific papers, as well as in official surveillance reports, Appendix I, Table AI-1. High prevalence of resistance against narasin was observed in enterococci from broilers in Norway, Table 3.3.2-1. Similar data have also been observed in Sweden (SWARM). No resistance in *C. perfringens* has been reported in spite of widespread use of this ionophore in many countries for several decades (Johansson et al., 2004; Lanckriet et al., 2010; Martel et al., 2004; Silva et al., 2009b; Watkins et al., 1997).

Cross-resistance between narasin and other antibacterial agents:

Cross-resistance between narasin and salinomycin was reported in enterococci isolated from animals, mainly broilers (Butaye et al., 2000; Butaye et al., 2001).

In the Norwegian surveillance program NORM-VET, narasin resistant enterococci have been isolated from monensin fed turkeys (Table 3.3.2-1), indicating that monensin might induce narasin resistance.

Using surveillance data from Norway from the years 2004-2014, a statistical association was observed between resistance against narasin and bacitracin in *E. faecium* from broilers (See NORM-VET report 2014 and additional calculations where a possible cross-resistance between narasin and monensin in turkey isolates was taken into account Appendix II) However according to the NORM-VET report 2014 (NORM/NORM-VET, 2014): " whether narasin resistance leads to cross-resistance with bacitracin or vice versa, or the presence of both resistances can be explained by some underlying cause(s) remains unclear. Consequently, in order to investigate if there is a possible causal relationship between the use of narasin and resistance to other antibacterials further studies are needed."

The number of vancomycin-resistant enterococci in Norwegian poultry is too low to be included in the statistics described above. However, studies have shown that former use of avoparcin, which induces cross-resistance to vancomycin, has selected for a reservoir of vancomycin resistant enterococci in Norwegian broiler production (NORM/NORM-VET, 2014). Avoparcin was routinely used as a growth promoter in Norwegian broiler and turkey production from 1986 until it was banned in 1995. The reservoir has persisted after the ban was implemented. An important question is whether the use of narasin has contributed to the persistence of this reservoir.

In the Norwegian surveillance programme NORM-VET, vancomycin resistant *E. faecium* has been identified by selective methods which favor the growth of these bacteria and subsequently been tested for resistance to other antibacterials, including narasin. This was done in 2009 and 2011 on bacteria isolated from broilers and in 2013 on bacteria isolated from turkeys, Table 2.2.1.1-1. The cut-off value for narasin resistance in *E. faecium* was changed in 2013 from 2 mg/L to 4 mg/L as suggested by EUCAST. In this report, an epidemiological cut-off of >2 mg/L is chosen for all isolates because >4 mg/L, according to the NORM-VET representatives, cuts through MIC distributions for *E. faecium* from some animal categories studied (e.g. broilers) in a manner not in agreement with the concept of wild-type distributions.

When applying a chi square test to the results, the number of narasin resistant *E. faecium* from broilers was significantly higher than expected ($p < 0.05$). On the other hand, the number of narasin resistant isolates from turkey was not higher than expected ($p > 0.05$).

Although these results may indicate a statistical relationship between narasin and vancomycin resistance, the number of sample is small. Furthermore, this is in contrast to an earlier Norwegian study, where the authors concluded: "a link between vanA genes and a narasin-resistance gene would seem unlikely because the vancomycin sensitive poultry enterococci did not express a lower MIC for narasin than the vancomycin-resistant enterococci did" (Sorum et al., 2004). Consequently, further studies on Norwegian isolates are required before conclusions can be drawn.

Table 2.2.1.1-1 Observed and expected numbers and percentages of narasin resistant isolates in groups of vancomycin resistant *E. faecium* from broiler and turkey faeces

Animal tested	Number of isolates tested	Narasin resistant isolates				p value**
		Observed no	Observed %	Expected no	Expected %*	
Broiler	57	57	100	40	71	<0.0001
Turkey	16	15	94	12	75	0.08

* Expected percentage is the percentage of narasin positive isolates among all isolates tested from broiler and turkey faeces.

** p value < 0.05 means that the observed number of narasin resistant isolated differs significantly from the expected number.

In a Swedish laboratory study, 26 vancomycin resistant enterococci isolated from poultry were separated into 11 clones. Vancomycin resistance was transferrable from the predominant and five minority clones in laboratory experiments. Resistance to narasin was co-transferred with vancomycin resistance in four of the six clones, including the predominant (Nilsson et al., 2012). Furthermore, according to a personal communication from Oskar Nilsson (National Veterinary Institute, Sweden) to one of the authors preparing this draft opinion, a putative resistance gene against narasin may have been identified at a plasmid also harboring a resistance gene against vancomycin in enterococci from Swedish poultry. However, as data is still preliminary, not peer reviewed and not published, further studies are needed before any conclusions can be drawn.

2.2.1.2 Lasalocid

Only 4 articles fulfilled the criteria to be included in this risk assessment. Only two studies were performed in broilers (Appendix I, Table AI-2).

Resistance to lasalocid:

C. perfringens and *Pediococcus acidilactici* and *Pervotella* (Bacteriodes) isolates were susceptible to lasalocid.

Cross-resistance between lasalocid and other antibacterial agents:

Lasalocid-and monensin-resistant adapted cultures of *Clostridium aminophilum* cultures were as susceptible to most antibacterials as non-adapted cultures. However, bacitracin displayed a 32-fold greater MIC value in the ionophore-adapted cultures (Houlihan and Russell, 2003).

2.2.1.3 Monensin

Thirteen original articles fulfilled the criteria to be included in this risk assessment. The published articles report susceptibility testing of monensin towards different bacterial species like *Enterococcus* spp, *Pediococcus* spp, *Clostridium* spp, *Prevotella* spp, *Fibrobacter* spp, *Veillonella* spp, and anaerobic bacteria isolated from different animal species (Appendix I, Table AI-3). The studies were performed in different animal species, but mainly in cattle. Only a single study included samples from poultry (Dutta et al., 1983).

Resistance to monensin:

No resistance was observed in *C. perfringens* isolates. Resistance against monensin was observed in *Clostridium aminophilum*, *Prevotella*, *Butyrivibrio*, *Fibrobacter*, *Veillonella*, and *Enterococcus*, and in anaerobe bacteria (Appendix I, Table A3).

Cross-resistance between monensin and other antibacterial agents:

Full cross-resistance between monensin and salinomycin was found in *E. faecium* from farm animals (Butaye et al., 2001). In a laboratory study using Gram negative rumen bacteria, increased resistance to one of these ionophores caused increased resistance to the other. Furthermore, cross-resistance to lasalocid and the antibacterial avoparcin was reported (Newbold et al., 1993). Enterococcal isolates from cattle fed monensin or monensin-tylosin displayed greater levels of resistance towards macrolides (erythromycin and tylosin), but there was no effect on the concentrations of the two macrolide resistance genes, *ermB* or *tetM*, in fecal samples (Jacob et al., 2008).

Monensin- and lasalocid-resistant adapted cultures of *Clostridium aminophilum* cultures were as susceptible to most antibacterials as non-adapted cultures. However, bacitracin displayed a 32-fold greater MIC value in the ionophore-adapted cultures (Houlihan and Russell, 2003).

In the Norwegian surveillance program NORM-VET, narasin resistant enterococci have been isolated from monensin fed turkeys, Table 3.3.2-1, indicating that monensin might induce narasin resistance

2.2.1.4 Salinomycin

Twenty-one original articles fulfilled the criteria to be included in this risk assessment. Resistance in different bacterial species like *S. aureus* and other staphylococcal species, *Clostridium perferingens*, *enterococci*, *Clostridium difficile*, *Listeria monocytognes*, anaerobe bacteria from cattle, *E. coli*, Gram-negative bacteria from pigs, *Salmonella* were examined. The studies were performed in different animal species, human, and foods.

Resistance to salinomycin:

E. faecium and *E. faecalis* are the most commonly reported bacterial species to have developed resistance against salinomycin (Butaye et al., 2000; Tremblay et al., 2011; Wiggins, 1996; Yoshimura et al., 2000). *C. perfringens* and *S. aureus* were susceptible to salinomycin in all studies. Anaerobe bacteria from cattle may also develop resistance against salinomycin. Enterococcal isolates from poultry in Denmark show high prevalence of resistance against salinomycin, which is the most common used coccidiostat in Denmark (DANMAP).

Cross-resistance between salinomycin and other antibacterial agents:

Full cross-resistance between salinomycin and narasin was evident, whereas no cross-resistance between these two ionophores, monensin and lasalocid was detected in one of the studies (Appendix I, Table AI-4, (Butaye et al., 2000)).

2.2.1.5 Maduramicin

Only one study was identified.

Resistance to Maduramicin

The study performed by Lanckriet et al. (2010) found that *C. perfringens* isolates, found in poultry were uniformly susceptible to all ionophore agents, including maduramicin (Appendix I, Table A5)

2.2.1.6 Semduramicin

No study was identified.

According to FDA: Semduramicin, an ionophorous agent had no remarkable activity against Gram-negative microorganisms tested, and activity against selected Gram-positive control organisms was minimal

2.2.1.7 EFSA´s evaluation

Several scientific opinions have been prepared by EFSA, regarding use of coccidiostats in poultry (EFSA, 2004a; EFSA, 2004b; EFSA, 2004c; EFSA, 2004d; EFSA, 2004e; EFSA, 2004f; EFSA, 2007; EFSA, 2010). Terms of reference in all of these opinions were regarding development of resistance in bacteria. The EFSA´s opinion from 2004 (EFSA, 2004c) concluded the following regarding resistance in bacteria:

“The MICs of narasin for common intestinal bacterial species such as *Enterococcus* spp. and *Clostridium perfringens* are basically low but enterococci may develop resistance to narasin. There is no cross-resistance to other antimicrobials except to salinomycin. Narasin may increase *Salmonella*-shedding, but there is no reason to believe that narasin is different from other polyether ionophores in this respect. There are no data on the influence of narasin on the intestinal microflora other than on *Clostridium perfringens* and *Salmonella*. Narasin, at the levels used for treatment of coccidiosis, is also effective in the prevention of necrotic enteritis in chickens.”

The same information was reflected in the opinion from (EFSA, 2007). EFSA´s opinion from 2010 (EFSA, 2010) refers to the same literature and databases, which have been reviewed in this risk assessment: “Occurrence of enterococcal resistance to narasin has been observed in monitoring programs (NORM-VET, 2006; SVARM 2007, FINRES-vet 2005 - 2006), and complete cross-resistance between narasin and salinomycin has also been reported (Butaye et al., 2000). The latter opinion concludes that: Resistance development in *Enterococci* against narasin, including cross-resistance to clinical relevant antibacterials, should be monitored.

2.2.1.8 Non-ionophores anticoccidal agents

Due to lack of antibacterial activity of non-ionophore anticoccidal agents, development of resistance against these agents in bacterial species is not expected.

2.2.2 Resistance to coccidiostats in coccidia

2.2.2.1 Resistance and cross-resistance

Sulfonamides were the first modern anticoccidials, and in 1939 Levine (1939) demonstrated anticoccidial activity of sulphonamide either used alone or in combination with other non-ionophore coccidiostats for coccidiosis control. However, a crucial important question related to the use of coccidiostats is the development of resistance. The first to suggest that resistance might develop to anticoccidial drugs was Horton-Smith in 1952 in abstract presented at the 9th World´s Congress, Paris, France, cited in Chapman (1993). However, Joyner et al. (1963) stated that resistance do not represent a serious problem in the control

of coccidiosis in the field. Nowadays, this is no longer the case, as many drugs have been introduced and resistance has arisen to all of them. In their review devoted to anticoccidial drug resistance, Abbas et al. (2011) discussed the following topics; what is resistance, type of resistance, factors involved in resistance, geographical variations in resistance development and resistance management. According to the authors, numerous anticoccidial drugs have been used, and resistance to all of them has been reported in different parts of the world since the first study was published by Waletzky et al. (1954) (Table 2.2.2.1-1). However, it is suggested that ionophores develop resistance at a slower rate than the non-ionophore non-ionophore coccidiostats. An explanation for this slow acquisition of resistance to ionophores may be that they allow for some leakage of sensitive oocysts. This leads to a less stringent resistance selection than with non-ionophore non-ionophore coccidiostats. <http://www.noah.co.uk/issues/briefingdoc/13-anticoccidials.htm> Previously it was a general opinion that in order to minimize the occurrence of resistance it was of crucial importance to shorten the exposure time to anticoccidial drugs as much as possible and to rotate the use of various coccidiostats with a different mode of action with successive flocks, combine non-ionophore coccidiostats and ionophore treatments, or employ shuttle programs during a flock grow out (Allen and Fetterer, 2002; Peek, 2010).

Coccidiostats may be included in different feeds as the sole drug during the life of a flock (single-drug program) or in so-called shuttle programs in which several coccidiostats (ionophore or synthetic compounds) are used in different feeds in a single flock. Studies conducted in which birds were given monensin alone or in shuttle programs with various synthetic drugs, gave equivocal results (Gard et al., 1978; Kohls, 1974; Watkins and Bafundo, 1993).

Table 2.2.2.1-1 Reports of resistance in fowl coccidia against coccidiostats included in this assessment. After Chapman (1997) and Abbas et al. (2011) with some additional citation marked with *

Drug	Country	Resistance described by	Year
Narasin	USA	Weppelman et al.	1977
Lasalocid	USA	Weppelman et al.	1977
	USA	Ruff et al.	1985*
	China	Li et al.	2004*
Monensin	USA	Jeffers	1974a
	China	Tsang	1980*
	Gt. Britain	Chapman	1982
	USA	Ruff et al.	1985*
	USA	Austine et al.	1986*
	Germany	Stephan et al.	1997
	China	Li et al.	2004*
Salinomycin	USA	Ruff et al.	1985*
	USA	Jeffers	1989
	Germany	Stephan et al.	1997
	India	Yadav and Gupta	2001
	China	Li et al.	2004*
	Pakistan	Abbas et al.	2008a
	Iran	Arabkhazaeli et al.	2013*
	USA	Chapman and Jeffers	2015*
Maduramicin	USA	McDougald et al.	1987
	Germany	Stephan et al.	1997
	China	Li et al.	2004*
	Pakistan	Abbas et al.	2008a,b
Semduramicin	China	Li et al.	2004*
Robenidine	USA	Jeffers	1974a
	USA	McLoughlin and Cute	1979*
	Germany	Stephan et al.	1997
Diclazuril	Czechoslovakia	Bedrnik et al.	1991*
	Brazil	Kawazoe and Fabio	1994
	Germany	Stephan et al.	1997
	Pakistan	Abbas et al.	2009*
	Iran	Arabkhazaeli et al.	2013*
Decoquinat^a			
Halofuginone	France	Hamet	1986
	Germany	Stephan et al.	1997

Drug	Country	Resistance described by	Year
Nicarbazin	Britain	Hemsley	1964
	India	Gill and Bajwa	1979
	USA	McLoughlin and Cute	1979*
	Germany	Stephan et al.	1997
	USA	Bafundo et al.	2008*

Readers with further interest on drug resistance in avian are referred to the review papers of (Chapman, 1984; Chapman, 1993; Chapman, 1997; Smith et al., 1981), the Ph.D. thesis of Peek (2010) and the research paper of Stephan et al. (1997).

One of the main debates still ongoing amongst coccidiologists is the ability for acquiring resistance to one drug by the use of another drug, the so-called cross-resistance (Chapman, 2007). According to Chapman (1998), "multiple resistance is resistance to more than one drug, even though they have different mode of action". It is therefore of importance to evaluate that cross-resistance and multiple resistance are not similar. In an early study, Jeffers (1974) displayed that two strains of *E. tenella* differing in resistance to amprolium and decoquinate. Based on their cation selectivity, transport rate capacity and structure, three classes of ionophores can be discriminated (Pressman, 1976; Westley, 1982), monovalent, monovalent glycoside and divalent ionophores. Several papers indicate that cross resistance is less obvious between products of different classes, for instance between maduramicin and monovalent ionophores or between lasalocid and monovalent ionophores (Bedrnik et al., 1989; Marien et al., 2007; McDougald et al., 1987). Evidence of incomplete cross-resistance within a certain ionophore class is illustrated by the fact that, after years of use of the monovalent ionophore monensin, resistance to narasin in United States was encountered before the product was commercially launched (Weppelman et al., 1977). However, cross-resistance may not always occur between different compounds with similar mode of action. (Smith et al., 1981) revealed inconsistent results of cross-resistance in *Coccidia* isolates to lasalocid, monensin, narasin or salinomycin having similar mode of action. Weppelman et al. (1977) revealed no differences in efficacy between narasin and monensin against *E. acervulina*, *E. tenella* and *E. maxima*. In contrast, lasalocid had an effect towards some strains that were not well affected by either narasin or monensin. Augustine et al. (1987) addressed the effect of monensin, salinomycin and lasalocid and revealed that all three coccidiostats markedly inhibited invasion of cecal tissues by sporozoites of ionophore sensitive (IS) *E. tenella*. As data suggest difference in ionophore accumulation by IS and resistant isolates of *E. tenella*, the authors suggested that the differences might be related to differences in membrane permeability. Bedrnik et al. (1989) investigated the effect of monensin, narasin, salinomycin, maduramicin and lasalocid on two field isolates of *E. tenella* obtained from a farm with a long-term occurrence of coccidiosis and revealed that monensin, narasin and salinomycin had no effect. In contrast, maduramicin and lasalocid controlled them effectively. In an early study, Ryley et al. (1980) evaluated cross resistance

between monensin and lasalocid and two coccidia strains, one sensitive and one resistant. Each treatment starting the day before inoculation and based on observations on mortality, fecal score and weight gain it was revealed that optimal control of the sensitive strain was revealed at 240 ppm monensin, while 150 ppm lasalocid was efficacious. With the resistant strain, results with 240 ppm monensin were less effective, but a bit more effective to those achieved with 120 ppm on the sensitive strain. Based on the results, the author suggested approximately a 2-fold degree of resistance. With regard to lasalocid, 300 ppm was required for the resistant strain to achieve similar degree of control as 150 ppm on the sensitive strain, again a 2-fold degree of resistance. The results of Ruff et al. (1985) are of interest, as the results revealed that a field strain of *E. tenella* was markedly less sensitive to narasin, lasalocid and monensin than a laboratory isolate that has never been exposed to coccidiostats. Abbas et al. (2008) evaluated multiple resistance in three field isolates of *E. tenella* collected from poultry farms - with a history of prophylactic anticoccidial medication failure, and revealed that only one isolate displayed multiple resistance to salinomycin, maduramicin and clodolol.

There may be several reasons for the development of multiple resistance in field isolates; altered permeability of the cell membrane so that the drug is no longer taken up or is rapidly pumped out of the cell, use of alternative biochemical pathway, modification of the target sites in the coccidia (Abbas et al., 2011) as well as genetic recombination (Stephan et al., 1997).

2.2.2.2 Resistance to different coccidiostats and combination of drugs

In an early study, Waletzky and Hughes (1946) revealed that only a few of the 45 sulfanilamides tested; the halogenated sulfapyrimidines, sulfamethazine and sulfapyrazine, which were ten, five and four times as active as sulfaguanidine against *E. tenella*, respectively. Varga and Sréter (1996) investigated the coccidiostat feed supplementation efficacy of a combination of monensin (8 p.p.m.) and duokvin (120 p.p.m.) with that of only monensin at recommended level of 100 p.p.m. against a field isolate of *E. acervulina*. The authors revealed no significant difference in the chemoprophylactic activity of the treatments. In a study with ten *Coccidia* field isolates from north Germany, Stephan et al. (1997) revealed that partial or complete resistance to nicarbazin was noticed in eight isolates, maduramicin and halofuginone in seven, to monensin in six, to salinomycin in five, to diclazuril in two and to toltrazuril in one field isolate. However, the authors stated that for *Coccidia* strains with low resistance the results can be misinterpreted, as in spite of high oocyst index, very good overall results can be obtained due to growth promotion.

The effect of different coccidiostats supplemented alone or in combination with duokvin (120 p.p.m.) against *Cryptosporidium bailey* were investigated by Varga et al. (1996) and revealed the following efficacy percentages; lasalocid 45, monensin 37, semduramicin 29, narasin 23

and salinomycin 21. However, the efficacy could increase 36-80% by combination with duokvin, with the exception of lasalocid.

When discussing the resistance to coccidiostats, it may also be of importance to notice that Kochansky and Pettis (2005) demonstrated that several of the polyether ionophores revealed high activity against American foulbrood, caused by *Paenibacillus* larvae. Narasin was most active, followed by lasalocid and salinomycin.

2.2.3 Effects of coccidiostats on intestinal microbiota

Antibacterial growth promotion in agricultural animal production has been practiced for nearly 70 years in the United States and other countries. Early indications of a beneficial effect on production efficiency in poultry was reported by Moore et al. (1946) and numerous investigations have reported modulation of the gut microbiota of poultry by antibacterial supplements e.g. (Barnes and Goldberg, 1962; Butaye et al., 2003; Ford et al., 1981; George et al., 1982). Modulation of the intestinal microbiota towards a “healthy” community by feeding probiotics, prebiotics and lactoferrin are investigated e.g. (Alloui et al., 2013; Apajalahti et al., 2004; Ducatelle et al., 2015; Geier et al., 2011; Giannenas et al., 2012; Oviedo-Rondón et al., 2010; Pourabedin and Zhao, 2015; Saleh et al., 2014; Zhao et al., 2013) as such supplements improve gastrointestinal health and suppress known intestinal and food-borne pathogens.

Several studies have been performed on the effects of coccidiostats on intestinal microbiota in chickens and these studies revealed modulation of the gut microbiota. The effects of coccidiostats in focus on in the present assessment on the intestinal microbiota are shown in Table 2.2.3-1. To our knowledge, no information is available on the effect of semduramicin, robenidine, diclazuril and decoquinate on gut microbiota. One study, (Bailey et al., 1988) investigated the effect of nicarbazin in combination with other antimicrobials, but no information is available on only nicarbazin administration.

Table 2.2.3-1 Effect of coccidiostats on gut microbiota

Coccidiostats	Effect on gut microbiota	References
Narasin	Significant reduction of <i>C. perfringens</i> counts	(Kaldhusdal et al., 2012)
Lasalocid	Higher total anaerobe counts, <i>Enterobacter</i> spp., <i>Enterococcus</i> , ratio of <i>E. coli</i> : <i>Lactobacillus</i> spp. No effect on total aerobes, <i>C. perfringens</i> , <i>Bifidobacterium</i> spp.	(Giannenas et al., 2012)
Monensin	No effect on cecal colonization of <i>Salmonella</i>	(Manning et al., 1994)
	Crop. No effect on lactobacilli counts. At day one, significant reduced numbers of coliforms and enterococci. At day five, significant increase in number of coliforms, but numbers of enterococci were not affected Caeca. Lactobacilli counts were significantly increased at day one, but no effect was noticed at day five. Coliforms were significantly reduced at day one, but no effect was revealed at day five. Enterococci counts were not significantly affected at day one or day five	(Rada and Marounek, 1997)
	Significant effect of microbiota. Rich in Clostridia (<i>Clostridium irregularis</i> and <i>C. lituseburens</i>) and <i>Lactobacillus crispatus</i> , but low in <i>Lactobacillus acidophilus</i>	(Liu and Reynolds, 1999; Liu et al., 2005)
	Reduced numbers of sequences and numbers of operational taxonomic units (OTUs) Shannon (diversity) was unaffected Depletion of <i>Roseburia</i> , <i>Lactobacillus</i> and <i>Enterococcus</i> Enrichment of <i>Copracoccus</i> and <i>Anaerofilum</i>	(Danzeisen et al., 2011)
Salinomycin	No resistance selection in coliforms and streptococci.	(George et al., 1982)
	Significantly reduced the incidence of <i>Salmonella</i> shedding at week 6, but no effect on <i>Campylobacter</i> shedding	(Bolder et al., 1999)
	Significantly lower counts of <i>Campylobacter perfringens</i> and <i>Lactobacillus salivarius</i> . No effect on counts of anaerobic bacteria, <i>C. perfringens</i> , coliforms and lactose-negative enterobacteria	(Engberg et al., 2000)
	Scanning electron microscopy revealed fewer bacteria in the ileum	(Chichlowski et al., 2007)
	Reduced counts of <i>Lactobacillus</i> -, Enterobacteriaceae- and <i>Clostridium</i> -like bacteria in the lumen of ileum	(Olsen et al., 2008)

Cocciostats	Effect on gut microbiota	References
	Ileal digesta. Reduced total numbers of bacteria and <i>Lactobacillus/Enterococcus</i> caecal digesta. Increased total numbers of bacteria, but reduced <i>Lactobacillus/Enterococcus</i>	(Czerwiński et al., 2012)
Maduramicin	Crop. No effect on lactobacilli counts. At day one, significantly reduced numbers of coliforms and enterococci. At day five, significant increase in number of coliforms, but numbers of enterococci were not affected Caeca. Lactobacilli counts were significantly increased at day one, but no effect was noticed at day five. Coliforms were significantly reduced at day one, but no effect was revealed at day five. Enterococci counts were not significantly affected at day one or day five	(Rada and Marounek, 1997)
Halofuginone	Chicken fed halofuginone at 3 ppm revealed no significant increase in excretion rate of <i>Salmonella typhimurium</i> . However, animals fed 6 ppm halofuginone showed a significant increase in excretion of <i>S. typhimurium</i>	(Barrow et al., 1988b)

2.2.4 Transfer of genes mediating resistance to coccidiostats

2.2.4.1 Bacteria

The literature searches performed identified only one original scientific paper addressing horizontal transfer of resistance against coccidiostats. The paper described transfer of horizontal transfer of phenotypic narasin resistance between different clones of *E. faecium* in a laboratory experiment (Nilsson et al., 2012). However, horizontal transfer of a large variety of other antibacterial resistance genes is known to occur in bacteria.

2.2.4.2 Coccidia

Until recently, the impact of horizontal gene transfer on eukaryotic evolution was thought to be limited. However, the rapid increase in publicly available eukaryotic genomic data has changed the views on the frequency and subsequent important roles horizontal gene transfer may play in eukaryotic evolution (especially unicellular organisms) (Keeling and Palmer, 2008). Studies on a number of parasitic microbial eukaryotes (including coccidia, but not *Eimeria*) indicate that these have been significantly affected by prokaryote-to-eukaryote lateral gene transfers during evolution (Alsmark et al., 2013; Alsmark et al., 2009; Hirt et al., 2015). A majority of the genes were involved in cell metabolism. A broad range of prokaryotic donors has been involved in such transfers, but in particular bacterial groups that

share the same habitats as the parasites investigated, including the host microbiota. Possible eukaryote-to-prokaryote and eukaryote-to-eukaryote gene transfers have also been identified in these parasites. However, the data indicate resistance to coccidiostats being horizontally transferred between coccidia strains or between coccidia and bacteria is not relevant in a short or medium term perspective, but rather in long term evolution.

Vertical gene transfer is the transfer of genes from parent to offspring. Studies on *E. tenelli* indicate that strains resistant to different coccidials may produce offspring carrying resistance genes from both parent strains due to gene recombination during the sexual multiplication stage in the chicken intestine. This means that two parent strains being resistant to one coccidiostat each can produce offspring with reduced resistance to both coccidiostats (Chapman, 1984).

2.3 Alternatives to in-feed antimicrobials

Coccidiostats are approved because they protect the birds against intestinal coccidiosis. The most obvious alternative to coccidiostats in the feed is vaccination against coccidia. Coccidia are intracellular parasites and are highly immunogenic, which has led to the development of vaccines based on live coccidia. Some of these vaccines are based on strains that are still pathogenic ('non-attenuated' vaccines). Such poultry vaccines are used in the US, but not in Europe. Increasing consumer demands for chickens raised without in-feed antimicrobials has driven an increasing proportion of the US broiler industry to transition from conventional to 'antibacterial-free' production practices. These practices require no in-feed antibacterial growth promoters, no ionophorous coccidiostats and no non-ionophore coccidiostats. Recent experience from the USA suggests that presently used non-attenuated vaccines and/or administration methods may not yet be fully satisfactory in conventional broiler rearing. A significant problem appears to be emergence of necrotic enteritis during the 3rd week of age (Schaeffer et al., 2015).

Research on vaccines based on subunits of coccidial antigens has so far not succeeded in producing sufficient protection against coccidiosis. Such killed vaccines would be beneficial because of lower production costs and a lower risk of contamination by other pathogens (Peek and Landman, 2011).

Alternatives included in this assessment are vaccines, eradication and feed additives other than coccidiostatica. The Panel has not assessed possible effects of other types of management changes.

2.3.1 Vaccines used in Europe

The immunogenicity of coccidia can be retained in artificially selected, non-pathogenic strains, which has led to the development of live anticoccidial vaccines that are inherently non-pathogenic (Chapman, 2012). Until recently such vaccines have been used mainly in chickens reared for egg production, but they are now increasingly used also in the broiler industry (Chapman and Jeffers, 2014). This type of anticoccidial vaccine is now used increasingly in commercial Norwegian broiler farms, instead of in-feed coccidiostats. So far clinical coccidiosis has not been experienced as a significant problem in this transition process to broiler rearing without in-feed coccidiostats (A. Løvland, personal communication).

Although there are conflicting experiences with the use of anticoccidial vaccines in broilers, it seems clear that such vaccines do induce a protective response against coccidiosis (maybe not always early enough?) but do not induce specific immunity against *C. perfringens* and necrotic enteritis.

As opposed to anticoccidial vaccines, no vaccines against necrotic enteritis caused by *C. perfringens* are yet commercially available. Major challenges in ongoing research on vaccine development include selection of the proper combination of antigens as well as the identification and implementation of an optimal administration strategy (Mot et al., 2014). The lack of commercially available vaccines against necrotic enteritis suggests that the abolishment of in-feed ionophorous coccidiostats (with an antibacterial effect against *C. perfringens* causing necrotic enteritis) may be more problematic with regard to necrotic enteritis than with regard to coccidiosis.

2.3.2 Eradication

An ideal alternative to preventive medication would be eradication from the birds' environment of the causative bacterium *C. perfringens* and the parasites (*Eimeria spp.*) causing coccidiosis, which is an important predisposing factor for necrotic enteritis. Neither of these goals is easy to reach. The coccidia form oocysts, which survive outside the host and resist commonly used disinfectants. However, effective products are commercially available and their potential may not have been fully utilised up to now. A study based on data from 2000 to 2004 produced data suggesting that at least 25 % of Norwegian broiler flocks were coccidia negative, but the data also suggested an increasing trend in the prevalence of infected flocks (Haug et al., 2008). Eradicating *C. perfringens* from the broiler environment is even more challenging than eradication of coccidia. *C. perfringens* forms spores which can survive even more harsh environmental conditions than the coccidia.

2.3.3 Other feed additives

The EU ban on antibacterial growth promoters prompted increased efforts at developing additives which could replace the banned products. Acid-based products, probiotics, prebiotics, synbiotics, yeast-based products, plant-derived products, combinations of these, and other products have been developed and marketed as feed additives with claimed positive effects on the digestive system of broilers and fattening turkeys (Abbas et al., 2012; Bozkurt et al., 2013; Dahiya et al., 2006; Dibner and Buttin, 2002; Geier et al., 2010; Stanley et al., 2014; Vidanarachchi et al., 2013). These products have been tested for efficacy against coccidia with conflicting, non-consistent or non-convincing results (Peek and Landman, 2011). Most products developed appear to target the bacterial microbiota rather than coccidia.

2.3.3.1 Acid based products

The most commonly used organic acids are propionic and formic acids, but at least 12 different acids have been included in acid-based products (<http://www.thepoultrysite.com/articles/2372/organic-acidbased-products-market-evaluation-and-technical-comment>). A majority of products contain more than one acid. Organic acids are often combined with salts of organic acids, because such products are easier to handle and less corrosive. Organic acids are rapidly metabolised from crop to gizzard, which limits their effect on performance and intestinal microbiota, but use of double salts (Luckstadt and Mellor, 2011) and/or coating/encapsulation are used to overcome these problems. Butyric acid and potassium diformate have been shown to improve broiler performance and decrease the incidence of necrotic enteritis caused by *C. perfringens* (Huyghebaert et al., 2011) (Luckstadt and Mellor, 2011). However, organic acids may also impair broiler performance, depending on acid type and inclusion levels in the feed (Patten and Waldroup, 1988).

2.3.3.2 Plant products

Plant products (phytogenic additives) vary widely with respect to botanical origin, processing and composition. Many products are based on blends of various active compounds. Phytogenic compounds may be classified into herbs (flowering, nonwoody, and nonpersistent plants), spices (herbs with an intensive smell or taste commonly added to human food), essential oils (volatile lipophilic compounds derived by cold expression, steam or alcohol distillation) or oleoresins (extracts derived by non-aqueous solvents) (Windisch et al., 2008). The number of *in vivo* studies on the efficacy of plant products is still quite limited. Production efficiency results are contradictory, although a majority of published studies may seem to indicate improved performance (Hashemi and Davoodi, 2010; Hippenstiel et al., 2011). Blends of thymol and cinnamaldehyde (Bento et al., 2013) and of thymol, carvacol,

eugenol, curcumin and piperin (Mitsch et al., 2004) have been demonstrated to reduce intestinal counts of *Clostridium perfringens*.

2.3.3.3 Prebiotics, probiotics and synbiotics

Prebiotics. Gibson and Roberfroid (1995) defined prebiotics as an "ingredient of the nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and / or activity of a limited number of bacterial species already resident in the colon, and thus attempt to improve host health". Oligosaccharides are commonly used in commercial products. Mannan-oligosaccharides have been shown to reduce *C. perfringens* levels in turkeys (Alloui et al., 2013).

Probiotics are defined by FAO/WHO as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Kabir, 2009). This definition excludes products based on killed bacteria, which is in agreement with the lack of solid documentation of the efficacy of non-viable forms of probiotic strains (Aureli et al., 2011; Nurmi and Rantala, 1973) detected that gut contents from adult, healthy hens could protect young birds against *Salmonella* infection in the same way as the normal intestinal microbiota of adult chickens (Schneitz, 2005). The term 'competitive exclusion' was coined to designate this phenomenon. Competitive exclusion (CE) products have been associated primarily with undefined products, i.e. products based on a high number of partially unknown strains of intestinal microbes from healthy individuals. The interest in probiotics as promoters of gastrointestinal broiler health is more recent than their use against *Salmonella spp.* Regulatory agencies have been reluctant to approve undefined microbial products due to the uncertainty of a consistent composition of the products. This concern has paved the way for defined products based on one or a few known strains (Kerr et al., 2013). Defined probiotic products marketed for broilers can be categorized as a) Non-spore forming bacteria (usually lactobacilli, enterococci, bifidobacteria) and (b) Bacterial spore formers (*Bacillus spp.*). Probiotic bacteria are vulnerable to long-lasting and high temperatures during broiler feed processing. A potential advantage of spore formers is their ability to survive feed processing and the gastric barrier, although non-spore forming bacteria are usually protected by various types of coating intended to ensure delivery to the intestine. The hypothesized mechanism of **synbiotics** is that they increase the levels of beneficial bacteria, compared to the administration of probiotics or prebiotics alone (Mookiah et al., 2014; Shimizu et al., 2013) and results are sometimes difficult to evaluate due to a poor design (Alloui et al., 2013). Partly undefined competitive exclusion products have been demonstrated to reduce levels of caecal *C. perfringens* and improve production results (Schneitz, 2005). A multi-strain *Lactobacillus spp.*-based probiotic has been reported to improve average daily weight gain of turkeys (Kabir, 2009). Among single strain probiotics, a product based on a *Bacillus subtilis* strain have been shown to reduce intestinal *C. perfringens* counts and improve feed efficiency in a challenge experiment (Jayaraman et al., 2013).

2.4 Summary of hazard identification and characterisation

Resistance to coccidiostats in bacteria

- All five coccidiostats approved for use in Norway are ionophores that also display an antibacterial effect, mainly against Gram-positive bacteria. Resistance to four of them in enterococci and a few other bacteria has been reported.
- Resistance in *C. perfringens*, the causative bacterium for the poultry disease necrotic enteritis, has not been reported.
- A limited amount of data may indicate an association between narasin and resistances against bacitracin, as well as between narasin and vancomycin.
- The additional six coccidiostats which are approved in the EU display little or no antibacterial effect, and antibacterial resistance is therefore not considered to be a relevant subject.

Resistance to coccidiostats in coccidia

- Resistance to all the eleven coccidiostats in question has been reported.
- It has been suggested that ionophores might develop resistance at a slower rate than the non-ionophore coccidiostats.
- Various shuttle and rotation programmes are used to try to avoid or delay development of resistance. Information in the scientific literature on the effect of such programmes is scarce.
- Cross-resistance between different ionophores has been reported.
- Coccidia may develop resistance to more than one coccidiostat that is not due to cross-resistance, either after exposure to several coccidiostats or by gene recombination during the sexual multiplication stage in the chicken intestine.
- Horizontally transferred resistance against coccidiostats between Coccidia strains or between Coccidia and bacteria is not expected to be of importance in a short term perspective.

Alternatives to in-feed antimicrobials

- Eradication from the birds' environment of coccidia (*Eimeria spp.*) causing coccidiosis is difficult to achieve because the coccidia form oocysts which survive outside the host and resist commonly used disinfectants.
- Vaccination with non-pathogenic vaccines is now used increasingly in commercial Norwegian broiler farms, instead of in-feed coccidiostats. So far (October 2015)

coccidiosis has not been reported as a problem in this transition process to broiler rearing without in-feed coccidiostats in Norway.

- Non-antimicrobial feed additives, i.e. acid-based products, probiotics, prebiotics, synbiotics, yeast-based products, plant-derived products, combinations of these, and other products have been developed and marketed as feed additives. These products have been tested for efficacy against coccidia with conflicting, non-consistent or non-convincing results. The majority of these products appear to target the bacterial microbiota rather than coccidia.
- The Panel has not assessed possible effects of other types of management changes.

3 Exposure

3.1 Literature

Human exposure to resistant bacteria

After preliminary searches including multiple combinations of the terms antimicrobial, antibacterial, resistance, resistant bacteria, enterococci, humans, farmers, workers, infection, transfer, colonization, two review papers from 2006 and 2011 was chosen as the main sources of information.

Human exposure to coccidiostats through handling of feed preparations and feed

Relevant terms used in database searches using Web of Science, Google Scholar and PubMed: TOPIC (Ionophor OR Narasin OR Monensin OR Salinomycin OR Lasolocid OR Maduramicin) AND feed AND (occupational exposure OR occupation OR exposure OR workers); Timespan: All years

Human exposure to coccidiostats through handling contaminated manure

Web of Science: TOPIC: manure AND narasin AND exposure. A similar literature search with use of same terms, but replacing narasin with Salinomycin / Monensin / Lasolocid / Maudoramycin / Non-ionophore / Diclazuril / Decoquinat / Halofuginon / Nicrabazin was included. Also searched for: TOPIC: manure AND antibiotic AND exposure AND human, and same terms replacing manure with litter, human with farmer and antibiotic with anticoccidial.

Human exposure to coccidiostats in poultry products

Relevant terms used in database searches using Web of Science, Google Scholar and PubMed: TOPIC (Ionophore OR Narasin OR Monensin OR Salinomycin OR Lasolocid OR Maduramicin) AND (food OR meat OR eggs); Timespan: All years. The Norwegian Food Safety Authority's surveillance reports were also used.

Environmental exposure to coccidiostats

A literature search using relevant terms such as; Narasin AND degradation AND soil OR manure OR litter, Narasin AND plant uptake, Antibiotic AND plant uptake, Anticoccidial AND soil OR manure OR compost using the Advanced Search Builder provided by PubMed or same topic word in Web of Science was performed. A similar literature search with use of same terms, but replacing narasin with Salinomycin / Monensin / Lasolocid / Maudoramycin / Non-ionophore / Diclazuril / Decoquinat / Halofuginon / Nicrabazin was also performed.

Consumption of therapeutic antibacterials

The following search strings were used to shed light on the risk of increased use of therapeutic antimicrobials in poultry production under current production practices if anticoccidials with antibacterial effects are replaced by anticoccidials without such effects:

Web of Science: TOPIC: ((chemical coccidiostat*) OR (chemical anticoccidial)) AND TOPIC: (*C. perfringens*). Timespan: All years. TOPIC: (nicarbazin) AND TOPIC: (*C. perfringens*). Timespan: All years. TOPIC: (from ionophor* to (non-ionophor* OR chemical)) AND TOPIC: (broiler OR chick* OR poult*) AND TOPIC: ((therapeutic antibiotic*) OR therap*). Timespan: All years.

PubMed: ((((((ionophore or chemical coccidiostat) OR (ionophore or chemical anticoccidial))) AND therap*) AND antibiotic*)) AND (broiler OR chick*).

Resistance against narasin

The following search strings were used for literature on resistance in coccidia and *C. perfringens* against narasin:

Web of Science: TOPIC: (*Eimeria*) AND TOPIC: (narasin) AND TOPIC: (resistance OR tolerance). Timespan: All years. TOPIC: (*Eimeria*) AND TOPIC: (narasin OR monensin OR lasalocid OR salinomycin) AND TOPIC: (resistance OR tolerance) AND TOPIC: (Norwegian OR Norway). Timespan: All years. TOPIC: (*C. perfringens*) AND TOPIC: (narasin) AND TOPIC: (resistance OR tolerance). Timespan: All years.

3.2 Statistics related to in-feed coccidiostats and Norwegian poultry production and consumption

3.2.1 Use of in-feed coccidiostats in broiler rearing

Conventional broiler rearing has up to 2015 been based on continuous use of in-feed coccidiostats from the day of hatch until a few days prior to slaughter. During the last few days before slaughter the broilers are offered a feed without coccidiostats. Narasin is the only coccidiostat that has been used in Norwegian broiler rearing during the last 15-20 years. The in-feed inclusion rate of narasin is 70 mg/kg. The switch to narasin was prompted by outbreaks of severe necrotic enteritis in 1995, emerging shortly after the abolishment of in-feed antibacterial growth promoters. The antibacterial growth promoter that had been use in Norway was avoparcin from 1987, and the ionophorous coccidiostat used to most broilers from 1988-1995 was monensin. The reason narasin was chosen was probably the fact that this ionophore had been used with success and without antibacterial growth promoters in Sweden since 1990.

Up to 2015 only a small fraction (less than 10 %) of Norwegian broilers has been reared without in-feed coccidiostats. In 2014 two major distributors of broiler meat (Nortura and Norsk kylling) declared as their intention to abolish all use of in-feed coccidiostats gradually during 2015 and 2016.

3.2.2 Use of in-feed coccidiostats in turkey rearing

Norwegian slaughter turkeys are offered in-feed coccidiostats from day of hatch until they are approximately seven to nine weeks old. Female turkeys are slaughtered at approximately 11 weeks of age, male turkeys at approximately 17-18 weeks of age. Hence, female and male turkeys are raised without coccidiostats approximately 30 and 50 % of their rearing period respectively. Because feed consumption is highest during the last part of the rearing period, the fractions of feed without coccidiostats are higher than 30 and 50 %.

3.2.3 Feed statistics reported by NFSA

In NFSA's annual report on feed statistics, coccidiostat levels in commercial feeds are reported following random samplings conducted during NFSA's surveillance program each year. For 2013, for example, coccidiostat levels were analysed in a total of 81 random samples. Among these, 26 samples contained coccidiostat levels in compliance with declared amounts by the feed producers and the minimum-maximum concentration ranges specified in the Norwegian and Commission regulations (see Tables AIII-1 and AIII-2 in Appendix III). Deviations were observed in one sample, in which the level measured was below the declared level by the feed producer and the deviation was greater than the method's level of uncertainty (NFSA, 2014).

Table 3.2.3-1 Use of coccidiostats in poultry feeds from 2009 to 2014 shown as tonn feed added coccidiostats and kilogram active substance of the coccidiostats

Type of coccidiostat	Lasalocid		Monensin		Narasin		Total coccidiostats	
	Feed (ton)	active substance (kg)	Feed (ton)	active substance (kg)	Feed (ton)	active substance (kg)	Feed (ton)	active substance (kg)
2009	700	63	11034	885	126781	8621	138516	9569
2010	0	0	9417	805	133672	9080	143143	9885
2011	0	0	11325	1060	137320	9394	148645	10454
2012	0	0	12309	1080	150503	10378	162812	11458
2013	0	0	13360	1174	178233	12345	191593	13519
2014	0	0	15824	1313	182790	12409	198614	13722

3.2.4 Minimum and maximum content of coccidiostats allowed in complete diet formulations for poultry

The minimum and maximum levels of various coccidiostats in complete diet formulations are given in Appendix III's Table AIII-1 for those authorized for use in Norway and the EU and Table AIII-2 for those authorized in the EU but not Norway. It is important that the feed producers comply with these ranges to aid in avoiding development of antimicrobial resistance that may occur when insufficient doses are added and to avoid intoxications that may result when excessive doses are added. It is for these reasons the surveillance includes monitoring of coccidiostat levels following random sampling of broiler and turkey feeds. As indicated in section 3.2.6, in the last five years, non-compliance was observed in one of 26 samples (3.8%) in 2013 (NFSA, 2014).

3.2.5 Animal exposure to coccidiostats

Mean daily feed intake estimate for broilers (5 week production cycle) was calculated to be 95 g, ranging from 13 to 186 g for day 1 and 35, respectively. Thus, mean daily Narasin exposure would be in the range of 5.7-6.7 mg per day, ranging from a minimum of 0.8 to a maximum of 13 mg for day 1 and 35, respectively. Estimated cumulative exposures during a production cycle as they pertain to current practices in Norway are given in Table 3.2.5-1.

Mean daily feed intake estimate for turkeys during the first 7-9 week production cycle when they receive Monensin-supplemented diets was calculated to be as much as 92 g (range 11-190 g from week 1 to week 9) for hens and 110 g (range 12-235 g from week 1 to week 9) for toms. Thus, mean daily Monensin exposure would be in the range of 6-9 mg (range 0.7-19 mg) for hens and 7-11 mg for toms (range <1-24 mg). Estimated cumulative exposures during a production cycle as they pertain to current practices in Norway are given in Table 3.2.5-1.

Table 3.2.5-1 Estimates of target animal exposure (broilers and turkeys coccidiostat intake) over a production cycle according to current practices in Norway (Source: Animalia pers. Comm., 2015)

Active substance	Species	Dose range mg/kg feed	Production period* weeks	Cumulative feed intake kg	Cumulative coccidiostat intake mg
Monensin	Broilers	100-125	5	3.3	330-413
Monensin	Turkeys	60-100	9	5.8 for females	348-580
Monensin	Turkeys	60-100	9	6.9 for males	414-690
Narasin	Broilers	60-70	5	3.3	198-231

* For broilers, in-feed coccidiostat supplementation is practiced during the entire production cycle, with the exception of the day(s) just prior to slaughter as prescribed by the applicable withdrawal period for each coccidiostat. For turkeys, in-feed coccidiostat supplementation is only practiced during the first 7-9 weeks of the total production cycle.

3.2.6 Cross-contamination during feed production

During feed production, the coccidiostat-containing premix is added with other feed ingredients during feed formulation. Following production of the coccidiostat-containing feed, low levels of coccidiostats are retained within the production line, which is a source of cross-contamination of the next feed produced, in some cases for a non-target species or category of animal, e.g. a mammal or layer hens. Especially the initial quantities of feeds that come off the production line can contain high levels of coccidiostats and should be discarded. Yet some cross-contamination still occurs in feeds for non-target animals, as reflected by surveillance data reported by NFSA (see below).

EFSA has conducted risk assessments of cross-contamination of non-target feedstuffs with the registered coccidiostats (EFSA, 2007; EFSA, 2008a; EFSA, 2008b; EFSA, 2008c; EFSA, 2008d; EFSA, 2008e; EFSA, 2008f; EFSA, 2008g; EFSA, 2008h; EFSA, 2008i; EFSA, 2008j). The physicochemical properties of the coccidiostat-containing pre-mixes appear to be of relevance for the degree cross-contamination that can occur between batches of feeds made in multi-product feed plants, i.e. those that produce feeds for a range of animal categories/species. These pre-mix properties will affect the levels of active ingredients that are retained within the production line and consequently appear in other feeds.

In NFSA's feed surveillance reports (Föranalyser) over the last five years, all but one feed and pre-mix sample for target animals, mainly broilers and turkeys for fattening, tested for declared coccidiostat levels have shown compliance (see Table 3.2.6-1). However, numerous feed samples for non-target species or categories of animals have contained coccidiostats at trace levels, i.e. above or below maximum residue levels (MRLs) as specified by the Norwegian "Forskrift om förvarer" (Regulation for feedstuffs) of May 2, 2012, indicating low levels of cross-contamination. In 70 random samples analysed in 2014, 12 of the samples

(17%) contained trace amounts of coccidiostats (NFSA, 2015). In 2013, 17 of 53 (32%; NFSA, 2014); in 2012, 12 of 44 samples (27%; NFSA, 2013), in 2011, 15 of 31 samples (48%; NFSA, 2012); and in 2010, 1 of 13 samples (8%; NFSA, 2011) contained trace levels of coccidiostats. Of the 53 samples analysed in 2013, 11 samples contained traces of narasin, three contained traces of narasin and monensin, one contained traces of narasin and nicarbazin, and one contained traces of narasin, monensin and salinomycin.

Table 3.2.6-1 Monitoring coccidiostat content (narasin, monensin, robenidine, salinomycin, lasalocid, nicarbazin, diclazuril and maduramycin) in feedstuffs for target (those meant to receive in-feed coccidiostats) and non-target (those not meant to receive in-feed coccidiostats) animals under NFSA's surveillance program for feeds (Fôranalyser) for 2012-2014 (NFSA, 2013; NFSA, 2014; NFSA, 2015)

Type of animal feedstuffs	Number of samples			
	Total sampled	Total sampled with declared coccidiostat content for target animals	Non-compliant feeds for target animals	Non-compliant feeds for non-target animals ⁴
2012	52	8	0	12 (9/3)
Ruminants ¹	0	-	-	-
Pigs ¹	18	0	0	0/2 ⁵
Poultry ¹	18	1	0	7/1 ⁶
Premixes ²	16	7	0	2/0
Other feeds ³	0	-	-	-
2013	81	28	1	16 (16/0)
Ruminants ¹	11	0	0	0
Pigs ¹	19	0	0	5/0
Poultry ¹	49	26	1	11/0
Premixes ²	2	2	0	0
Other feeds ³	0	-	-	-
2014	70	23	0	13 (11/2)
Ruminants ¹	6	0	-	1/0
Pigs ¹	20	0	0	5/0
Poultry ¹	41	21	0	4/2 ⁷
Premixes ²	3	2	0	0
Other feeds ³	0	-	-	-

¹ The feeds for ruminants, pigs and poultry are formulated feeds for the respective animals; ² Premixes are generally blended feed ingredients containing single nutrients, such as vitamins and minerals, as well as other feed additives; ³ "Other feeds" pertain to formulated feeds for animal species other than those specified. ⁴ Feeds for non-target animals refers to those that are not meant to contain coccidiostats; values given in the column are number of analysed samples containing trace levels of coccidiostats as a result of cross-contamination that are below MRL/above MRL; MRL = maximum residue level; as set by the Norwegian regulations concerning feedstuffs (Forskrift om

förvarer); ⁵ Of the 2 pig feeds containing trace levels of coccidiostats above declared values of 0 in 2012, both contained narasin levels above MRL – 2.3 and 2.4 mg/kg; ⁶ Of the 8 poultry feeds containing trace levels of coccidiostats above declared values of 0 in 2012, one sample contained narasin levels above MRL – 0.75 in a feed for other poultry; ⁷ Of the 6 poultry feeds containing trace levels of coccidiostats above declared values of 0 in 2014, 2 contained narasin levels above MRL – 7.3 mg/kg in a broiler finishing feed and 1.6 mg/kg in a feed for layers.

3.2.7 Consumption of poultry products in Norway

The Norwegian production is entirely consumed nationally. In addition to this, there is some import of chicken and turkey, but this is at present marginal compared to domestic production. The annual production of chicken meat was in 2013: 91 931 000 kg and of turkey meat 9 856 000 kg. This corresponds to an annual real consume (eaten) of 52 353 000 kg poultry meat (<http://animalia.no/>), approximately 10 kg per person.

In the Norkost 3 consumption study based on two 24-hour recalls by telephone at least one month apart, food amounts were presented in household measures or estimated from photographs (Totland et al., 2012). The study was conducted in 2010/2011, and 1787 adults (925 women and 862 men) aged 18-70 participated. Mean poultry consumption was 30 g/day for all participants and 82g/day among those who reported to eat poultry.

Table 3.2.7-1 Consumption of poultry in Norkost 3 consumption study (n=1787), gram/day

Type of product consumed	All (n=1787)		Women (n=952)		Men (n=862)	
	Mean	P95 ¹	Mean	P95 ¹	Mean	P95 ¹
Broiler meat, g/day	25	131	22	112	28	150
All broiler products, g/day	27	131	23	113	30	150
All poultry, g/day	30	136	26	120	34	162

¹ 95 percentile

Table 3.2.7-2 Consumption of poultry in Norkost 3 consumption study, gram/day per dose who consume poultry (consumers only)

Type of product consumed	All			Women			Men		
	n	mean	P95 ¹	n	mean	P95 ¹	n	mean	P95 ¹
Broiler meat, g/day	530	84	188	281	71	150	249	99	258
All broiler products, g/day	576	83	195	304	71	150	272	96	235
All poultry, g/day	650	82	199	343	71	162	307	94	232

¹ 95 percentile

Explanation of the tables: The variable named "Broiler meat" is just meat and skins from whole broiler, i.e. whole broiler, broiler legs and wings. The variable named "All broiler products" is broiler meat plus all products of broiler, e.g. sausages, pies, burgers, schnitzel and

the like. The last variable is called "Poultry" and here is all consume of broiler, turkey, duck and goose merged.

3.3 Human exposure to resistant bacteria

3.3.1 How can humans be exposed to resistant bacteria from animal production chains?

Humans may be exposed to coccidiostat resistant bacteria from poultry in a number of ways, e.g. by handling live animals, their manure, though slaughtering and processing, and by preparation and consumption of meals from poultry products (Figure 3.3.1-1). Routes of exposure are by direct contact, ingestion and inhalation.

A number of studies indicate that animal-human transfer of bacteria resistant to various antibacterials has occurred (Reviewed by Marshall and Levy (2011)). The studies focus on bacteria in the gastrointestinal tract, and do not include data on bacterial resistance against coccidiostats. In general, the studies show that farm and slaughterhouse workers, veterinarians, and those in close contact with farm workers are directly at risk of being colonized or infected with resistant bacteria through close contact with colonized or infected animals, or their manure and litter. Likewise, human consumption of food carrying antibacterial-resistant bacteria may result, either directly or indirectly, in acquisition of antibacterial-resistant infections. For example in the United States, where gentamicin remains the most commonly used antibacterial in broiler production, a revelatory study in 2007 found that the risk for carrying gentamicin-resistant *E. coli* was 32 times higher in poultry workers than in other members of the community: half of all poultry workers were colonized with gentamicin-resistant *E. coli*, while just 3% of nonpoultry workers were colonized (Luangtongkum et al., 2006). In a Dutch study, identical resistance patterns of *E. coli* were found in turkeys, turkey farmers and turkey slaughterers and in broiler, broiler farmers and broiler slaughterers, strongly indicating transmission of resistant clones and resistance plasmids of *E. coli* from poultry to humans (Van den Bogaard et al., 2001). In Denmark, similar resistance patterns and genes were detected in *E. faecalis* and *E. faecium* strains from humans, broilers, and swine (Aarestrup et al., 2000).

Regarding colonization and gene transfer in enterococci, the Panel is not aware of any data on coccidiostat resistance genes. However several experiments have been performed with bacteria resistant to other antibacterials (reviewed by Angulo et al. (2006)). Only transient intestinal carriage was observed in 18 volunteers after ingestion of antibacterial-resistant *E. faecium* from chicken and pork, and all stool samples were negative after 35 days (Sørensen et al., 2001). Interestingly, when *E. faecium* strains of human origin were fed to human volunteers, the same duration of colonization was observed (Lauková et al., 2004). This suggests that host specificity is not the main issue that determines prolonged enterococcal

colonization of the gut. Furthermore, typing of the most common enterococci in the intestine of human volunteers showed that the enterococcal flora of the human intestine often changed, which may indicate that enterococci in general do not colonize for extended periods of time (Gelsomino et al., 2003). However, it has been shown that transfer of resistance genes from enterococci of animal origin to enterococci of human origin did occur in healthy humans (Lester et al., 2006). Likewise, resistance genes were transferred between enterococci of animal and human origin *in vivo* in the intestinal tracts of mice (Bourgeois-Nicolaos et al., 2006; Mater et al., 2005). These studies show that, although enterococci can be placed in an environment different from their origin where they may be reluctant to colonize, transfer of resistance genes may occur *in vivo*, in animals as well as in humans. The significance of this, however, will depend on the frequency of horizontal transfer and on the availability of donor strains.

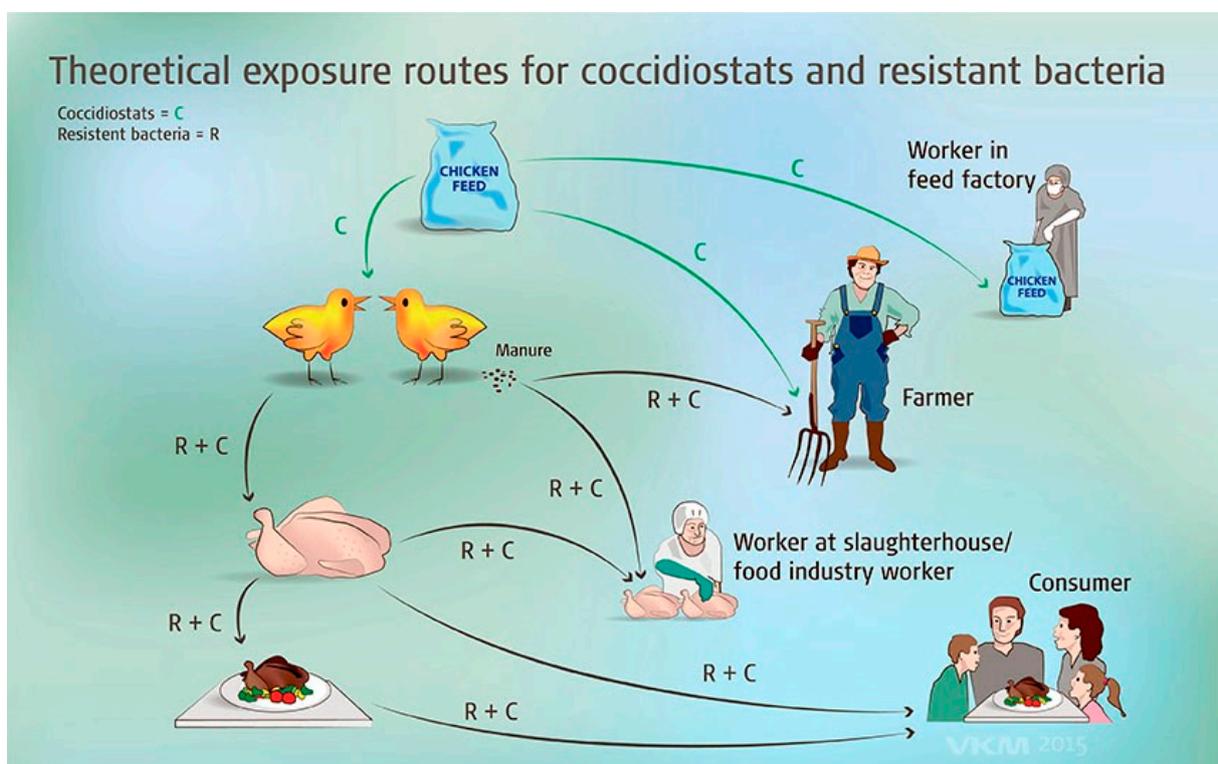


Figure 3.3.1-1 Theoretical routes for human exposure to coccidiostats and coccidiostat resistant bacteria in the poultry production food chain.

3.3.2 Coccidiostat resistant bacteria in the Norwegian poultry production chain

The Norwegian surveillance programme NORM-VET has been monitoring the prevalence of coccidiostat resistant enterococci in the poultry production chain since 2002. However, only resistance against narasin has been investigated. Narasin resistant enterococci have been

identified in samples from broilers and turkeys, as well as from broiler and turkey meat, as summarized in Table 3.3.2-1. In the years not included in the table, narasin resistance was not investigated. Each sample taken represents one flock or one piece of meat. Details of the sampling procedures are described in Appendix IV. If enterococci were present in a sample, one randomly selected strain being either *E. faecalis* or *E. faecium* was subjected to resistance testing. The cut-off value for narasin resistance in *E. faecium* was changed in 2013 from 2 mg/L to 4 mg/L as suggested by EUCAST. In this report, the cut-off of 2 mg/L is chosen for all isolates because 4 mg/L, according to the NORM-VET representatives, cuts through MIC distributions for *E. faecium* from some animal categories studied (e.g. broilers) in a manner not in agreement with the concept of wild-type distributions. When using the cut-off value of 2 mg/L, percentages are approximate twice as high as those resulting from a cut-off of 4 mg/L.

The data show in general a low percentage of narasin resistant *E. faecalis*, whereas the percentage of resistant *E. faecium* isolates is high. The total percentage of narasin resistant enterococci in the samples is high for both broilers and turkeys. Enterococci (both non-resistant and resistant) were isolated from more than 90 % of the faecal samples, and *E. faecium* was the dominant species from both broilers and turkeys. Consequently, the total percentage of narasin resistant enterococci was also high in faeces, i.e. 62 % in broilers and 67 % in turkeys. In the meat samples from broilers, the percentage of enterococci isolated was lower (82 %) and only half of these were *E. faecium*. Therefore the total percentage of resistant enterococci was lower than in faeces, i.e. 36 %. The percentage in turkey meat was even lower, but this was only tested one of the years, which makes the data less reliable.

Monensin, but not narasin, is used as a coccidiostat in turkeys. The narasin resistance observed in enterococci from turkeys is probably due to cross-resistance between monensin and narasin. These two coccidiostats belong to the same class of ionophores, and cross-resistance between the two has been observed in coccidia (Westley, 1982). Very few narasin resistant isolates were identified in faeces from layers (1.5 % of all samples), as can be expected since coccidiostats are not used in layer feed.

In addition, *E. hirae*, which is a pathogen, was isolated from specimens collected in disease outbreaks in broiler production in 2010. Resistance to the narasin was detected in 27 of 41 these isolates (66 %). This disease emerged as a problem in Norwegian broilers around year 2000, but does not appear to have been of major significance during recent years.

Table 3.3.2-1 Narasin resistant isolates identified in the Norwegian surveillance programme Norm-Vet during the years 1999-2014

Source	Year	Total no. of samples ¹	Samples with enterococci		Samples with resistant enterococci	
			No.	%	No.	%
Broiler faeces	2002	166	149	90	87	53
	2004	91	84	92	72	79
	2006	219	205	94	165	75
	2011	252	238	94	126	50
	All years	728	676	93	450	62
Turkey faeces	2007	58	55	95	35	60
	2013	131	128	98	89	68
	All years	189	183	97	124	66
Broiler meat	2002	212	175	83	43	20
	2004	100	79	79	49	49
	2006	126	103	82	68	54
	All years	438	357	82	160	37
Turkey meat	2007	107	72	67	24	22
	All years	107	72	67	24	22

¹ Each sample represents one flock or one piece of meat. One enterococcal isolate from each sample was tested for resistance.

3.3.3 Exposure of workers to coccidiostat resistant bacteria

Broiler: In the NORM-VET surveillance programme enterococci resistant against coccidiostats were identified 62 % of the samples (Table 3.3.2-1). Each sample represents one flock. The number of flocks per farm per year is usually between six and eight. The Panel has no information on whether all sampled flocks are from different farms. However, for the purpose of this risk analysis, the Panel assumes that each flock sampled represents one farm. Furthermore, the Panel assumes that if a farm has one flock with resistant bacteria, there is a high probability that the other flocks on this farm will harbour such bacteria. Consequently, the Panel assumes that the incidence of samples positive for resistant enterococci is approximately the same for farms as for flocks. According to the NORM-VET reports, sampling is performed one to three weeks before slaughter. As broilers are slaughtered at age 28 – 32 days, this implies that they may harbour narasin resistant bacteria from quite a young age.

The bacteria can also survive outside the animal host, e.g. in manure. Furthermore, narasin will also be excreted to the manure (see chapter 3.4.3), meaning that in addition to the

narasin resistant bacteria from the broilers, other bacteria in the manure may also develop narasin resistance. In addition, resistance genes may be transferred between the bacteria in the manure by horizontal transfer, thus increasing the number of resistant bacteria. Various treatments, e.g. composting, of the manure may reduce the number of enterococci present. Assessment of the effects of such methods has not been performed.

Consequently, when regularly handling manure, as well as equipment, clothing and anything else that has been in contact with this including animals, the farmers and other production workers will be exposed to resistant bacteria if they are present in the flock. Main route of exposure is direct contact. Exposure through inhalation is also possible. Adequate hygienic protection procedures will reduce the risk of exposure, e.g. restricted access, protective clothing and hygienic barriers, proper procedures for disposal of manure and litter, as well as cleaning and disinfection. As far as the panel knows, there is extended use of such procedures on Norwegian broiler farms today. The same considerations apply to workers in slaughteries and the food processing industry.

Turkeys: For Norwegian turkeys, the coccidiostat feed additive is not narasin, but monensin. It is not known from literature that monensin can induce resistance against narasin in enterococci, but indications of cross-resistance between these two coccidiostats have been observed in coccidia. There is no information on the prevalence of monensin resistant enterococci in the Norwegian turkey production chain. However, 66 % narasin resistant enterococcal isolates from Norwegian turkeys is reported. As this is most probably due to cross-resistance between narasin and monensin, the cross-resistance may be incomplete, indicating that the level of monensin resistance is at least as high as that of narasin. Turkeys are slaughtered at ages 11-12 weeks (hens) and 18-20 weeks (roosters) and sampled for monitoring of narasin resistant enterococci one to three weeks before. Therefore, it is not known how early in life they acquire or develop resistant bacteria. However, they are only offered in-feed coccidiostats from day of hatch until they are approximately seven to nine weeks old, meaning that resistance development must have occurred within this time period. Apart from this, the panel assumes that the same considerations apply for turkey as for broiler production.

3.3.4 Exposure of consumers to coccidiostat resistant bacteria

Data from the NORM-VET surveillance programme showed that 37 and 22 % of the pieces of raw meat from broiler and turkey, respectively, harbored enterococci resistant to narasin. Main routes of consumer exposure are direct contact by handling of fresh and frozen raw poultry meat and ingestion of non heat treated products. The bacteria will not survive the heat treatment of normal cooking of poultry meat and products. The same hygienic procedures that are recommended for handling raw meat in general will reduce the risk of

exposure, e.g. hygienic barriers between the meat and other food products, proper heat treatment, hand wash and general kitchen hygiene.

3.4 Human exposure to resistance development

3.4.1 Development of resistance to coccidiostats in the human microbiota

Bacteria of the human normal microbiota may develop resistance if they are exposed to coccidiostats. All human skin and mucosal surfaces are covered by bacteria, i.e. the normal microbiota which consists of ten times as many bacterial cells as the human body's own cells. The bacteria of the normal microbiota may be exposed to coccidiostats by direct contact, ingestion and inhalation. The Panel is not aware of any information on the level of exposure, e.g. the amount of coccidiostats or the time period, necessary for the various bacteria to give rise to resistant variants.

3.4.2 Human exposure to coccidiostats through handling of pre-mix preparations and feed

Instructions for safe handling of veterinary medicinal products and feed additives are generally provided with the products, including coccidiostats. Adherence to such instructions should minimize the probability of exposure of workers who handle the products during pre-mix and feed preparation and handling via various routes, including over the skin and airways. In the scientific literature, however, occupational exposure of feed mill and farm workers has received little attention and certainly as it pertains to the contribution to development of antibacterial resistant bacteria in humans. Measures have been implemented to reduce human occupational exposure to and absorption of coccidiostats. To reduce dusting, and thereby primarily skin and airway exposure but also indirect oral exposure, coccidiostat-containing pre-mixes are now in the form of granules rather than powders. Both feed mill and farm workers are advised to wear protective equipment, such as masks and gloves. However, studies in which occupational exposure to coccidiostats is quantified were not found in the peer-reviewed scientific literature and therefore the significance of skin and airway exposure to total exposure cannot be quantified accurately. Without the use of protective masks and garments it can, however, be assumed that the risk of exposure is high, but will be substantially reduced if protective measures are used. In any case, workers, including farmers, who prepare and handle coccidiostat-containing pre-mixes and feed should be treated as "high consumers" and appropriate measures should be reinforced to reduce the risk of development of antibacterial resistant bacteria in these human populations.

The coccidiostats on the market are available in pre-mixes with concentrations in the range of 10-200 g/kg for the those registered in Norway (see Table AIII-1 of Appendix III) and 5-

250 g/kg for those registered in the EU (see Table AIII-2 of Appendix III), depending on the active ingredient and producer. Other diluting, inactive ingredients are included in the pre-mixes at variable levels as technical aids, such as various oils (e.g. soya or mineral oil), minerals, starch, sugars, fibre sources (e.g. wheat bran, rice or soybean hulls). The pre-mix is incorporated into the formulated feed during the mixing stage.

The physicochemical properties of the pre-mixes appear to be of relevance for the degree and route of exposure of workers. Narasin, for example, can cause irritation to the eyes, has been shown to be toxic in dogs when inhaled, and has sensitisation potential with skin contact and by inhalation. Due to the sensitising properties of Narasin, EFSA's FEEDAP Panel recommends the use of appropriate personnel protective equipment for the workers (EFSA, 2004c). In addition, the pre-mixes containing narasin are in the form of granules with a low dusting potential. Narasin is not, however, considered as toxic via the oral route and less attention has perhaps been directed toward the prevention of oral ingestion. On the other hand, when protecting against inhalation, masks protecting both the nose and mouth are generally used.

For some of the registered coccidiostats, safety provisions are provided in the Norwegian and Commission Regulations governing their use (Table 3.4.2-1).

Table 3.4.2-1 Summarizes of the safety precaution specified for each registered coccidiostat according to the Norwegian and Commission Regulations governing their use

Type of coccidiostats	Respiratory protection	Protective clothing (gloves etc.)	Eye/face protection
Narasin	X	X	X
Monensin-Na	(X)	X	X
Lasalocid-Na			
Salinomycin-Na			
Maduramicin ammonium alfa	X	X	X
Nicarbazin			
Narasin+ Nicarbazine	X		
Robenidine HCl			
Decoquinate			
Semduramicin			
Diclazuril	X	X	X
Halofuginone			

3.4.3 Human exposure to coccidiostats through handling contaminated manure

Workers can be exposed to manure (manure is here used as term for excreta and litter, be aware of different use of terminology in the literature) at different stages; i) during slaughter, ii) transporting manure out of poultry house – normally with use of tractor-, iii) cleaning the poultry house (after slaughtering and starting up with new stock) where residues of manure will be removed manually, iv) during handling stored manure or during composting, v) spreading manure at agricultural fields, and vi) handling of manure for production of commercial poultry fertilizer products. The extent of exposure depends on which stage workers are in contact with manure, which measures are taken during the work, and the coccidiostat residue levels in manure. In an EFSA report regarding environmental risk assessment of additives, products and substances used in animal feed (EFSA, 2007), estimation of residue levels in manure was based on the assumption 100% excretion of unchanged parent coccidiostats. This approach is an overestimation and in order to obtain a more realistic exposure concentration in excreta or manure, pharmacokinetics data for the given coccidiostat in addition to their reduction rate during storage/treatment of manure is needed.

3.4.3.1 Excretion of coccidiostats and residue levels in excreta and manure

Excretion studies with use of ¹⁴C-labeled coccidiostats have been performed. As shown in section 3.4.4, narasin excretion more than 85 % of ¹⁴C-labelled narasin within 48 h (FAO, 2009b) and 81.9 % within 3 days (Catherman et al., 1991) have been measured. Another study reported by EFSA (1991) where nicarbazin and narasin were given in combination, showed that 80 % and 99% of recommended dosage was excreted, respectively, whereas 30 % of excreted narasin was parent narasin and 20 % as hydroxylated metabolites. Six major metabolites which incorporated the dihydroxy and trihydroxynarasin structure were identified and they accounted for 20 % of the parent antibacterial activity and for a very weak ionophoric activity. The fate of the remaining 50 % is not known, but the antimicrobial activity of the excreted product is low. The data reported by FAO (2009b) showed that narasin was extensively metabolized by chicken liver and that oxidation was the primary metabolism pathway. Fifteen metabolites and the parent narasin were found in the excreta and that both distribution and relative magnitude of radioactivity from liver and excreta were similar.

A comprehensive experimental study investigating chicken excretion kinetics of six coccidiostats (non-labeled substances), their reduction during storing and composting and plant uptake, has been performed by Gent University – Laboratory of Food Analysis and Institute for Agricultural and Fisheries research (Ghent, 2012). Of the five coccidiostats approved in Norway, only maduramicin was not included in this study. The selection of

substances was based on chemical structure, lipid solubility, working mechanisms and their application in Europe. For instance, for the coccidiostats approved in Norway the octanol-water coefficient (log Kow) ranged from 2.4 for salinomycin (EFSA, 2004d) to 6.74 for lasalocid (Swan, 2012). These two substances reflect more than twenty thousand times difference in hydrophobicity which again reflects the difference in bioavailability and water solubility.

In this study (Ghent, 2012), manure was defined as wet excreta only and litter defined as excreta, bedding material, feathers, wasted feed and wasted water. In order to avoid confusion, the term manure will be used for excreta in bedding material in the present risk assessment. The excreta without bedding, collected from broilers kept in digestibility cages, represented the worst-case scenario residue concentration of the coccidiostats. In this study a wood shaving thickness of approximately 10 cm was used (and more added when manure became too wet). A summary of the coccidiostats concentration measured in feed, excreta and manure is shown in Table 3.4.3.1-1. Narasin concentration in manure in the same range as found in the Ghent study have been observed in commercial poultry farms (average 12.7 mg/kg dm)(Furtula et al. 2010) and in an experimental study (13 mg/kg dm) (Eggen et al., 2011; Østensvik, 2008).

The residue concentration of coccidiostats in fresh manure will in addition to excretion kinetics be influenced by the production practice such as room- and floor temperature and the depth of bedding layer. In Norwegian poultry production it is recommended to use approximately 1 and 2-3 cm layer of bedding material for broiler and turkey, respectively Bagley (2002). Thus, it might be expected a lower dilution effect which again might result in higher residue level of coccidiostats in manure with the Norwegian practice than the 10 cm bedding layer used in the study at Ghent University.

It was recently observed that many pharmaceuticals which are excreted by humans as conjugates are re-conjugated back to active parent compounds in wastewater treatment plants (Verlicchi et al. 2012). The extent of conjugation of coccidiostats in poultry is not known, neither the possibility for re-conjugation during e.g. storage or composting.

Tab 3.4.3.1-1 Coccidiostat concentration in feed, excreta and manure (Ghent, 2012). Measured concentrations in excreta and manure are given in fresh weight (fw) and dry matter (dm)

Substance	Dosage in feed		Reported mean residue levels in excreta and manure before storage/composting (mg/kg)			
	Expected	Measured (mg/kg)	Excreta (fw)	Excreta (dm)	Manure (fw)	Manure (dm)
Narasin ¹	50	46 ± 10	6.2 ± 2.0	21.8	7.0 ± 2.0	14.3
Monensin ¹	120	137 ± 30	23.6 ± 5.7	78.9	12.0 ± 0.6	24.2
Salinomycin ¹	70	80 ± 6	3.6 ± 0.3	10.4	3.8 ± 0.9	7.3
Lasalocid ¹	100	109 ± 28	20.9 ± 2.7	102.7	15.9 ± 6.2	36.8
Nicarbazin ²	50	43 ± 5	18.8 ± 1.5	66.5	26.3 ± 2.6	53.4
Diclazuril ²	1	1.160 ± 0.003	0.28 ± 0.02	0.9	0.29 ± 0.01	0.6

¹regulated in Norway; ²regulated in Europe and not in Norway

3.4.3.2 Disappearance of coccidiostats during storage and composting

The term 'disappearance fate' includes several removal processes. In addition to abiotic and biotic degradation, physical trapping in nanopores in soil particles, immobilization due to covalent binding to organic matter (also called unextractable residue) (Kästner et al., 2014), and evaporation of volatile substances are part of this term. The disappearance fate of substances will depend on their physicochemical properties and the present biotic and abiotic environment; such as the microbial community and activity, temperature, redox conditions and water content to mention some factors. There are some studies on disappearance of coccidiostats in manure during storage and composting (e.g. (Ghent, 2012; Sun et al., 2014; Žižek et al., 2014), but high variation in disappearance fates are observed.

In the study by Ghent University, manure was first stored in bins for two months without any composting was initiated (< 40°C). Then the compost process was speeded up and performed in small scale vessels for 1.5 month (41-62°C). The average reduction during the storage period (< 40°C) was 22% for diclazuril and for the other coccidiostats in the range of 68-96% (Table 3.4.3.2-1). During the composting process (> 40°C) the reduction efficiency of diclazuril increased to 79%, which was a 57% additional reduction. A storage study of fresh excreta at room temperature during one month was also performed. The average reduction was 4% for diclazuril, 33-39% for lasalocid and nicarbazin, and 78% and 83% for salinomycin and narasin, respectively. The coefficient of variation for the manure data was high. For narasin it was 88%, and the measured concentration of monensin was higher after storage than before. This demonstrates the challenge of representative sampling due to the inhomogeneity of manure which forms clumps and which also might lead to different environmental conditions, e.g. redox-conditions, within a sample. The coefficients of variation in fresh manures were much lower and ranged from 8.1 to 24.3%.

In addition to the inhomogeneity of manure, the abiotic and biotic factors mentioned above determine the rates of the different disappearance processes and are also part of the explanation of the observed high difference in reduction rates.

Sun et al. (2014) performed a degradation study in broiler manure (excreta and bedding material) which demonstrated high influence of water content and temperature; reduction of salinomycin and narasin in manure was stimulated with enhanced water content, and at optimal water content, reduction occurred both at 35 and 45 °C but was inhibited at 60 °C. Independent of water content and temperature, no monensin reduction in manure occurred. However, abiotic reduction of monensin in soil microcosms was observed in the same study.

Composting has been demonstrated to enhance the reduction rates; e.g. for salinomycin half-life a few days (Hansen et al., 2012; Ramaswamy et al., 2010), for monensine half-lives from 22 day in manure and 11 day during composting (Dolliver et al., 2008) and for lasalocid during aging manure and composting half-lives 62 day and 18 days, respectively, (Žižek et al., 2014). There exist a numbers of papers regarding composting of therapeutic veterinary and human antibacterials but since reduction fate is so dependent on substances', properties of these data are not useful for this risk assessment.

To summarize, it is reasonable to assume that workers in contact with well composted manure will be exposed to lower coccidiostat levels than workers in contact with stored manure. Workers in contact with fresh excreta or manure have highest probability for exposure of highest residue levels of coccidiostats. There are few studies of coccidiostats excretion in chicken, both concerning reliable residue levels of parent compounds in excreta, and which metabolites are excreted. Furthermore, the ability of such metabolites to induce resistance in microorganisms is not known. Due to lack of knowledge, it is difficult to perform a realistic exposure evaluation for workers handling poultry manure.

Table 3.4.3.2-1 Reported residue concentrations of coccidiostats, given in µg/kg fresh weight (fw) and dry matter (dm), in excreta and manure before and after storage or composting at different temperatures (Ghent University, 2012)

Substance	Residue concentration ± standard deviation (SD) in manure and litter before storage or composting (µg/kg)				Reported mean residue levels in manure and litter after storage/composting (µg/kg)								
	Manure		Litter		Manure after 1 month storage (room temp)			Litter after 2 months composting (<40°C)			Litter after 2 months composting (>40°C)		
	fw	dm	fw	dm	fw	dm	% red.	fw	dm	% red.	fw	dm	% red.
Narasin¹	6155 ± 956	21749	7019 ± 1975	14252	1370 ± 1206	3795	83	335±332	1614	89	155±78	467	97
Monensine¹	23615 ± 5741	78848	11982 ± 569	24231	43015 ± 9502	110436	0	1256±755	5255	78	438±211	1375	94
Salinomycin¹	3586 ± 327	10364	3827 ± 906	7303	1144 ± 585	2304	78	73±10	320	96	43±6	160	98
Lasalocid¹	20891 ± 2741	102658	15921 ± 6212	36812	22964 ± 10834	68652	33	1055±223	5718	84	328±116	1262	97
Nicarbazin²	18823 ± 1517	66512	26291 ± 2590	53383	14548 ± 6069	40299	39	3533±1134	17027	68	2081±983	6268	88
Diclazuril²	278 ± 24	890	294 ± 11	602	325 ± 42	856	4	91±8	467	22	38±3	129	79

¹ approved for use in Norway; ² approved for use in Europe and not in Norway

3.4.4 Human exposure to coccidiostats in poultry products

One question that arises is whether humans are exposed to coccidiostats when consuming chicken flesh or other products. Traditionally, all veterinary medicinal products administered to food-producing farm animals must be discontinued for a set period of time to avoid human exposure to the drugs when they consume the animal products. This time period is called the withdrawal period. Continuous surveillance measures of animal products are an important task of national regulatory agencies to control compliance of farmers and veterinarians to these withdrawal periods. The length of the withdrawal period is dependent on the length of time drug residues remain in the animal body, which may differ considerably depending on the animal tissue and drug in question. Thus so called pharmacokinetic investigations of drugs are important to map the bioavailability, absorption, metabolism and elimination of drug residues, which include both the parent drug and any bioactive metabolites, in various animal products meant for human consumption, and thus determine withdrawal periods for specific drugs. Maximum residue limits (MRLs) in animal tissues are set for each drug for various practical and scientific reasons. One important reason is that the sensitivity of detection for a given drug may vary, depending on the method employed. This sets constraints on the ability of regulating bodies to set a zero tolerance for any drug residues in any animal product.

Because coccidiostats 1) are regulated as feed additives rather than as medicinal products, and 2) the principal effect of coccidiostats is on the coccidia and microbiota within the lumen of the intestinal tract, relatively few exhaustive reports from conventional pharmacokinetic studies reporting concentrations of coccidiostats in animal tissues exist in the peer-reviewed scientific literature. However, the metabolism and elimination of most ionophore coccidiostats have been reported to be rapid from breast and leg/thigh muscle tissue from broilers fed rations containing coccidiostats (Catherman et al., 1991; FAO, 2009a; FAO, 2009b; Henri et al., 2012; Olejnik et al., 2014; Peippo et al., 2005), as reflected by the short withdrawal times (0-5 days) registered for each coccidiostat (see Table AIII-1 and AIII-2 in Appendix III). MRLs in animal tissues following the withdrawal times are at the $\mu\text{g}/\text{kg}$ wet weight level, also indicating generally rapid metabolism and elimination from tissues. Most coccidiostats, however, are lipophilic, which means that residues accumulate at higher levels in tissues with higher fat content, such as the fat, skin, and liver. This is taken into consideration, however, with these tissues generally used as marker tissues when setting MRLs, as well as for testing during surveillance.

One major challenge, which has received more recent attention by the scientific community, is the accumulation of coccidiostats and other drug residues in eggs (see review by Goetting et al. (2011)). Human exposure to ionophore coccidiostats due to the consumption of eggs was generally considered nearly non-existent as mature, egg-laying hens are not fed coccidiostat-containing rations. However, the accidental exposure of laying hens to

coccidiostats due to cross-contamination that can occur at feed mills (see section 3.2.6) cannot be completely ruled out, which also makes it nearly impossible for farmers and veterinarians to comply with withdrawal periods accordingly. As exemplified in a recent study by Olejnik et al. (2014), low level exposure of laying hens to rations supplemented with 0.27 mg/kg semduramicin, which normally has a 5 day withdrawal time, led to detection of semduramicin levels well above MRL (set at 2 µg/kg) in three of six liver samples (mean for the 6 samples 2.57; SD 2.47 µg/kg) and all ovarian yolk samples (mean for the 5 samples 19.5; SD 8.9 µg/kg). Undetectable levels (<0.1 µg/kg) or values under the MRL were observed in spleen, heart, breast muscle, thigh muscle, gizzard and ovarian tissue. A recent review (Goetting et al., 2011) mapped the coccidiostat residues found in eggs from hens exposed to both therapeutic and below therapeutic levels, such as levels that may occur as a result of cross-contamination, in rations. The number of days from last treatment until residues was no longer detected were reported, as far as the exhaustive review of the literature would allow. With the exception of monensin, all other coccidiostats required substantially longer periods of time (>3 [for salinomycin] to >60 [for nicarbazin] days) than the set withdrawal periods for residues of each coccidiostat to clear whole eggs and/or egg yolks.

Thus human exposure to coccidiostats with the consumption of eggs due to cross-contamination of feeds for laying hens may be higher than with consumption of muscle tissue from broilers and other poultry fed rations containing coccidiostats. This becomes particularly apparent from data reported in the Norwegian Residue Monitoring Programme, summarised in Table 3.4.4-1. Coccidiostats have not been detected in poultry meat in the last five years of surveillance. In the same period, however, numerous eggs (of the 133-150 sampled per year) have been found to contain trace levels of coccidiostats and 1-3 eggs have contained levels above MRLs nearly every year. In 2014, Norwegians were reported to consume on average 12.5 kg eggs per year. In 2012, 61 758 metric tonnes of eggs were produced in Norway, increasing steadily from a level of 47 400 metric tonnes in 2002.

Table 3.4.4-1 Summary of coccidiostat residues reported in foods of animal origin from the 'Residue monitoring programme' in the last five years. The number of non-compliant poultry samples in which levels were above the maximum residue levels (MRLs) are reported, as well as the number of egg samples in which anti-coccidials were detected at trace levels (above or below MRL). Total number of samples analysed are given in parentheses

Year	Food item ¹		
	Poultry ²	Eggs	Other ³
2014	0 (67)	1 above MRL (N) (150)	0
2013	0 (63)	0 above MRL; 8 under MRL (N) (140)	0
2012	0 (47)	3 above MRL (2N, 1M); 11 under MRL (N) (133)	0
2011	0 (51)	1 above MRL (N); 12 under MRL (N) (140)	0
2010	0 (59)	2 above MRL (N) (133)	0

¹ MRLs for narasin (N) and monensin (M) is 2 µg/kg; and for lasalocid (L) 150 µg/kg;

² "Poultry" is muscle tissue from broilers, turkeys and layers (hens);

³ "Other" indicates anti-coccidials analysed in meat from other terrestrial animals, including imports

A toxicological risk assessment of human exposure to coccidiostats via the food chain has also recently been conducted (Dorne et al., 2013) and a risk characterization of coccidiostats for human health after intake of animal products from non-target species fed cross-contaminated diets at 2%, 5% and 10% of the maximum levels authorized in target species, and related to acceptable daily intakes (ADIs) from a toxicological perspective. This data can also give information regarding human exposure as it relates to the development of antibacterial-resistant bacteria in humans. However, a full risk characterization from this data is not possible at this time.

Table 3.4.4-2 Risk characterization of coccidiostats for human health from a toxicological perspective after intake of animal products from non-target species fed cross-contaminated diets at 2%, 5% and 10% of the maximum levels authorized in target species, and related to acceptable daily intakes (ADIs). Adopted from Dorne et al. (2013)

Coccidiostat	ADI ($\mu\text{g}/\text{kg}$ BW per day)	Animal products	% ADI after cross-contamination		
			2%	5%	10%
Ionophoric coccidiostats					
Lasalocid	5	Eggs/liver/skin	3	13	27
Maduramicin	1	Liver/skin/fat and muscle	0.7	1.9	3.7
Monensin	3	Liver and eggs	0.1	0.3	0.6
Narasin	5	Liver and eggs	0.07	0.35	0.17
Salinomycin	5	Liver and eggs	0.2	0.4	0.8
Semduramicin	1.25	Muscle/liver	2	5	10
Non-ionophoric coccidiostats					
Decoquinate	75	Eggs/liver/kidney/muscle/skin/fat	0.14	0.38	0.75
Diclazuril	29	Eggs/liver/kidney/muscle/skin/fat	0.016	0.04	0.08
Halofuginone	na	Eggs/liver/kidney/muscle/skin/fat	na	na	na
Nicarbazin	770	Eggs/liver/muscle	0.04	0.09	0.18
Robenidine	37.5	Eggs/liver/kidney/muscle/skin/fat	0.9	2.2	4.3

The question arises whether coccidiostats will be sufficiently denatured during heat treatment of poultry meat and eggs consumed by humans to eliminate the danger of development of antibacterial-resistant bacteria. The effect of processing on numerous veterinary residues in foods was reviewed by Moats (1999). The conclusions drawn were that 1) normal cooking procedures for meat, even to "well-done", may not be sufficient to inactivate even the more heat sensitive compounds, and 2) the relevance to human exposure is uncertain since the activities of the various degradation products are largely unknown. See also section 3.3 for effects of heat treatment on coccidiostat denaturation.

Pharmacokinetic data

Narasin

Peippo et al. (2005) fed 30 male and 30 female broiler chickens (Ross 508-hybrid) unmedicated starter rations from 1-14 days of age before feeding grower ration supplemented with 0, 3.5 or 70 mg narasin per kg feed until slaughter. Tissues were sampled from three birds fed the 70 mg/kg narasin ration following withdrawal times of 0, 3 or 5 days, while four birds fed 0 and 3.5 mg/kg narasin rations tissues were sampled without any withdrawal time. Birds were fed unmedicated rations during the withdrawal time. Birds had free access to feed and water during the entire feeding trial. The tissues sampled were

plasma, leg muscle and breast muscle. Narasin was determined by time-resolved fluoroimmunoassay and results shown in Table 3.4.4-3.

Table 3.4.4-3 Concentration of narasin in plasma and muscle tissues of broilers fed rations containing 0, 3.5 or 70 mg narasin/kg feed (from Peippo et al. (2005))

Ration group and withdrawal time	Bird number	Narasin concentration		
		Plasma (µg/L)	Leg muscle (µg/kg)	Breast muscle (µg/kg)
0 mg/kg narasin; no withdrawal time	1	ND	ND	ND
	2	ND	ND	ND
	3	ND	ND	ND
	4	ND	ND	ND
3.5 mg/kg narasin; no withdrawal time	1	1.6	0.7	1.2
	2	1.8	0.6	0.7
	3	4.2	1.7	0.6
	4	3.4	1.6	1.3
70 mg/kg narasin; no withdrawal time	1	39.8	2.4	2.1
	2	59.3	4.2	2.3
	3	70.2	6.2	4.5
70 mg/kg narasin; 3 d withdrawal time	1	ND	ND	ND
	2	ND	ND	ND
	3	ND	ND	ND
70 mg/kg narasin; 5 d withdrawal time	1	ND	ND	ND
	2	ND	ND	ND
	3	ND	ND	ND

ND: not detected; limit of detection (LOD): 0.6 µg/kg; limit of quantification (LOQ): 1.8 µg/kg

As indicated by the data when narasin was present in the diet (i.e. no withdrawal time) it was efficiently absorbed from the intestinal lumen and entered the blood stream of chickens, with concentrations closely reflecting the ration levels. In the muscle, however, narasin concentrations were considerably reduced compared to plasma levels and the muscle levels did not follow ration levels as closely. Following 3 and 5 day withdrawal periods, no narasin was detected in any tissues, indicating that it is rapidly metabolized.

The above conclusions were generally confirmed by other, HPLC-based data reported by FAO (2009b). This method was reportedly somewhat less sensitive with an LOD and LOQ of 10 and 25 µg/kg, respectively. Narasin residues were not detected in muscle at any time following ration withdrawal (0, 6, 12 and 24 h), while kidney samples contained <25 µg/kg (the LOQ) at 0 h and not detectable levels thereafter. Liver and skin/fat samples contained 46.2 and 67.1 µg/kg, respectively, at 0 h. Six hours following withdrawal no narasin was detected in liver, while 39.1 µg/kg was detected in skin/fat, which was reduced to <25 µg/kg and none detected at withdrawal times of 12 and 24 h, respectively.

The fate of narasin following exposure to higher ration levels than the 80 mg/kg used in the two above-mentioned broiler studies have also been reported FAO (2009b). This has practical importance, e.g. following possible over-dosing that may occur unintentionally at feed mills. Broilers exposed to 80 or 160 mg/kg narasin in rations fed from day 0 to day 42 (approximately one production cycle). Narasin residues were analysed in tissues at 2, 24, 72, 120 and 168 h following withdrawal of narasin-containing feed using bio-autography using *Bacillus stearothermophilus* var. *calidolactis* C-953 as the indicator organism (LOQ 25 µg/kg). For both the 80 and 160 mg/kg rations, narasin was detected 2 h following withdrawal in skin and fat and in one skin sample at 24 h following withdrawal in the 160 mg/kg group. No narasin was detected in any muscle, liver or kidney samples at any time following withdrawal or in skin and fat samples following the 24 h withdrawal period.

In a study reported with rats, radioactivity following a single oral dose of 2.3 mg (0.596 µCi/mg) ¹⁴C-labelled narasin was followed to more closely evaluate the absorption and elimination of narasin (FAO, 2009b). Using rats in metabolism cages, elimination via urine versus faeces could be observed, which is generally difficult to accomplish with broilers. An average of 75% of the radioactive dose was eliminated 52 h post-dosing, in which only 1.1% of the excreted radioactivity was found in the urine and the remainder (98.9%) in the faeces. Rats equipped with a catheter in the bile duct provided data indicating that 15% of the dose was absorbed and eliminated through the hepatic system. Narasin was also reported to be rapidly eliminated from tissues of broilers (FAO, 2009b). Four 8-week old broiler chickens fed a ration containing 80 mg narasin/kg feed were given a single, oral (encapsulated) dose of ¹⁴C-labelled narasin. Excreta were collected from the chickens daily and analysed for radioactivity. More than 85% of the radioactive dose was excreted within 48 hours.

Similar results were obtained in a third narasin study with broilers and quail (Catherman et al., 1991), however in that study ¹⁴C-labelled narasin was injected by cardiac puncture and therefore not considered as applicable to farming conditions and human dietary exposure. In this latter study, it took 3 days for an average of 81.9% of the radioactive dose to be excreted in broilers, while 75% was excreted in quail. Some tissue differences in elimination were also reported, with 80% and 92% of the dose clearing the plasma of broilers and quail, respectively, whereas in both broilers and quail, the liver, heart, fat, skin and ovarian tissue contained traces of radioactivity 1 d post-injection, while muscle and kidney contained no traces of radioactivity 1 d post-injection. Radioactivity was detected in excreta up to day 12 post-injection, but at narasin-equivalent levels well below the MRL of 50 µg/kg set for broiler tissues (see Appendix III, Table AIII-1).

Narasin metabolites have been reported in both liver and in excreta, with at least some similar metabolites observed in both (FAO, 2009b). Unchanged narasin represents ca. 5% of total narasin residues in hepatic tissue. Narasin metabolites have at least twenty times less antimicrobial activity (tested with *Bacillus subtilis*) than the narasin A.

The data summarized above confirms that a withdrawal period of 1 day is sufficient to reduce narasin levels to below MRLs, but possibly not completely eliminate trace levels of narasin and/or its metabolites, from poultry tissues consumed by humans.

Monensin

In turkeys and chickens fed rations containing 100 mg/kg monensin, Henri et al. (2012) reported 30% bioavailability in chickens and 1% in turkeys. Residues were rapidly eliminated from plasma, liver and muscle tissue, and following 8 h of withdrawal, monensin was not detected in liver, breast or thigh muscle. Fatty tissue retained monensin longer, but was near or below MRL at 24 h and below detection levels 72 h following withdrawal. Data supporting this has been reported by FAO (2009a). The data summarized above confirms that a withdrawal period of 1 day is sufficient to reduce monensin levels to below MRLs, but possibly not completely eliminate trace levels of monensin and/or its metabolites, from poultry tissues consumed by humans.

Salinomycin

In chickens fed rations containing 70 mg/kg salinomycin, Henri et al. (2012) reported 15% bioavailability. Residues were rapidly eliminated from plasma and muscle tissue, and following 8 h of withdrawal, salinomycin was not detected in plasma or breast muscle. Fatty tissue retained salinomycin longer, but was not detected in any tissues 24 h following withdrawal.

The data summarized above confirms that a withdrawal period of 1 day is sufficient to reduce salinomycin levels to below MRLs, but possibly not completely eliminate trace levels of salinomycin and/or its metabolites, from poultry tissues consumed by humans.

3.4.5 Environmental exposure to coccidiostats

Poultry manure is a valuable fertilizer and poultry manure-based compost or poultry manure is either applied directly to agricultural fields by farmers, stored for later land application or transported for further treatment (composting, heating etc.) and preparation for commercial fertilizers. Poultry manure-based fertilizers are used in agriculture as well as gardens and kitchen gardens.

As discussed under section 3.4.3, the coccidiostats concentrations in manure when applied as fertilizer will vary and depend on the excretion kinetics, their chemical physicochemical properties, and the manure history (fresh, stored, treated). In soil the coccidiostats might undergo different processes; abiotic and biotic degradation, trapping in nanopores, immobilization to non-extractable residues, leachates and runoff from soil to nearby water recipients or taken up by plants and soil-living organisms. These disappearance processes

depend on the environmental condition and factors such as temperature, redox, light exposure, as well as microbial activity.

Even there are several papers related to environmental fate of pharmaceuticals, also veterinary medicine from manure (Boxall et al., 2003; Du and Liu, 2012; Hailling-Sørensen et al., 1998; Sarmah et al., 2006), the coccidiostats have been scarcely included. It is only during the last decade research related to environmental occurrence and fate of the ionophore coccidiostats has been performed. These substances' physicochemical properties and interaction with other elements and the influence of pH related to their pKa values is complicated, and there is no consensus on their environmental behavior as discussed by Hansen et al. (2012). However, there are some environmental measurements of ionophores in different matrixes, including agriculture runoff water, sediments in recipients and agriculture soil (e.g. (Bak et al., 2013; Hansen et al., 2012; Sun et al., 2013a).

Monensin, salinomycin and narasin have been measured in runoff from manure-fertilized lands in a US study (water samples n=17) in maximum concentrations 2389, 9022 and 348 ng/L, respectively (Sun et al., 2013b). In top soil from the same field (soil samples n=10), only monensin was detected over analytical detection limit and in the range of 5-183 µg/kg (average 101 µg/kg). The median and maximal concentration of these coccidiostats in poultry manure from farms in the area were 291, 4607, and 237 µg/kg and 4057, 21878 and 3310 µg/kg, respectively (Sun et al., 2013b). In real samples from Denmark performed related to establishing an analytical methodology and not an environmental survey, monensin was detected in soil and manure in ng/kg-level, salinomycin only in the sediment sample, while narasin was detected in soil and sediment sample (Bak et al., 2013). Lasalocid was not detected above limit of detection level.

It is well known that plants take up pharmaceuticals, possible also as active uptake for certain structures (Eggen and Lillo, 2012), that uptake and further translocation within plants is related to the physicochemical properties, and there are significant differences between plant species (e.g. (Eggen and Lillo, 2012; Sallach et al., 2015)). There are several studies related to plant uptake of therapeutic pharmaceuticals (Boonsaner and Hawker, 2015; Boxall et al., 2006; Wu et al., 2014), including antibacterials, but few with coccidiostats (Broekaert et al., 2012; Eggen et al., 2011; Ghent, 2012). In the Ghent University study, two different coccidiostats application approaches were used for studying uptake of coccidiostats in carrot, potato, lettuce, zucchini and tomato; one was addition of manure (see Table 3.4.3.1-1 for coccidiostat concentrations) reflecting a field dose of 10 tons/ha and the other using excreta spiked with premix coccidiostats to mimic a worst-case scenario.

In the manure-added soil, only nicarbazin and monensin were detected in carrot and lettuce, respectively. Nicarbazin residue in unpeeled carrot was in range of 8.7-10.7 µg/kg dw (1.1-1.4 µg/kg fw) and the monensin in lettuce in range of 9.6-49.3 µg/kg dw (0.4-2.0 µg/kg fw). In the worst-case scenario, excreta spiked with pre-mix, nicarbazin was found in unpeeled

potato and courgette, lasalocid in unpeeled potato and carrot, and monensin in lettuce and unpeeled potato (used only ¼ doses due to phytotoxic effects on potato growth). Due to phytotoxic properties, possible uptake of monensin and nicarbazin in carrot were not available. No uptake was measured in tomatoes.

Humans might be exposed to coccidiostats via plants grown in manure-based compost or manure-amended soil; but only relevant for crops with significant uptake of coccidiostats via roots (e.g. root vegetables) or leave-vegetables with manure transferred to leaves via rain splash (e.g. lettuce). The measured monensin in lettuce grown in manure amendment soil but no uptake in root vegetables found in the same study indicates that transfer via leave is a transfer pathway to consider. Based on uptake data from the comprehensive study by Gent University, and related to food consumption data and acceptable daily intake, it was claimed unlikely to pose a direct threat to public health (Broekaert et al., 2012).

Environmental risk or exposure of non-target organisms is not part of this risk assessment and not discussed. Runoff of none-ionophore veterinary antibacterials and uptake from aquatic plants should not be ignored when determining human exposure to antibacterials (Boonsaner and Hawker, 2015).

Risk due to spreading ionophores in the environment has last years received more scientific attention. How to optimize treatment of manure before land application and sustainable manure storage are now also put on the agenda. Measures to reduce transfer of coccidiostats to the environment through controlled and optimized storage of manure stimulating degradation of coccidiostats is possible and should be considered.

Research related to antibacterial resistance genes from application of manure has recently been published (Furtula et al., 2010; Joy et al., 2013; Zhang et al., 2014; Zhu et al., 2013) but there are no knowledge available for evaluation such risk today.

3.5 Use of therapeutic antibacterials

3.5.1 Use of therapeutic antibacterial agents for poultry in Norway

In Norway, veterinary medicinal products (VMPs) are prescription only and have to be dispensed to veterinarians and animal owners through pharmacies. An exemption from this is medicated feed – i.e. feed containing premixes of antimicrobial agents that are dispensed through authorised feed mills; presently this applies only for antimicrobials used in fish farming. Both pharmacies and feed mills have to purchase VMPs through licenced wholesalers. The wholesalers are mandated to report their sales to the Norwegian Institute of Public Health (NIPH).

The Norwegian Veterinary Institute gather detailed data on sales of antibacterial veterinary medicinal products (VMPs) from NIPH for the NORM-VET report. The data are collected at package level and include among others information on the dosage form for each antibacterial VMP.

Commercial poultry is treated against bacterial infections by adding the antibacterial VMP through drinking water. Currently three VMPs applicable for treatment through drinking water are sold in Norway (NORM-VET, unpublished data).

Until recently the major sales in Norway of dosage form applicable for treatment through drinking water represented an amoxicillin VMP approved for poultry only (Figure 3.5.1-1). As from 2012, phenoxymethylpenicillin (authorised for poultry only) is used increasingly. Statistics for 2014 and 2015 have not been available, but it is likely that phenoxymethylpenicillin is the predominant compound presently used to treat bacterial disease in commercial Norwegian poultry. The reason for this change is an increased awareness of the presence of extended spectrum betalactamase (ESBL)-producing Gram-negative bacteria in poultry. Minor amounts of an enrofloxacin VMP (authorised at EU level for use in the target species chickens, turkeys and rabbits) are also sold (presumed mainly to non-commercial poultry and pet birds).

Although these products might be used in other animal species as well, the data presented in Figure 3.5.1-1 is thought to give a valid estimate on therapeutic use of antibacterial VMPs in poultry in Norway.

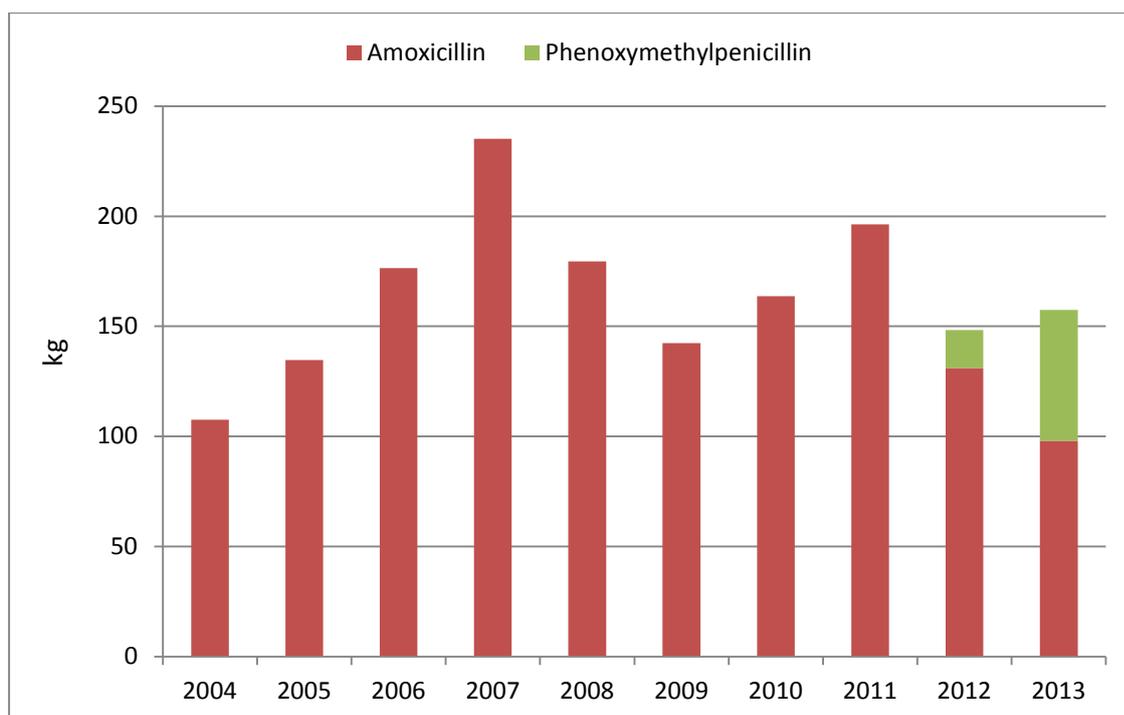


Figure 3.5.1-1 Sales, in kg active substance, in Norway of antibacterial veterinary medicinal products applicable for treatment of poultry during the years 2004-2013 (NORM-VET, unpublished data). In addition 0.1 to 0.7 kg enrofloxacin is sold annually during this period

3.5.2 Use of therapeutic antibacterials in broilers

Therapeutic antibacterials are rarely used in Norwegian broilers. Data from Animalia (Norwegian Meat and Poultry Research Center, Thorbjørn Refsum, personal communication 2015) indicate that only one single conventional (i.e. using in-feed coccidiostats) broiler flock was treated during the time span from January 2013 until October 2014. Because 8000 flocks were slaughtered during this time period, this figure corresponds to a treatment frequency of 0.01 %. During the same time span a total of 800 organic (no in-feed coccidiostats) broiler flocks were slaughtered, and 1.5 % of these flocks (corresponding to 12 flocks) were treated. This difference between conventional and organic flocks is statistically significant, suggesting an increased probability of using therapeutic antibacterials in flocks reared without in-feed coccidiostats.

A Norwegian study collecting data from a total of 120 conventional broiler flocks reared during 2003-2006 indicated use of therapeutic antibacterials in 2.0 % (1/49) of flocks offered feed with coccidiostats and in 5.6 % (4/71) of the flocks offered feed without coccidiostats (Kaldhusdal, 2006). Although these figures suggest a difference between flocks with and without coccidiostats, the low numbers of flocks in this study preclude any firm conclusions about the relationship between uses of coccidiostat and use therapeutic antibacterials.

3.5.3 Use of therapeutic antibacterials in turkeys

Data from Animalia (Norwegian Meat and Poultry Research Center, Thorbjørn Refsum, personal communication 2015) indicate that 22 of 159 turkey flocks (13.8 %) were treated with therapeutic antibacterials during 2014. Most of these cases were associated with clinical necrotic enteritis. The occurrence varies substantially with time and will probably be lower in 2015. Such cases are usually treated with phenoxymethylpenicillin, a compound with an optimal combination of efficacy and narrow spectrum of activity. The majority of cases of treatment with therapeutic antibacterials take place in flocks that are offered feed supplemented with coccidiostats. Turkey feeds cannot be supplemented with narasin and salinomycin. In particular narasin has been assumed to be more efficient than other ionophores in preventing necrotic enteritis, an assumption which is supported by MIC data on *C. perfringens* strains from broilers (Martel et al., 2004). Probability of increased use of therapeutic antibacterials for poultry associated with changed usage of in-feed coccidiostats.

3.5.4 Probability of increased use of therapeutic antibacterials associated with changed usage of in-feed coccidiostats

The coccidiostat presently used in Norwegian broiler rearing (narasin) is a polyether antibacterial (also called an ionophore) which has an effect against the bacterium causing necrotic enteritis (*C. perfringens*) as well as against coccidiosis, which is a predisposing factor for necrotic enteritis. If this coccidiostat is replaced with an coccidiostat without the antibacterial effect e.g. nicarbazin or diclazuril, see (Lanckriet et al., 2010; Lensing et al., 2010) and no other factors are changed, the specifically growth-suppressing activity against *C. perfringens* is removed, which will enable this bacterium to proliferate more easily in the broiler intestine. If we assume that the new coccidiostat are equally efficient against relevant coccidia as narasin, the risk of necrotic enteritis, and therefore also the risk of use of therapeutic antibacterials, is likely to increase. If the new coccidiostat is more effective against relevant coccidia, the risk of necrotic enteritis is more dependent upon other predisposing factors than coccidiosis and can therefore vary with the presence or absence of such factors.

The net outcome depends on the efficacy of narasin and the new coccidiostat against relevant strains of coccidia present in Norwegian broiler farms. No data on resistance to ionophores in coccidial strains from Norwegian poultry farms have been found. The evaluation therefore must be based on general knowledge. Development of resistance in coccidia against ionophores (Augustine et al., 1987; Chapman, 1993; Peek and Landman, 2003) and reduced activity against coccidia shown by ionophores as compared with non-ionophore coccidiostats (Peeters et al., 1994) has been documented from other countries. However, development of resistance in coccidia has been demonstrated for all 11 EU-authorized coccidiostats, including the non-ionophore coccidiostats. Because ionophores

(narasin from 1996, other ionophores from 1988) have been used almost continuously in Norwegian farms since 1988, it is possible that the anticoccidial efficacy of the ionophores may have been reduced. However, no reports of increasing problems with clinical or subclinical coccidiosis in Norwegian poultry has been found, which suggests that resistance to ionophores in coccidia has been of minor importance up to now.

Coccidiosis is not the only predisposing factor for necrotic enteritis, and other factors (e.g. feed factors) will change under commercial conditions. If these changes favour *C. perfringens* proliferation and toxin production, the lack of a specific antibacterial effect of a non-ionophorous coccidiostat will involve an increased risk. The same will apply if coccidia strains develop tolerance or resistance to the new coccidiostat.

No data indicating a development of resistance in *C. perfringens* against narasin has been documented (Johansson et al., 2004; Lanckriet et al., 2010; Martel et al., 2004; Silva et al., 2009a; Watkins et al., 1997), in spite of widespread use of this ionophore in many countries for several decades.

Provided these considerations are valid, a replacement of narasin (without any other changes) over time is likely to lead to intermittently or continuously higher levels of use of therapeutic antibacterials. This is particularly likely to be the case in broilers, which up to now have been offered feeds supplemented with narasin. The situation is slightly different in turkeys, which are offered feeds supplemented with ionophores that are not considered as efficient against necrotic enteritis as narasin, although data on lasalocid are contradictory (Lanckriet et al., 2010; Martel et al., 2004).

3.5.5 Probability of increased use of therapeutic antibacterials for poultry if in-feed coccidiostats are replaced by anticoccidial vaccines

The literature on the effects of replacing in-feed coccidiostats with anticoccidial vaccines is so far scarce. Preliminary reports from the US suggest that this strategy may be associated with outbreaks in commercial farms of necrotic enteritis at 2-3 weeks of age. These outbreaks appeared to be associated with suboptimal vaccine delivery preventing sufficiently early development of immunity to coccidia, thus allowing pathogenic coccidia strains (environmental and possibly also the non-attenuated vaccine strains that are used in the US but not in Europe) to act as a predisposing factor for necrotic enteritis (Mozisek et al., 2015; Schaeffer et al., 2015). An experimental study conducted in an initially coccidia-free environment indicated that intestinal *C. perfringens* counts were considerably higher in vaccinated birds than in unvaccinated birds given narasin (Waldenstedt et al., 1998), which is not surprising given the fact that narasin (but not anticoccidial vaccines) exerts a directly suppressing effect on *C. perfringens*.

Up to now conventional broiler rearing has been based on the use of in-feed coccidiostats. Production of broiler meat without such additives has constituted a small fraction of the total production volume, and has been based on a less intensive production system (less concentrated feeds, slower growth rate, lower stocking density and so forth) associated with higher production costs.

This situation is now changing. Two major Norwegian broiler meat distributors have declared their intention to abolish the use of in-feed coccidiostats before 2017, and an increasing number of broiler flocks have been raised without coccidiostats in 2015. These flocks have been treated at day-old with a live attenuated anticoccidial vaccine, and such vaccination is likely to become an integrated part of a combination of alternative measures replacing in-feed coccidiostats. In addition to these industry efforts, a research project (2015-2018) initiated by the industry and lead by the Norwegian Veterinary Institute will provide new data on the preventive effect of non-antibacterial in-feed additives on gastrointestinal health in broilers. These projects will provide a lot of data and knowledge about the consequences of abolished use of in-feed coccidiostats in conventional broiler rearing, but so far we do not have enough data to fully evaluate the impact on usage of therapeutic antibacterials.

Initial experience with conventional commercial flocks (treated with an anticoccidial vaccine and offered feeds without narasin) reared without any other specific management changes suggests that clinical necrotic enteritis may occur sporadically. However, outbreaks of necrotic enteritis will not necessarily be treated with therapeutic antibacterials. The possibility of using in-feed narasin as a measure to control clinical cases of necrotic enteritis is now being explored by the industry (personal communication, Atle Løvland). If this treatment strategy is successful, an increase in the use of therapeutic antibacterials may turn out to be unnecessary.

Anticoccidial vaccines can potentially prevent coccidiosis and thereby remove an important predisposing factor for necrotic enteritis. However, such vaccines do not induce specific immunity against necrotic enteritis, which is caused by the bacterium *C. perfringens* and may appear even in the absence of coccidiosis. It is therefore likely that if in-feed coccidiostats are replaced with anticoccidial vaccines and no other changes are done, the likelihood of necrotic enteritis will increase. On this background it is important to identify other changes in management, including non-antibacterial feed additives that can contribute to gastrointestinal health in broilers reared without in-feed coccidiostats.

3.6 Summary of exposure

Human exposure to narasin resistant bacteria in Norway

- In the Norwegian surveillance programme NORM-VET 2002 - 2013, the percentage of flocks tested with narasin resistant enterococci in faecal samples was 62 % for broilers and 67 % for turkeys.
- The percentage of meat samples contaminated with narasin resistant enterococci was 36 % for broiler meat and 22 % for turkey meat.
- **Farmers and other production workers** that without proper protective measures regularly handle animals and manure from flocks with resistant bacteria are likely to be exposed to narasin resistant bacteria.
- **Consumers** that without proper kitchen hygiene measures handle contaminated fresh or frozen raw poultry meat may be exposed to narasin resistant bacteria.
- **Consumers** are not likely to be exposed to narasin resistant bacteria by eating properly heat treated poultry meat and poultry products.

Human exposure to coccidiostats in Norway

- **Workers** that without protective measures prepare coccidiostat premixes, and prepare or handle coccidiostat containing feeds, are likely to be exposed to coccidiostats.
- **Workers** that without protective measure handle manure from coccidiostat fed poultry, either directly or after storage, or prepare commercial fertilizer products based on such poultry manure, are likely to be exposed to residue levels of coccidiostats.
- **Consumers** of poultry meat and meat products from coccidiostat fed poultry are not likely to be exposed to narasin.
- Manure from coccidiostat fed poultry might be a source of transfer of coccidiostats to the environment, both during improper storage/composting and run-off of coccidiostats to nearby recipients, and after manure is applied as fertilizer to soil.

Use of therapeutic antibacterials

- If the ionophore coccidiostats used in Norway is replaced by one or more coccidiostats with no antibacterial effect and no other changes are done, this is over time likely to lead to intermittently or continuously higher levels of use of therapeutic antibacterials due to increased incidence of necrotic enteritis.

- If coccidiostats are replaced with anticoccidial vaccines and no other changes are done, the risk of necrotic enteritis will increase which will also lead to increased use of therapeutic antibacterials.

4 Risk characterisation

4.1 Risk characterisation

The probabilities and risks are characterized as:

- Negligible (extremely low)
- Low (possible, but not likely)
- Medium (likely)
- High (almost certain)
- Not assessable

4.1.1 Resistance to coccidiostats in bacteria

Available data show that the enterococci isolated from poultry given feed with the coccidiostat ionophores narasin, monensin and salinomycin may become resistant to these agents. In particular, the prevalence of resistant *E. faecium* has been found to be high. Data regarding enterococcal resistance against the ionophores lasalocid and maduramicin are lacking. However, lasalocid was reported to induce resistance to *Clostridium aminophilum* in a laboratory experiment. On the other hand, resistance in the poultry pathogen *C. perfringens* has not been reported against any of the ionophores. Data regarding resistance in other Gram-positive bacterial species of the normal microbiota of animals and humans is scarce. It is probable that resistance can be transferred between bacteria of the same species, and possibly also between bacteria of different species.

Present information indicates that the non-ionophore coccidiostats do not induce resistance in bacteria.

Furthermore, the Panel has no information on possible differences between the ionophores regarding their probability to induce resistance, and is not aware of any such data from clinical trials or practical use of rotation or shuttle programmes. However, due to similarity in the chemical structure and mode of action of ionophore agents, it can be suggested that rotation between ionophores may not have any impact to minimize the development of resistance in bacteria. This is supported by the fact that cross-resistance between ionophores has been shown. Data regarding rotation between ionophore and non-ionophore agents is also lacking. Therefore, the Panel has no evidence to claim that such programmes may contribute to reduce antibacterial resistance.

A few studies may indicate a possible association between resistance against narasin and resistance against other antibacterials like vancomycin and bacitracin in enterococci. However, there is at present no conclusive evidence that the ionophore coccidiostats induce resistance against other antibacterial agents, but this should be further studied.

4.1.2 Resistance to coccidiostats in coccidia

All the coccidiostats have been shown to induce resistance in coccidia. Resistance testing of coccidia is less standardized and more complicated than for bacteria, thus explaining the general lack of surveillance data. It is suggested that ionophores induce resistance development at a slower rate than non-ionophore coccidiostats. An explanation for this slow acquisition of resistance to ionophores may be that they allow for some leakage of sensitive oocysts which leads to a less stringent resistance selection than with non-ionophore coccidiostats. After introduction of monensin, the first ionophore on the market in the 1970's, these drugs are still predominant in the prevention of coccidiosis, supporting the hypothesis of slow resistance development.

Cross-resistance is observed, e.g. between narasin, monensin and salinomycin. Even though numerous papers have reported coccidiostat resistance and cross-resistance, these topics merit further investigations especially related to those having different mode of action. Furthermore, there is little information available on the effect of exchange of coccidiostats on coccidiostat resistance development.

4.1.3 The effect of coccidiostats on intestinal microbiota

Compared to the numerous papers published on intestinal microbiota, the topic effect of coccidiostats on composition and development of the normal intestinal microbiota has received less attention. Consequently, the Panel is not aware of any information on whether coccidiostats may exert changes in the composition of the intestinal microbiota and thereby indirectly affect the prevalence of bacteria resistant to other antimicrobials.

4.1.4 Human exposure to antimicrobial/coccidiostat resistant bacteria

Surveillance data may indicate that as many as 2/3 of the broiler and turkey farms at given may harbor narasin resistant bacteria. Without risk-reducing measures, farm workers who are frequently in direct or indirect contact with manure are therefore considered to have a high probability of exposure to narasin resistant bacteria. This is also in agreement with data from the literature. Various treatments, e.g. composting, of the manure may reduce the number of resistant bacteria present. Assessment of the effects of such methods has not been performed. Production workers that through transport, slaughter and processing are in contact with manure from a large number of flocks without risk-reducing measures may also have a high probability of exposure to narasin resistant bacteria. However, the data indicate that the probability associated with handling of carcasses and meat is medium, as narasin resistant enterococci were found in only 1/3 of the meat samples. The main route of exposure for farm and production workers is direct contact, but inhalation of contaminated aerosols or dust particles is also possible. Various risk-reducing measures are normally

applied in all parts of production, meaning that the probabilities in real life are lower than the presented theoretical ones.

The main routes of exposure for consumers are direct contact by handling raw fresh or frozen meat and ingestion of food that is not heat-treated. Heat-treated poultry products constitute a negligible probability of exposure to narasin resistant bacteria, both by handling and by ingestion. Without risk-reducing measures the consumer probability of exposure to resistant bacteria when handling raw meat is considered to be low to medium. The probability will be lower if risk-reducing measures are applied. However, the Panel does not know to which extent such measures are applied in the private households.

Although intestinal colonization of humans with various antimicrobial resistant bacteria has been reported in literature, there is no such available information regarding narasin resistant enterococci. Nor is there any information on the probability of transfer of narasin resistance genes from poultry enterococci to bacteria in the normal microbiota of humans or to human pathogens. One important reason for the lack of such studies is that a gene or genes coding for narasin resistance have not yet been identified.

Consequently, it is not known to what extent exposure to narasin resistant enterococci from poultry will lead to colonization in humans, and if so, under what conditions and for how long. Furthermore, the consequences of such colonization are unknown, as ionophore coccidiostats are not used to treat infectious diseases in humans, and cross- or co-resistance with resistance to antibacterials considered important in human medicine has so far not been confirmed.

4.1.5 Human exposure to coccidiostats

Bacteria of human normal microbiota may theoretically develop resistance if they are exposed to coccidiostats. All human skin and mucosal surfaces are covered by bacteria, i.e. the normal microbiota that consists of ten times as many bacterial cells as the human body's own cells. The bacteria of the normal microbiota may be exposed to coccidiostats by direct contact, ingestion and inhalation. Little relevant information has been found regarding the amount and/or time period of exposure of bacteria to ionophores that is needed for them to develop coccidiostat resistance. For example, no information exists regarding the practical implications of human or non-target animals' intermittent exposure to low (at or just above MRLs) or trace (below MRLs) levels of coccidiostat residues for antimicrobial resistance development among their endogenous microbiota.

4.1.5.1 Human exposure to coccidiostats in feed

Without risk reducing measures, the probability of coccidiostat exposure is high for workers preparing pre-mixes, feeds at feed mills as well as farmers handling feeds at poultry farms.

Use of protective clothing including masks and gloves, as well as normal hygienic precautions such as washing hands and equipment following pre-mix/feed handling should minimize the probability of exposure to low.

Based on current knowledge it is not possible to estimate the probability of real life occupational exposure of feed handlers to coccidiostats. These groups may, however, be considered “high consumers” and monitoring them for coccidiostat exposure and subsequent antimicrobial resistance development among their endogenous bacterial populations may be indicative of the upper risk situation that the current use of coccidiostats in poultry feeds may have for the human population.

4.1.5.2 Human exposure to coccidiostats in manure

Without risk reducing measures, the probability for exposure of coccidiostats is high for workers handling manure; during slaughter, transport of manure out of the poultry house and during cleaning the poultry house (poultry production), during the spread of manure on agricultural fields. For workers in contact with manure after long-term storage, composting or other treatment, lower probability of exposure to coccidiostats is expected. The probability for exposure for workers producing commercial poultry manure-based fertilizers is unknown. The probability of exposure will depend on the measures taken. With good guidelines for use of protection equipment the probability for human exposure should be very low.

Based on today's knowledge, it is not possible to evaluate probability for transfer of coccidiostats to edible crops. A worst case scenario could be applying fresh manure (using appr.1 cm bedding) or short term stored manure to agricultural fields. From the study by Ghent University, uptake of nicarbazin, monensin and lasalocid in the root vegetables and lettuce were observed, but the risk evaluation from Ghent University based on their own results was that it is unlikely to pose a direct threat to public health (Broekaert et al., 2012).

4.1.5.3 Human exposure to coccidiostats in poultry carcasses and products

Adherence to withdrawal periods before slaughter apparently minimizes the probability of exposure from consumption of poultry meat to negligible, as indicated by surveillance data (see section 3.4.4).

Cross-contamination of feeds meant for laying hens with coccidiostats in feed mills may occur, and surveys have shown coccidiostat levels above MRLs in 0.7-2.3% of eggs on the Norwegian market. Since narasin appears to be relatively heat tolerant up to 70°C, which is a temperature that may be expected for eggs following commonly used/practiced heat treatment, it is likely that high consumers of eggs may be intermittently exposed to coccidiostats at levels at or above MRLs.

Based on current knowledge it is not possible to perform a risk evaluation for consumer exposure to low levels (at or somewhat above MRLs) of coccidiostats in eggs. High consumers of eggs may experience intermittent exposure to low levels of coccidiostats, but it is currently not known whether such exposure will contribute to antimicrobial resistance development among the consumers' endogenous bacterial populations.

4.1.6 Probability of increased use of therapeutic antibacterials for poultry when using alternative measures of coccidiosis control

Probability of increased use of therapeutic antibacterials for poultry if coccidiostats with antibacterial effects are replaced by coccidiostats without such effects: Ionophorous coccidiostats suppress the growth of *C. perfringens*, the cause of necrotic enteritis. If the ionophores are replaced by coccidiostats without antibacterial effect and nothing else is changed, the probability of increased use of therapeutic antibacterials to treat necrotic enteritis is high (i.e. almost certain). The magnitude of the increase is difficult to predict.

Probability of increased use of therapeutic antibacterials for poultry if in-feed coccidiostats are replaced by anticoccidial vaccines: Anticoccidial vaccines do not induce specific immunity against necrotic enteritis, which is caused by the bacterium *C. perfringens*. The probability of increased use of therapeutic antibacterials to treat necrotic enteritis is therefore high (i.e. almost certain) if in-feed coccidiostats are replaced with anticoccidial vaccines and no other changes are made. The magnitude of the increase is difficult to predict.

It is, however, likely that other changes will be made following a replacement of ionophorous coccidiostats with anticoccidial vaccines or non-ionophore coccidiostats, in particular if the increase in use of therapeutic antibacterials is substantial. The Norwegian industry is already exploring the possibility of using in-feed narasin to treat cases of clinical necrotic enteritis. If this treatment strategy succeeds, an increased use of therapeutic antibacterials may not become necessary even if the number of clinical cases increases. Further, numerous alternative non-antibacterial products for feed supplementation have been developed and marketed. Some of these products have been demonstrated to reduce intestinal counts of *C. perfringens* and may therefore be able to reduce the number of cases requiring treatment. None of these individual products appear to be as efficient as narasin, and more work is needed to compare the effect of combinations of alternative products with the effect of narasin. Other types of management changes are also likely to be encouraged if the use of narasin and other ionophores is abolished.

4.2 Summary of risk characterisation

Resistance in bacteria and coccidia

- All coccidiostats approved in Norway are ionophores which may induce resistance in both bacteria and coccidia.
- The probability of bacteria developing resistance varies between the bacterial species, whereas the probability of coccidia developing such resistance is unknown.
- Cross-resistance between ionophores is observed in both bacteria and coccidia.
- In bacteria, a possible association between resistance to ionophores and resistance to other antibacterials is indicated, but further studies are required to validate these findings.
- The Panel is not aware of any information on whether coccidiostats may indirectly affect the prevalence of bacteria resistant to other antibacterials by exerting changes in the composition of the intestinal microbiota.
- The additional six coccidiostats approved in the EU may induce resistance in coccidia, but have no effect on bacteria.

Human exposure to coccidiostat resistant bacteria or coccidiostats in Norway

A risk assessment cannot be performed as the consequences of human exposure to coccidiostats or to narasin resistant enterococci from poultry are unknown. Consequently, only the probability of exposure has been assessed.

The probabilities are characterized as:

Negligible (extremely low), Low (possible, but not likely), Medium (likely), High (almost certain) or Not assessable

All probabilities are assessed under the assumption that risk-reducing measures are not applied. Risk-reducing measures will lower the probabilities

Table 4.2-1 Probability of human exposure to narasin or narasin resistant bacteria in Norway if risk-reducing measures are NOT applied.

Population groups	Activity	Coccidiostats Level ¹	Narasin resistant bacteria Level ¹
Feed factory workers	Handling coccidiostat premix	H	Not applicable
	Handling feed with coccidiostats	H	
Farm workers and other production workers	Handling feed with coccidiostats	H	Not applicable
	Handling fresh manure from poultry given feed with coccidiostats	H	H
	Handling live animals and equipment contaminated with manure from poultry given feed with coccidiostats	N to L	H
	Handling composted manure from poultry given feed with coccidiostats	M	Not assessed
	Handling commercial poultry manure-based fertilizers	Not assessed	Not assessed
	Handling carcasses and meat	N	M
Consumers	Handling raw meat	N	L to M
	Ingestion of sufficiently heat-treated meat	N	N

¹ Probability levels: N = Negligible (extremely low), L = Low (possible, but not likely), M = Medium (likely), H = High (almost certain)

Probability of increased use of therapeutic antibacterials for poultry when using alternative measures of coccidiosis control

Ionophorous coccidiostats suppress the growth of *C. perfringens*, the cause of necrotic enteritis. If the ionophores are replaced by coccidiostats without antibacterial effect or anticoccidial vaccines and nothing else is changed, increased use of therapeutic antibacterials to treat necrotic enteritis is likely. The magnitude of the increase is difficult to predict.

It is, however, likely that other changes will be made following a replacement of in-feed coccidiostats with anticoccidial vaccines, in particular if the increase in use of therapeutic antibacterials is substantial. The Norwegian industry is already exploring the possibility of using in-feed narasin to treat cases of clinical necrotic enteritis. If this treatment strategy succeeds, an increased use of therapeutic antibacterials may not become necessary even if the number of clinical cases increases. Further, numerous alternative non-antibacterial products for feed supplementation have been developed and marketed although none of these individual products appear to be as efficient as narasin. Other types of management changes are also likely to be encouraged if the use of narasin and other ionophores is abolished.

5 Uncertainties

Development and transfer of resistance to coccidiostats in bacteria

The number of scientific publications on the effect of ionophores on the development of antimicrobial resistance is small for lasalocid and maduramicin, and lacking for semduramicin. More data is available for narasin, monensin and salinomycin. Since narasin and monensin are used in Norwegian poultry farming the panel considers the scientific basis strong enough to conclude that anti-microbial resistance is induced by ionophores in conditions found in Norway.

The panel has chosen to consider it likely that horizontal transfer of resistance against coccidiostats may occur since transfer of a large variety of other antibacterial resistance genes is known to occur in bacteria. However, only one scientific study describes such transfer, reporting horizontal transfer of resistance against narasin *in vitro*. Furthermore, as none of the a coccidiostat resistance genes have been described in scientific literature, it is not known to which degree these genes are located on transferrable gene elements. Consequently the frequency of such gene transfer is not known.

Cross- or co-resistance between coccidiostats and other antimicrobials in bacteria

A statistical association was found between resistance against narasin and bacitracin, and between narasin and vancomycin. In addition, two publications report laboratory studies that may support these data; one studying narasin resistance and vancomycin resistance and enterococci, and one studying lasalocid resistance and bacitracin resistance in *Clostridium aminophilum*. However, the amount of data is very limited, and the level of uncertainty is high. More studies are needed before any firm conclusions can be drawn, and especially the characterization of resistance gene(s) against narasin would elucidate a possible link between resistance against narasin and resistance against bacitracin or vancomycin in enterococci.

Probability of increased use of therapeutic antibacterials in poultry production under current production practices if coccidiostats with antibacterial effects are replaced by coccidiostats without such effects, or replaced by anticoccidial vaccines

The conclusion that this probability is high is based on the assumption that other predisposing factors for necrotic enteritis than coccidiosis are important and prevalent in the broiler environment. Our knowledge about the relative importance of coccidia and other predisposing factors for necrotic enteritis is limited. Although this risk is high, the degree of increase in use of therapeutic antibacterials is uncertain as data on this is limited. Also, if the

use of therapeutic antibacterials increases significantly, other management changes are likely to be implemented in the field in order to counteract such a development.

Human exposure to coccidiostat resistant bacteria

Estimation of probability levels of exposure to resistant bacteria are based on data from the Norwegian surveillance program NORM-VET. Concerning data from faeces of live animals, the sampling unit of concern is flock. The Panel has assumed that the prevalence of positive farms equals that of positive flocks. In addition, there may have been relatively large differences between the age of the chicken flocks at the time of sampling, and sampling procedures have been changed over the years; all adding to the uncertainty of the estimated prevalences.

Furthermore, the estimated prevalences of resistant bacteria, both in faeces and on raw meat, are based on investigations on two species of bacteria only, i.e. *E. faecium* and *E. faecalis*. There are no surveillance data on prevalences in other bacterial species of the normal microbiota of poultry. Thus, the prevalences of may be underestimated.

Probability of human exposure and resistance development among endogenous microbiota of workers and consumers exposed to coccidiostats

Real life exposure probabilities in humans handling coccidiostats (feed mill workers, farmers etc.) has not been quantified because such exposure will to a large extent depend on the use of protective measures. The Panel does not have information on the actual use of protective measures. Therefore the probabilities are given according to a worst case scenario. In real life where people are likely to use some kind of protective measures the probabilities will be lower. Without such measures, absorption across the skin and mucous membranes of the respiratory tract may occur, but the subsequent risk to development of antibacterial resistant strains of bacteria among endogenous bacterial populations is not known.

Nor have the consequences of intermittent, low level exposure of coccidiostats by consumers at or somewhat above MRLs in poultry products for development of antibacterial resistant strains of bacteria among endogenous populations been investigated. The temperature stability of various coccidiostats merit documentation as well, as does the ability of denaturation products following heat treatment to induce the development of antibacterial resistant strains of bacteria in consumers.

These uncertainties do not allow the Panel to conclude more specifically regarding the probabilities and risks of human coccidiostat exposure among workers or consumers with subsequent resistance development among their respective endogenous microbiota.

Probability of exposure of coccidiostats for workers handling poultry manure

Several studies confirm residual coccidiostats in poultry excreta and manure. The concentration levels depend on numbers of factors; e.g. the age and treatment of the manure, the properties of the substances and the environmental conditions. The risk for exposure of workers handling manure is higher in contact with fresh or short-time stored manure than composted manure. A study performed by Ghent University on request by EFSA gives a reliable indication of the residue levels which is expected to find in excreta and manure during storage, composting and transfer to certain crops of the studied coccidiostats. However, due to differences in practical aspects and the high variation in disappearance fate, the concentration levels the workers might be exposed for can be either lower or higher than observed in this study. The challenge of sampling of non-homogenous matrix, such as manure, might influence the results, and should be considered during interpretation of data.

6 Answers to the terms of reference

The probabilities and risks are characterized as:

- Negligible
- Low (Unlikely, but possible)
- Medium (Likely)
- High (Almost Certain)
- Not assessable

6.1 To what extent can the 11 EU-authorized coccidiostats induce resistance and/or cross-resistance in bacteria?

Among these 11 coccidiostats six are ionophores and five are non-ionophore coccidiostats.

Five of the ionophores are reported to display antibacterial effects. Four of these, i.e. Narasin, Lasalocid, Monensin and Salinomycin, have been reported to induce resistance in various bacteria. However, there are large variations between bacterial species tested regarding the prevalence of resistance, e.g. resistance is often identified in the enterococci of the normal microbiota, but not in the pathogen *C. perfringens*. Whether these variations are due to differences between the bacteria, the coccidiostats and/or the magnitude of the coccidiostat exposure is not known.

The ionophore Maduramicin also exhibits antibacterial activity. Only one study addresses the development of resistance to this coccidiostat in bacteria. This was in *C. perfringens*, where resistance development was not observed. However, based on its antibacterial effect it is reasonable to believe that bacteria also may develop resistance against this agent.

The ionophore Semduramicin sodium is reported to have limited effect on bacteria, and data on its ability to induce resistance in bacteria has not been found.

The five non-ionophore coccidiostats, i.e. Robenidine, Diclazuril, Decoquinone, Halofuginon and Nicarbazine, do not display antibacterial effect, and are therefore not expected to induce resistance in bacteria.

Cross-resistance between ionophores has been reported, e.g. between narasin and salinomycin, and between salinomycin and monensin.

There is at present no confirmed cross- or co-resistance in bacteria between coccidiostats and other antibacterial agents. However, an association is suggested between ionophore resistance and resistance against the antibacterials vancomycin and bacitracin, and the possibility that such an association will be confirmed by future research cannot be excluded.

In conclusion, resistance to and cross-resistance between ionophore coccidiostats has been reported in a few bacterial species, mainly enterococci. Studies of resistance development in *C. perfringens* have not shown such development. Resistance against other antibacterials induced by ionophores has been suggested but not confirmed.

6.2 To what extent can the 11 EU-authorized coccidiostats induce resistance in coccidia?

All the coccidiostats have been shown to induce resistance in coccidia. Resistance testing of coccidia is less standardized and more complicated than for bacteria, thus explaining the general lack of surveillance data.

It has been suggested that ionophores induce resistance development at a slower rate than non-ionophore coccidiostats, possible due to ionophores allowing for some leakage of sensitive oocysts that leads to a less stringent resistance selection than with non-ionophore coccidiostats. After the introduction of monensin, the first ionophore on the market in the 1970's, these drugs are still predominant in the prevention of coccidiosis, supporting the hypothesis of slow resistance development.

Most of the initially marketed non-ionophore coccidiostats have disappeared from the market due to their rapid induction of resistance development. However, the ones in use today may appear to induce resistance less rapidly.

Cross-resistance is observed between ionophores, e.g. between narasin, monensin and salinomycin.

In conclusion, all the coccidiostats have been shown to induce resistance in coccidia, but cross-resistance has only been demonstrated between ionophores.

6.3 Are there advantages or disadvantages associated with the development of resistance in bacteria under the current practice in Norway with only five coccidiostats available compared to the 11 EU authorised coccidiostats?

All five coccidiostats approved in Norway are ionophores that display antibacterial effect. Bacterial resistance against four of them, i.e. Narasin, Lasalocid, Monensin and Salinomycin, has been reported. Data is lacking for the fifth ionophore, i.e. Maduramicin, but based on its antibacterial effect it is reasonable to believe that bacteria also may develop resistance against this agent. The Panel has no information on possible differences between these five

coccidiostats regarding probability to induce resistance, cross-resistance or co-resistance in different bacteria.

One of the six coccidiostats, which is only authorized in the EU, i.e. Semduramicin, is also an ionophore. However this ionophore is reported to have a limited effect on bacteria, and data regarding its potential ability to induce resistance in bacteria has not been found. The rest of the coccidiostats, i.e. Robenidine, Diclazuril, Decoquinate, Halofuginon and Nicarbazin, are non-ionophore coccidiostats which do not display antibacterial effect and are therefore not believed to induce resistance in bacteria. Consequently, these coccidiostats offer coccidiosis prevention without the risk of inducing resistance in bacteria.

Although it is generally accepted that antibacterial rotation or cycling may reduce the risk of resistance development in bacteria, this may not apply to ionophores. Due to similarity in the chemical structure and mode of action of ionophores, rotation between them will most likely not contribute to minimizing the development of resistance in bacteria. This is supported by data showing cross-resistance between the ionophores narasin and salinomycin, as well as between salinomycin and monensin. The Panel is not aware of any data from clinical trials or practical use of rotation of different coccidiostats aiming to minimize development of resistance in bacteria, either with rotation between different ionophores, or between ionophores and non-ionophore coccidiostats. Consequently, the effect of rotation of coccidiostats on resistance development in bacteria is not known.

It must be noted that when responding to this term, the Panel has not considered any other properties of the coccidiostats than the ability to induce resistance in bacteria.

In conclusion, if all eleven coccidiostats were approved in Norway, coccidiostats without antibacterial effect would be available, thus offering coccidiosis prevention with a negligible risk of inducing resistance in bacteria.

6.4 Are there advantages or disadvantages associated with the development of resistance in coccidia under the current practice in Norway with only five coccidiostats available compared to the 11 EU authorised coccidiostats?

All five coccidiostats approved in Norway are ionophores with similar mechanisms of action. When resistance against one of them is developed, the probability of cross-resistance against several of others is high. Five of the remaining six coccidiostats that are only authorized in EU are non-ionophore coccidiostats with different modes of action, and cross-resistance between these and ionophores are not expected.

To reduce development of resistance, different shuttle and rotation programmes are used. In shuttle programmes, two or more coccidiostats are used during the grow-out of a flock, e.g. one for starter and others for grower and finisher. In rotation programmes, the coccidiostats used are changed at regular intervals, e.g. between flocks. In both programmes, the alternation is usually between non-ionophore coccidiostats and ionophores. Furthermore, coccidiocidal non-ionophore coccidiostats are reported to be used to reduce the infection pressure of coccidiosis, in a so-called clean-up program. There is little information in the scientific literature on the effectiveness of such programmes. None of these programmes can be used in Norway as non-ionophore coccidiostats are not available.

It should be noted that when responding to this term, the Panel has not considered any other properties of the coccidiostats than the ability to induce resistance in coccidia.

In conclusion, if all eleven coccidiostats were approved in Norway, coccidiostats without cross-resistance to ionophores would be available, thus offering the possibility to use shuttle and rotation programmes to reduce development of resistance in coccidia.

6.5 What are the risks of antibacterial resistance being developed in and/or transferred to people (workers) handling coccidiostat preparations, feed, poultry, poultry meat or manure from poultry production using coccidiostat feed additives? If so, what risk-reducing measures are available?

A risk assessment cannot be performed as the consequences of development of resistance or colonization of resistant bacteria in the human normal microbiota are unknown. Ionophore coccidiostats are not used to treat infectious diseases in humans, and no cross- or co-resistance with antibacterials considered important in human medicine have been confirmed. Consequently, only the probability of exposure has been assessed. The probabilities are characterized as: Negligible (extremely low), Low (possible, but not likely), Medium (likely), High (almost certain) or Not assessable.

Transfer of coccidiostat resistant bacteria to people. If risk-reducing measures are not applied, the probability of exposure to coccidiostat resistant bacteria is considered to be high for handling of manure, as well as equipment, clothing and anything else that has been in contact with this, including live animals. Various treatments, e.g. composting, of the manure may reduce the number of resistant bacteria present. Assessment of the effects of such methods has not been performed. The probability is considered to be medium for handling poultry carcasses and raw meat, as well as equipment, clothing and anything else that has been in contact with this. Direct contact and inhalation are the most probable routes for

exposure of workers to coccidiostat resistant bacteria. Risk-reducing measures are e.g. restricted access, protective clothing, hygienic barriers between “contaminated” and “clean” zones and equipment, proper procedures for handling of manure, regular and thorough cleaning and disinfection, and regular monitoring of bacterial contamination of the production premises.

The Panel has no information on whether transferred bacteria will colonize the human body, either transiently or permanently. Furthermore, there is no information on the probability of exchange of resistance genes from transferred bacteria to bacteria of the human natural microbiota or to pathogens.

Development of coccidiostat resistant bacteria in people. Bacteria of the human normal microbiota might develop resistance if they are exposed to coccidiostats. Little relevant information has been found regarding the level of exposure, e.g. the amount of coccidiostats, or the time period necessary for the various bacteria to give rise to resistant variants.

If risk-reducing measures are not applied, the probability of exposure to coccidiostats is considered high for workers handling of pre-mix feed preparations and feed. The probability is medium to high for workers handling manure. The probability is negligible for handling carcasses and raw meat. Most probable routes of exposure for workers are direct contact and inhalation. Personal risk-reducing measures are protective clothing including masks covering nose and mouth. Automation, restricted access, regular cleaning, ventilation and waste control will also contribute to risk reduction.

6.6 What are the risks of antibacterial resistance being developed in and/or transferred to people (consumers) handling and eating meat from poultry production using coccidiostat feed additives?

A risk assessment cannot be performed as the consequences of any development of resistance or colonization of resistant bacteria in the human normal microbiota are unknown. Ionophore coccidiostats are not used to treat infectious diseases in humans, and no cross- or co-resistance with antibacterials considered important in human medicine have been confirmed. Consequently, only the probability of exposure has been assessed. The probabilities are characterized as: Negligible, Low (possible, but not likely), Medium (likely), High (almost certain) or Not assessable

Transfer of coccidiostat resistant bacteria to people. The probability of exposure to coccidiostat resistant bacteria is negligible for ingestion of heat treated meat and meat products, and low to medium for handling of fresh or frozen raw meat. The routes of

exposure to coccidiostat resistant bacteria are direct contact and ingestion. Risk-reducing measures are the same as recommended for handling raw meat in general, e.g. hygienic barriers between the meat and other food products, proper heat treatment, hand wash and general kitchen hygiene.

The Panel has no information on whether transferred bacteria will colonize the human body, either transiently or permanently. Furthermore, there is no information on the probability of exchange of resistance genes from transferred bacteria to bacteria of the human natural microbiota or to pathogens.

Development of coccidiostat resistant bacteria in people. Bacteria of the human normal microbiota may develop resistance if they are exposed to coccidiostats. Little relevant information has been found regarding the level of exposure, e.g. the amount of coccidiostats, or the time period necessary for the various bacteria to give rise to resistant variants. The probability of exposure to coccidiostats through handling or ingestion of poultry meat and meat products is negligible.

6.7 What are the risks of an increase in the therapeutic use of antibacterials in poultry production under current production practices if coccidiostats with antibacterial effects are replaced by coccidiostats without such effects?

The presently used coccidiostats are ionophores that suppress the growth of *C. perfringens*, the cause of necrotic enteritis. *C. perfringens* also plays a role in the severity of the Gizzard Erosion and Ulceration syndrome (GEU) in chicken. The majority of cases are subclinical with impaired production performance as the main consequence. Necrotic enteritis is very well controlled in broilers when the in-feed coccidiostat narasin is used. *C. perfringens* appears to be slightly less sensitive to the in-feed coccidiostat monensin which is used for turkeys, and this may explain the more frequent use of therapeutic antibacterials in this production.

If the ionophores are replaced by non-ionophore coccidiostats without antibacterial effect and no other management changes are implemented, there is a high probability of an increased frequency of outbreaks of these diseases and hence an increase in number of flocks that need treatment with antibacterials. The magnitude of the increase is difficult to predict. Coccidiosis is one of the predisposing factors of necrotic enteritis. If the new coccidiostats are more efficient against coccidia than the ionophores presently used, this may reduce the risk of necrotic enteritis, but probably not to the same level as with ionophores, as other risk factors may still be present. The Norwegian industry is currently exploring the possibility of treating outbreaks with antibacterials that are not used as therapeutic antimicrobials in human medicine. If this approach succeeds, the probability of

increased use of conventional therapeutic antibacterials with relevance to human medicine will be low.

In conclusion, increased use of therapeutic antibacterials is likely if currently used ionophores are replaced by non-ionophore coccidiostats or anticoccidial vaccines, but the magnitude of this increase cannot be predicted. Further, if disease in broiler chickens can be treated with antibacterials not considered important to human medicine, the impact of such an increase will be low.

6.8 Do alternative measures exist that can be employed to reduce the risk of coccidiosis in broiler chickens as effectively as coccidiostats?

Eradication of coccidia from the birds' environment is difficult to achieve because the coccidia form oocysts that survive outside the host and resist commonly used disinfectants. However, effective disinfectants are commercially available and their potential may not have been fully utilised up to now.

Vaccination is another option. Coccidia are intracellular parasites that are highly immunogenic, which has allowed the development of vaccines based on live coccidia. Some of these vaccines are based on strains that are still pathogenic ('non-attenuated' vaccines), others are based on non-pathogenic ('attenuated') strains. Both types of vaccines are able to induce protective immunity. Only non-pathogenic vaccines are used in Europe. This type of anticoccidial vaccine is now used increasingly in commercial Norwegian broiler farms, instead of in-feed coccidiostats. So far coccidiosis has not been reported as a problem in this transition process to broiler rearing without in-feed coccidiostats in Norway.

A third type of alternative measures is non-antibacterial feed additives. Acid-based products, probiotics, prebiotics, synbiotics, yeast-based products, plant-derived products, combinations of these, and other products have been developed and marketed as feed additives with claimed positive effects on the digestive system of broilers and fattening turkeys. These products have been tested for efficacy against coccidia with conflicting, non-consistent or non-convincing results. The majority of these products appear to target the bacterial microbiota rather than coccidia.

The Panel has not assessed possible effects of other types of management changes.

In conclusion, assessed alternative measures are, eradication of the coccidian, vaccination and non-antibacterial feed additives. Based on current knowledge, the panel finds vaccination to probably be the best alternative.

7 Data gaps

Consequences of human exposure to coccidiostats or coccidiostat resistant bacteria

The use of coccidiostats with an antibacterial effect in poultry feed can create a reservoir of resistant bacteria that may theoretically spread to humans both by direct contact with animals and manure, as well as through the food supply. Furthermore, bacteria of the human normal microbiota may theoretically develop resistance if they are exposed to coccidiostats, and farmers and other workers in the poultry production chain may be exposed to in-feed coccidiostats when handling feed and manure.

However, the risk of human exposure to coccidiostat resistant bacteria, as well as to coccidiostats, is not assessable as there is little information on the consequences of such exposure. Few relevant data have been found in scientific literature on the potential establishment of coccidiostat resistant bacteria from poultry in the human microbiota, either transitionally or permanently. Furthermore, there is no information on the probability of exchange of coccidiostat resistance genes from bacteria of poultry origin to bacteria of the human natural microbiota or to pathogens.

Likewise, information on the level of exposure, e.g. the amount of coccidiostats or the time period necessary for the various bacteria in the human normal microbiota to give rise to resistant variants is lacking. For example, the presumed regular and potentially high levels of occupational exposure among feed mill and farm workers to coccidiostats and the risk of development of antibacterial resistant bacteria among their endogenous bacteria populations is currently unknown but may be a source of valuable information to map such risk among "high consumers". Furthermore, the ability of intermittent exposure of consumers to low levels, particularly MRLs, of coccidiostat residues in poultry products to induce development of antibacterial resistant bacteria is unknown, but would be of practical importance to gauge the need for any change in regulations regarding these feed additives. Likewise, concrete information on the heat sensitivity of various coccidiostats and the ability of denaturation products to induce antibacterial resistant bacteria is of practical importance for consumers of poultry products that are generally heat-treated before consumption.

In order to risk assess workers' possibility for developing antibacterial resistance due to residual levels of coccidiostats in manure, more knowledge regarding the excretion kinetic of parent compounds and metabolites, their antibacterial resistance activity and their degradation and removal fate during storage and different treatment processes of manure is required.

The effect of narasin in poultry feed on development and prevalence of resistance to other antimicrobials

The coccidiostat narasin is used as additives in poultry feed to prevent coccidiosis caused by coccidian parasites. Narasin also inhibits or kills various bacterial species, and it is shown that bacteria in the normal microbiota of poultry, i.e. enterococci, develop resistance to narasin. Although narasin is not used to treat infectious diseases in humans, data may indicate an association with resistance to antibacterials considered important in human medicine. However, data addressing this question is scarce. Furthermore, the fact that genes conferring resistance to narasin have not been described in scientific literature makes confirmation of such information in practice impossible.

Narasin and other ionophore coccidiostats may theoretically also indirectly influence development of resistance to other antimicrobials by altering the composition of the normal intestinal microbiota of chicken. It is not unlikely that ionophores can favour Gram negative bacteria in general, because most of these compounds suppress the growth of Gram positive bacteria. However, it is not known whether the ionophores in this way can specifically favour any subgroup of resistant Gram negative bacteria.

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Appendix I

Scientific literature on ionophore coccidiostatic agents, approved and with marketing authorization in Norway as feed-additive to poultry (narasin, salinomycin, monensin, lasolocid, and maduramicin)

Table AI-1 Bacterial resistance against narasin

References	Country	Source	Bacterial species (number)	Tested antimicrobial agents	Conclusion
Nilsson <i>et al.</i> , 2012	Sweden	Broiler	<i>E. faecalis</i> , <i>E. faecium</i> (26)	Naracin, Vancomycin Bacitracin Ampicillin Virginamycin Streptomycin Gentamicin	Decreased susceptibility to narasin was co-transferred with the <i>vanA</i> gene in some clones.
Fard <i>et al.</i> , 2010	Australia	Pigs	Enterococci (192)	Ampicillin, Avilamycin, Avoparcin, Bacitracin, Favophospholipol, Gentamicin, Narasin, Tetracycline, Tamulin, Tylosin, Vancomycin, Virginiamycin, Copper and Zinc	All isolates were susceptible to narasin
Lanckriet <i>et al.</i> , 2010	Belgium	Broilers	<i>C. perferingens</i> (51)	Amoxicillin Kincomycin Tylosin and Ionophore anticoccidials:	The <i>C. perferingens</i> isolates examined were highly susceptible to the ionophore antibiotics Lasolocid, narasin,

References	Country	Source	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				lasalocid, salinomycin, maduramicin, narasin and a combination of narasin and nicarbazin	maduramicin, and salinomycin. Nicrabazin did not inhibit <i>C. perferingens</i> isolates even at a concentration of 128 microg/ml.
Silva et al, 2009	Brazil	Broiler chicken	<i>C. perferingens</i> (55)	Penicillin, narasin, monensin, avilamycin, lincomycin, tetracycline, bacitracin	All islates were susceptible to narasin and monensin.
Watkins et al., 2007	USA	Turkey and broiler chicken	<i>C. perferingens</i> (?)	Tilmocosin, tylosin, virginamycin, avilamycin, avoparcin, monnsin, narasin, penicillin, lincomycin, bacitracin	All islates were susceptible to narasin and monensin.
Sørum et al., 2004	Norway	Poultry, pocrine	<i>E. faecalis</i> (64), <i>E. faecium</i> (77)	Vancomycin	The MIC-values for narasin differed between the poultry (1-4 mg/liter) and the porcine (0.25-0.5 mg/liter) isolates, indicating a decreased susceptibility towards narasin among enterococci from poultry. -
Johansson et al., 2004	Sweden Demark Norway	Poultry	<i>C. perferingens</i> (102), Sweden (58) Norway (24) Denmark (20)	Ampicillin Avilamycin Bacitracin Narasin Avilamycin Erythromycin	All isolates were susceptible to narasin. Resistance to other antimicrobial agents were also low, except for high degree of resistance in isolates from

References	Country	Source	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				Vancomycin Oxytetracycline	Sweden.
Martel et al., 2004	Belgium	Broiler chicken	<i>C. perferingens</i> (47)	Avialmycin Tylosin Amoxicillin, Favomycin (babermycin) Chlortetracycline Oxytetracycline Ionophore coccidiostats: Narasin, monensin, lasalocid, salinomycin, mauduramycin	All strains were uniformly sensitive to the ionophore antibiotics monensin, lasalocid, salinomycin, maduramicin and narasin. Chlortetracycline and oxytetracycline were active at very low concentrations, but low-level acquired resistance was detected in 66% of the strains investigated. Sixty-three percent of the strains showed low-level resistance to lincomycin.
Butaye et al., 2002	Belgium	Pigenos	<i>E. faecalis</i> (17) <i>E. faecium</i> (13) <i>E. columbae</i> (48)	Ampicillin Babermycin Vancomycin Monensin Narasin Virginamycin Avilamycin Tetracycline	No resistance against bambermycin, vancomycin, monensin, narasin , virginiamycin, avilamycin, and ampicillin was seen. Tetracycline and tylosin was a frequent occurrence of resistance in all three enterococcal species tested. For

References	Country	Source	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				Tylosin Enerofloxacin Bacitracin Streptomycin Gentamicin	other antimicrobial agents, the occurrence of resistance was varied on the different enterococcal species.
Butaye <i>et al.</i>, 2001	Belgium	Farm and pet animals	<i>E. faecium</i> (146) and <i>E. faecalis</i> (166)	Ampicillin Babermycin Avopacin Monensin Narasin Virginamycin Avilamycin Bacitracin Oxytetracycline Tylosin Enerofloxacin Streptomycin Gentamicin	Resistance against antibiotics, like narasin, used solely for growth promotion was more prevalent in <i>E. faecium</i> strains than in <i>E. faecalis</i> strains. Narasin resistance, fully cross-resistant with salinomycin was found only in <i>E. faecium</i> from farm animals, mainly broilers.
Butaye <i>et al.</i>, 2000	Belgium	Meat poultry Cheese Raw pork	<i>E. faecium</i>	Ampicillin Babermycin Avopacin	Decreased susceptibility/resistance against growth promoter agents narasin, bacitracin, and virginamycin were

References	Country	Source	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				Narasin Virginamycin Avilamycin Bacitracin Minocycline Tylosin Avilamycin Streptomycin Gentamicin Dalfospristin/quinupristin Vancomycin	figh among strains from poultry meat.
Butaye et al., 2000	Belgium	Poultry	<i>E. faecium</i> (32) <i>E. faecalis</i> (33)	Salinomycin, Narasin, Monensin Lasalocid	Resistance against salinomycin and narasin in enterococci was frequent among poultry strains, whereas in pig strains, resistance was less common. No resistance was found against monensin and lasalocid. Full cross-resistance between salinomycin and narasin was evident. There was no cross resistance

References	Country	Source	Bacterial species (number)	Tested antimicrobial agents	Conclusion
					between these two ionophores and monensin and lasalocid.
Butaye et al., 2000	Belgium	Animals, foods	<i>E. faecium</i> (199) (47 from pet animals, 66 from farm animals, and 86 from foods) and <i>E. faecalis</i> (199)(53 from pet animals, 62 from farm animals, and 39 from foods).	Avilamycin Bacitracin Narasin Avoparcin Tylosin Virginamycin	Acquired resistance to bacitracin, narasin , tylosin, and virginiamycin was seen for both bacterial species. The MICs for the narasin-resistant strains were bimodally distributed, which indicates acquired resistance.
Lechtenberg et al., 1998	USA	Feedlot cattle (bovine hepatic abscesses)	<i>Fusobacterium necrophorum</i> (37)	Narasin and 18 different antimicrobial agents, which are used therapeutically and as growth promoters in animals in USA.	The isolates were resistant against ionophores antibiotics like Lasalocid, salinomycin, monensin and tetramas in, but susceptible to narasin .
Nagaraja & Taylor 1987	USA	Ruminant	24 different ruminal bacterial species	Narasin Lasalocid Monensin Salinomycin	Gram-positive bacteria were susceptible to ionophore and nonionophore compounds and Gram-negative bacteria were non/susceptible/res

References	Country	Source	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				Avoparcin Tylosin Virginamycin Thiopeptin	istant.

Table AI-2 Bacterial resistance against lasalocid

References	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
Cobos et al., 2011	Mexico	Lambs	<i>Pediococcus acidilactici</i> (the number has not been given)	Monensin Lasalocid	<i>P. acidilacti</i> was susceptible to both monensin and lasalocid
Lanckriet et al., 2010	Belgium	Broilers	<i>C. perferingens</i> (51)	Amoxicillin Kincomycin Tylosin and Ionophore coccidiostats: lasalocid, salinomycin maduramicin narasin and a combination of narasin and nicarbazin	The <i>C. perferingens</i> isolates examined were highly susceptible to the ionophore antibiotics Lasalocid, narasin, maduramicin, and salinomycin. Nicrabazin did not inhibit <i>C. perferingens</i> isolates even at a concentration of 128 µg/ml.
Martel et al., 2004	Belgium	Broiler chicken	<i>C. perferingens</i> (47)	Avialmycin Tylosin Amoxicillin, Favomycin (babermycin) Chlortetracycline Oxytetracycline Ionophore coccidiostats: Narasin, monensin, lasalocid,	All strains were uniformly sensitive to the ionophore antibiotics monensin, lasalocid, salinomycin, maduramicin and narasin. Chlortetracycline and oxytetracycline were active at very low

References	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				salinomycin, mauduramycin	concentrations, but low-level acquired resistance was detected in 66% of the strains investigated. Sixty-three percent of the strains showed low-level resistance to lincomycin.
Houblian and Russell 2001	USA	Cattle	<i>Clostridium aminophilum</i> F (number not stated)	Penicillin G, ampicillin, cephalosporin C, vancomycin, carbenicillin, tetracycline, chloramphenicol, erythromycin, streptomycin, lincomycin, rifampicin, trimethoprim, novobiocin, polymyxin B and bacitracin	Monensin- and lasalocid-resistant <i>C. aminophilum</i> F cultures were as susceptible to most antibiotics as non-adapted cultures. The only antibiotic that seemed to have a common mechanism of resistance was bacitracin, and the ionophore-adapted cultures had a 32-fold greater MIC.

Table AI-3 Bacterial resistance against Monensin

References	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
Jacob et al., 2014	USA	Cattle	<i>E. coli</i> O157 (21), <i>Salmonella</i> (21), commensal <i>E. coli</i> (188), <i>Enterococcus</i> (96)	Different class antimicrobial agents: aminocyclitols, aminoglycoside, beta-lactams, Cephalosporins, lincosamides, glycopeptides, macrolides, phenicols, quinolones, sulphonamides, tetracyclines	<i>Enterococcus</i> isolates from cattle fed monensin or monensin and tylosin had greater levels of resistance toward macrolides (erythromycin and tylosin). However, antimicrobial feed additives did not appear to increase the presence or concentration of either <i>ermB</i> or <i>teM</i> resistance elements in cattle
Silva et al., 2009	Brazil	Broiler chicken	<i>C. perferingens</i> (55)	Penicillin, narasin, monensin, avilamycin, lincomycin, tetracycline, bacitracin	All isolates were susceptible to narasin and monensin.
Watkins et al., 2007	USA	Turkey and broiler chicken	<i>C. perferingens</i> (?)	Tilmococin, tylosin, virginamycin, avilamycin, avoparcin, monensin, narasin, penicillin, lincomycin, bacitracin	All isolates were susceptible to narasin and monensin.
Cobos et al., 2011	Mexico	Lambs	<i>Pediococcus acidilactici</i> (the number has not been given)	Monensin Lasolocid	<i>P. acidilactici</i> was susceptible to both monensin and lasolocid
Martel et al., 2004	Belgium	Broiler chicken	<i>C. perferingens</i> (47)	Avialmycin Tylosin Amoxicillin, Favomyacin	All strains were uniformly sensitive to the ionophore antibiotics monensin, lasalocid, salinomycin, maduramicin and narasin.

References	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				(babermycin) Chlortetracycline Oxytetracycline Ionophore coccidiostats: Narasin, monensin, lasalocid, salinomycin, mauduramycin	Chlortetracycline and oxytetracycline were active at very low concentrations, but low-level acquired resistance was detected in 66% of the strains investigated. Sixty-three percent of the strains showed low-level resistance to lincomycin.
Houblian and Russell 2001	USA	Cattle	<i>Clostridium aminophilum</i> F (number not stated)	Penicillin G, ampicillin, cephalosporin C, vancomycin, carbenicillin, tetracycline, chloramphenicol, erythromycin, streptomycin, lincomycin, rifampicin, trimethoprim, novobiocin, polymyxin B and bacitracin	Monensin- and lasalocid-resistant <i>C. aminophilum</i> F cultures were as susceptible to most antibiotics as non-adapted cultures. The only antibiotic that seemed to have a common mechanism of resistance was bacitracin, and the ionophore-adapted cultures had a 32-fold greater MIC.
Butaye et al., 2001	Belgium	Farm and pet animals	<i>E. faecium</i> (146) and <i>E. faecalis</i> (166)	Ampicillin Babermycin Avopacin Monensin Narasin Virginamycin Avilamycin	Resistance against antibiotics, like narasin, used solely for growth promotion was more prevalent in <i>E. faecium</i> strains than in <i>E. faecalis</i> strains. Narasin resistance, fully cross-resistant with salinomycin was found only in <i>E. faecium</i> from farm animals, mainly broilers. Resistance against growth promoter agents monensin

References	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				Bacitracin Oxytetracycline Tylosin Enrofloxacin Streptomycin Gentamicin	and flavomycin was not detected.
Todd et al., 2001	USA	Cattle	<i>Prevotella</i> spp. (15)	Monensin	Strains that were repeatedly transferred with sublethal doses tolerated (resistant) more monensin than those that were un-adapted.
Callaway and Russell 1999	USA	Cattle	<i>Prevotella bryantii</i>	Monensin	<i>Prevotella bryantii</i> cultures treated with monensin grew more slowly than untreated cultures, but only if the monensin concentration was greater than 1 microM. Cultures that were repeatedly transferred (eight transfers or 25 doublings) with monensin always grew rapidly, even at a 10 microM concentration.
Todd et al., 1999	USA	Cattle	<i>Butyrivibrio fibrisolvens</i> 49, <i>Streptococcus bovis</i> JB1, <i>Clostridium aminophilum</i> F, S. <i>ruminantium</i> HD4, and <i>M. elsdenii</i> B159	Monensin	Gram-positive ruminal bacterial species are generally susceptible to monensin, but some bacteria can adapt and increase their resistance. This resistance could be explained by the ability of these low G + C Gram-positive bacteria to synthesize outer membranes
Newbold et al., 1993	UK	Cattle	<i>The ruman Gram-negative</i>	Monensin, tetronasin	<i>In vitro</i> study: All three species became

References	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
			<i>bacteria Fibrobacter succinogenes</i> S85, <i>Prevotella ruminicola</i> M384 and <i>Veillonella parvula</i> L59		more resistant to the ionophore with which they were grown. Increased resistance to one ionophore caused increased resistance to the other, and cross-resistance to another ionophore--lasalocid--and an antibiotic--avoparcin.
Morehead and Dawson 1992	USA	Ruminant	<i>Prevotella (Bacteroides) ruminicola</i> subsp. <i>ruminicola</i> 23 and <i>P. ruminicola</i> subsp. <i>brevis</i> GA33	Monensin	<i>Prevotella</i> (Bacteroides) <i>ruminicola</i> subsp. <i>ruminicola</i> 23 and <i>P. ruminicola</i> subsp. <i>brevis</i> GA33 by stepwise exposure to increasing concentrations of monensin. The resulting resistant strains (23MR2 and GA33MR) could initiate growth in concentrations of monensin which were 4 to 40 times greater than those which inhibited the parental strains. Resistant strains also showed enhanced resistance to nigericin and combinations of monensin and nigericin but retained sensitivity to lasalocid.
Dutta et al., 1983	Belgium	Pigs, cattle poultry	<i>Clostridium</i> spp.	chloramphenicol penicillin G, lincosamides tetracycline avoparcin, carbadox, monensin, nitrovin, virginiamycin bacitracin,	All strains were classified as susceptible to avoparcin, carbadox, furazolidone, monensin, nitrovin and ronidazole.

References	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				tiamulin Monensin	
Dawson and Boling 1983	USA	Heifers	Anaerobic bacteria	Monensin	Monensin-resistant bacteria may be present in greater numbers in the rumens of animals fed monensin-supplemented diets. However, greater proportions of monensin-resistant organisms were not necessarily associated with altered fermentation patterns.

Table AI-4 Bacterial resistance against salinomycin

Reference	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
Tremblay et al., 2011	Canada	Broiler chicken and turkey flocks	<i>E. faecalis</i> <i>E. faecium</i> (387) from five different processing plants	Bacitracin, Chloramphenicol, Ciprofloxacin, Erythromycin, Flavomycin, Gentamicin, Kanamycin, Lincomycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin-dalfopristin, Salinomycin, Streptomycin, Tetracycline, Tylosin, Vancomycin.	<i>E. faecalis</i> (7%) and <i>E. faecium</i> (12.8%) of the isolates were resistant to salinomycin.
Okada et al., 2011	Japan	Foods, environments, animals and human patients	<i>L. monocytogenes</i> (201)	Ampicillin, Chloramphenicol, Dihydrostreptomycin, Erythromycin, Enrofloxacin, Gentamicin, Kanamycin, Lincomycin, Nosiheptide, Salinomycin, Vancomycin, Virginiamycin	All isolates were susceptible to salinomycin.
Aarestrup and Tvede 2011	Denmark	Human with diarrhoea	<i>C. difficile</i> (65)	Avilamycin, Flavomycin, Monensin, and Salinomycin	Avilamycin, monensin and salinomycin had MIC-values comparable or slightly lower than those reported for metronidazole and vancomycin.
Lanckriet et al., 2010	Belgium	Broilers	<i>C. perferingens</i> (51)	Amoxicillin Kincomycin Tylosin and Ionophore	The <i>C. perferingens</i> isolates examined were highly susceptible to the ionophore antibiotics lasolocid, narasin, maduramicin, and salinomycin. Nicrabazin

Reference	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				coccidiostats: lasalocid, salinomycin, maduramicin, narasin and a combination of narasin and nicarbazin	did not inhibit <i>C. perferingens</i> isolates even at a concentration of 128 µg/ml.
Fluckey et al., 2009	USA	Cattle	<i>Enterococcus</i> spp (279)	Chloramphenicol Flavomycin Lincomycin Tylosin Erythromycin Tetracycline Quinupristin-dalfopristin Streptomycin Ciprofloxacin Linezolid Salinomycin	While all isolates were resistant to chloramphenicol, only 0.4% was resistant against salinomycin.
Diarra et al., 2007	Canada	Broiler	<i>E. coli</i> (197)	Bambermycin, Penicillin, Salinomycin, Bacitracin, Tetracycline, Amoxicillin Ceftiofur Spectinomycin, Sulfonamides Chloramphenicol, Gentamicin	The proportions of isolates positive for <i>sull</i> , <i>aadA</i> , and integron class 1 were significantly higher in salinomycin-treated chickens than in the control or other treatment groups.
Mcgowan et al., 2006	USA (Northern Georgia)	Vegetables, fruits, meat	<i>Enterococcus</i> spp. (209)	Salinomycin Lincomycin Penicillin Linezolid Vancomycin Nitrofurantoin	Only 2 isolates (1.1%) of the isolates were salinomycin-resistant. Resistant against linomycin and bacitracin were higher in the enterococci isolates.
Pringle et al., 2006	Sweden	Pig	<i>Brachyspira pilosicoli</i> (Gram-negative anaerobe bacteria) (103)	Salinomycin Tiamulin	The susceptibility to salinomycin and doxycycline was high but the MICs for avlosin varied

Reference	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				Tylosin Doxycycline Valnemulin Lincomycin Avilosisin	
Scalzo et al., 2004	UK	Poultry (experimentally infected) The effect of continuous in-feed administration of coccidiostats on antimicrobial sensitivity and the level of bacterial shedding in poultry experimentally infected with Salmonella.	Salmonella enterica subsp. enterica serotype Typhimurium definitive type 104 (DT104)	Salinomycin Monensin Semduramicin	Supplementation of the diet with an coccidiostat drug does not appear to affect antimicrobial resistance or the level of excretion of salmonellae.
White et al., 2003	Georgia, USA	Poultry	<i>S. aureus</i> (77)	Tetracycline Erythromycin Kanamycin Chloramphenicol Gentamicin Streptomycin Nitrofurantion Linezolid Quinupristin/dalfopristin Vancomycin Virginiamycin	All isolates were susceptible to salinomycin.

Reference	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				Salinomycin Flavomycin	
Martel et al., 2004	Belgium	Broiler chicken	<i>C. perferingens</i> (47)	Avialmycin Tylosin Amoxicillin, Favomycin (babermycin) Chlortetracycline Oxytetracycline Ionophore coccidiostats: Narasin, monensin, lasalocid, salinomycin, mauturamycin	All strains were uniformly sensitive to the ionophore antibiotics monensin, lasalocid, salinomycin, maduramicin and narasin. Chlortetracycline and oxytetracycline were active at very low concentrations, but low-level acquired resistance was detected in 66% of the strains investigated. Sixty-three percent of the strains showed low-level resistance to lincomycin.
Yoshimura et al., 2000	Japan	Chickens in boiler and layer farms	Enterococci (222)	Ampicillin, Clindamycin, Erythromycin, Streptomycin, Tetracycline Tylosin Ofloxacin Avilamycin, Salinomycin Virginiamycin	Resistance to salinomycin was detected in all enterococcal species, ranging from 12.4% of <i>E. faecium</i> isolates to 50% of <i>E. hirae</i> isolates.
Aarestrup et al., 2000	Denmark, Finland, Norway	Broilers, pigs	Enterococci	Avilamycin Avoparcin Bacitracin Flavomycin	Only a limited number of the isolates, all from Danish broilers were categorized as resistant against monensin or salinomycin.

Reference	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
				Monensin Salinomycin Spiramycin Tylosin Virginiamycin	
Butaye et al., 2000	Belgium	Poultry	<i>E. faecium</i> (32) <i>E. faecalis</i> (33)	Salinomycin, Narasin, Monensin Lasalocid	Resistance against salinomycin and narasin in enterococci was frequent among poultry strains, whereas in pig strains, resistance was less common. No resistance was found against monensin and lasalocid. Full cross resistance between salinomycin and narasin was evident. There was no cross resistance between these two ionophores and monensin and lasalocid.
Møller et al., 1997	Denmark	Swine Cattle Poultry	1) indicator bacteria (<i>E. coli</i> , <i>E. faecalis</i> , <i>E. faecium</i>), 2) zoonotic bacteria (<i>Campylobacter</i> , <i>Salmonella</i> , <i>Y. enterocolitica</i>), and 3) animal pathogens (<i>E. coli</i> , <i>S. aureus</i> , coagulase-negative staphylococci (CNS), <i>S. hyicus</i> , <i>A. pleuropneumoniae</i>)	Avilamycin, avoparcin (vancomycin), bacitracin, carbadox, flavomycin, monensin, olaquinox, salinomycin, spiramycin (erythromycin, lincomycin), tylosin (erythromycin, lincomycin), and virginiamycin (pristinamycin)	Acquired resistance to all currently used growth promoting antimicrobials was found. A frequent occurrence of resistance was observed to avilamycin, avoparcin, bacitracin, flavomycin, spiramycin, tylosin and virginiamycin, whereas resistance to carbadox, monensin, olaquinox and salinomycin was less frequent.
Wiggins 1996	Virginia, USA	Cattle,	Enterococci (previously identified as faecal streptococci)	Chlortetracycline, Halofuginone, Oxytetracycline, Salinomycin,	Resistance against salinomycin was high in enterococci of various sources, however no

Reference	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
		poultry, human, and wild-animal wastes	(1435)	Streptomycin	resistance were observed in isolates from human.
Devriese et al., 1993	Belgium	Poultry pigs calves	<i>C. perferingens</i> (94)	Bacitracin Tylosin Virginamycin Flavomycin Avilamycin Salinomycin	Acquired resistance against bacitracin was detected in some isolates from poultry and bovines and resistance to tylosin and virginiamycin in some strains from all species investigated. Overall, the prevalence of resistance was comparable to the low levels recorded in 1979 in <i>Cl. perfringens</i> isolates from the same animal host species.
Nagaraja & Taylor	USA	Ruminant	24 different ruminal bacterial species	Narasin Lasalocid Monensin Salinomycin Avoparcin Tylosin Virginamycin Thiopeptin	Gram-positive bacteria were susceptible to ionophore and nonionophore compounds and Gram-negative bacteria were non-susceptible/resistant.
Olumeyan et al., 1986	Kansas, USA	Cattles	Anaerobic bacteria	Salinomycin (cattle fed diets with and without salinomycin.	Salinomycin-resistant bacteria increased from 7.6 to 15.6% in salinomycin-fed steers but remained unchanged in control steers. Salinomycin had no effect on cellulolytic and lactate-utilizing bacteria, but the proportion of

Reference	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
					amylolytic bacteria was higher in salinomycin-fed steers than in control steers.
Kondo 1988	Colorado, USA	Broiler	<i>C. perferingens</i> (88)	22 different antimicrobial agents Bacitracin, monensin, Salinomycin, lasalocid	polyethers of monensin, salinomycin and lasalocid were generally adequate in low concentrations while there was a high level of resistance to three tetracyclines in 90 per cent of the strains and all isolates were insusceptible to streptomycin of the aminoglycoside antibiotics
George et al., 1982	USA	Chicken	Coliforms and streptococci (enterococci)	Chicken fed salinomycin	

Table AI-5 Bacterial resistance against maduramicin

References	Country	Food-producing animal	Bacterial species (number)	Tested antimicrobial agents	Conclusion
Lanckriet <i>et al.</i> , 2010	Belgium	Broilers	<i>C. perferingens</i> (51)	Amoxicillin Kincomycin Tylosin and Ionophore coccidiostats: lasalocid, salinomycin, maduramicin, narasin and a combination of narasin and nicarbazin	The <i>C. perferingens</i> isolates examined were highly susceptible to the ionophore antibiotics lasalocid, narasin, maduramicin, and salinomycin. Nicrabazin did not inhibit <i>C. perferingens</i> isolates even at a concentration of 128 µg/ml.

Appendix II

Investigations on possible associations between narasin resistance and resistance to other antimicrobial agents in *E. faecium* – as provided by the Norwegian Veterinary Institute



Investigations regarding possible associations between narasin resistance and resistance to other antimicrobial agents in *Enterococcus faecium*

Introduction

Possible associations between narasin resistance and resistance to other antimicrobial agents in *Enterococcus faecium* is discussed in NORM-VET 2014. However, after a request from the Norwegian Scientific Committee for Food Safety, the analyses have been repeated with some changes in the model and categorization of groups as described below.

Material and methods

As described in NORM-VET 2014, resistance data for all *E. faecium* isolates originating from all animal species included in NORM-VET during 2003-2013 were included in the analysis (N=1246). These isolates originated from cattle (n=49), swine (n=165), sheep (n=1), dogs (n=12), broilers (n=719), layers (n=103) and turkeys (n=197).

The data were reanalyzed using separate models categorizing the isolates into the following groups:

- "all species"
- "all species except broilers and turkey"
- "all species except poultry" (poultry = broilers + layers + turkey)
- "broilers"
- "turkey"

Further description of the statistical methods is described in NORM-VET 2014.

Results

As described in NORM-VET 2014, a significant positive association was observed between

- narasin and bacitracin resistance for the groups
 - "all species" (p<0.001)
 - "broilers" (p<0.001)
 - "all species except broilers and turkey" (p<0.02)
 - "all species except poultry" (p<0.03)
- narasin and gentamicin resistance for the group
 - "broilers" (p<0.05)
- narasin and streptomycin for the group
 - "broilers" (p<0.05)

Whereas a significant negative association was observed between

- narasin and streptomycin resistance for the groups
 - "all species" (p<0.01)
 - "all species except broilers" (p<0.005)

A separate analysis of isolates originating from turkey showed no significant association between narasin resistance and other resistances.



Conclusions

If cross resistance against narasin and other antimicrobials did occur, one could expect that there would be a positive association between narasin and other/specific antimicrobials for all/many different groups.

However, the fact that

- the observed association between the occurrence of resistance against narasin and streptomycin was positive (for “broilers”) and negative (for “all species” and “all species except broilers”) AND
- a positive association between the occurrence of resistance against narasin and gentamicin was observed only for “broilers”

indicates that cross-resistance does not occur.

Resistance against narasin and bacitracin occurred quite commonly. However, whether the narasin resistance increase the probability of bacitracin resistance or vice versa or both resistances can be explained by the same cause is unclear. In order to investigate if there is a possible causal relationship between the usage of narasin and resistance to other antimicrobials, further investigations are needed.

References

NORM/NORM-VET 2014. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo 2015. ISSN:1502-2307 (print) / 1890-9965 (electronic).

Appendix III

Minimum and maximum content of coccidiostats allowed in complete diet formulations for poultry

Table AIII-1 Overview of coccidiostats registered in Norway

<i>Generic name; Trade name(s), concentration in premix (producer)</i>	<i>Chemical formula</i>	<i>Species/ Production</i>	<i>Maximum age</i>	<i>Minimum content mg/kg complete feed with moisture content 12%</i>	<i>Maximum content</i>	<i>Other provisions, incl. withdrawal times, MRL- values in animal products</i>	<i>Regulation</i>
Lasalocid A natrium Avatec 150 G; 15 g/100 g (Zoetis Belgium SA)	C ₃₄ H ₅₃ O ₈ Na	Chickens: Layers Broilers Turkeys Pheasants, guinea fowl, quails, arable hens; but not egg-layers	16 weeks - 16 weeks -	75 75 75 75	125 125 125 125	No withdrawal time specified Shall not be mixed with other coccidiostats Dangerous for equines MRL (for chickens): Liver and kidney: 50 µg/kg wet wt Milk: 1 µg/kg wet wt Other: 5 µg/kg wet wt MRL (for turkeys, pheasants, etc.): Commission regulation (EU) no 37/2010	
Maduramicin ammonium alfa Cygro 10 g; 10 g/kg (Zoetic Belgium SA)	C ₄₇ H ₈₃ O ₁₇ N	Chickens: Broilers	-	5	6	Use prohibited at least 3 days before slaughter Shall not be mixed with other coccidiostats	

						Dangerous for equines MRL Liver and skin/fat: 150 µg/kg wet wt Kidney: 100 µg/kg wet wt Muscle: 30 µg/kg wet wt
Monensin-natrium Elancoban G100; 10 g/100g Elancogran 100; 10 g/100 g Elancoban G200; 20 g/100 g (Eli Lilly & Co Ltd)	C ₃₆ H ₆₁ O ₁₁ Na	Chickens: Layers Broilers Turkeys	16 weeks - 16 weeks	100 100 60	120 125 100	Use prohibited at least 1 day before slaughter Shall not be mixed with other coccidiostats MRL: Skin and fat: 2 µg/kg wet wt Liver, kidney and muscle: 8 µg/kg wet wt
Coxidin; 25 g/100 g (Huvepharma NV Belgium)		Chickens: Layers Broilers Turkeys	16 weeks - 16 weeks	100 - 60	125 - 100	Use prohibited at least 1 day before slaughter Shall not be mixed with other coccidiostats Dangerous for equines MRL: Skin and fat: 25 µg/kg wet wt Liver, kidney and muscle: 8 µg/kg wet wt Other animal products: 2 µg/kg wet wt
Narasin Monteban Monteban G100; 100 g/kg (Eli Lilly & Co Ltd)	C ₄₃ H ₇₂ O ₁₁	Chickens: Broilers	-	60	70	Use prohibited at least 1 day before slaughter MRL: Egg: 2 µg/kg wet wt Milk: 1 µg/kg wet wt Liver: 50 µg/kg wet wt Other animal products: 5 µg/kg wet wt
Salinomycin natrium Salinomax 120G; 120	C ₄₂ H ₆₉ O ₁₁ Na	Chickens:				Use prohibited at least 1 day before slaughter

g/kg (Zoetis Belgium SA)	Broilers	12 weeks	50	70	Dangerous for equines and turkeys MRLs: Egg: 3 µg/kg wet wt Liver: 5 µg/kg wet wt Other animal products: 3 µg/kg wet wt
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Sacox 120 microGranulat; ; 120 g/kg (Huvepharma NV Belgium)	Chickens: Broilers Layers	- 12 weeks -	60 50	70 50	Use prohibited at least 1 day before slaughter Dangerous for equines and turkeys MRL: Egg: 3 µg/kg wet wt Liver: 5 µg/kg wet wt Other animal products: 3 µg/kg wet wt
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Kokcican 120 G (KRKA d.d Novo mesto, Slovenia)	Chickens: Broilers	12 weeks	60	70	Use prohibited at least 3 days before slaughter Dangerous for equines and turkeys MRL: All animal products: 5 µg/kg wet wt

Source: Annex 1 and 2 of «Forskrift om tilsetningsstoffer til bruk i fôrvarer»

Table AIII-2 Overview of coccidiostats registered in the EU (Ref.: EU (2014) EU Register of Feed Additives pursuant to Regulation (EC) No 1831/2003; Appendix 3e & 4(I). Annex I: List of additives. Edition 185 released 12.05.2014)

Generic name; Trade name(s), concentrations in premix (producer)	Chemical formula	Animal species and category	Maximum age	Minimum content <i>mg/kg complete feed with moisture content 12%</i>	Maximum content	Other provisions, incl. withdrawal period, MRL-values in foodstuffs of animal origin
Decoquinat Deccox; 60,6 g/kg (Zoetis Belgium SA)	C ₂₄ H ₃₅ NO ₅	Chickens: Broilers	-	20	40	No withdrawal time specified MRL Liver and skin/fat: 1000 µg/kg wet wt Kidney: 800 µg/kg wet wt Muscle: 500 µg/kg wet weight
Diclazuril Clinacox 0.5 %; 0.50 g/100 g (Eli Lilly and Co. Ltd/Janssen Pharmaceutica NV)	C ₁₇ H ₉ Cl ₃ N ₄ O ₂	Chickens: Broilers Layers Turkeys Rabbits Guinea fowl	-	1	1	No withdrawal time specified MRL Liver: 1500 µg/kg wet wt Kidney: 1000 µg/kg wet wt Muscle and skin/fat: 500 µg/kg wet wt
Halofuginone hydrobromide Stenorol; 6 g/kg (Huvepharma NV)	C ₁₆ H ₁₇ BrClN ₃ O ₃ ,HBr	Chickens: Broilers Layers Turkeys	- 16 weeks 12 weeks	2 2 2	3 3 3	Use prohibited at least 5 days before slaughter MRL: ?
Lasalocid A sodium Avatec 150 G; 15	C ₃₄ H ₅₃ O ₈ Na	Chickens Broilers	-	75	125	Use prohibited at least 5 days before slaughter Dangerous for equines

g/100 g (Alpharma Belgium BVBA)		Layers	16 weeks	75	125	MRL: EU regulation no 37/2010
		Turkeys	12 weeks	75	125	
		Others				
Maduramicin ammonium alpha Cygro 10 G; 10 g/kg (Alpharma Belgium BVBA)	$C_{47}H_{83}O_{17}N$	Chickens: Broilers Turkeys	-	5	6	Use prohibited at least 3 days before slaughter Shall not be mixed with other coccidiostats Dangerous for equines MRL Liver and skin/fat: 150 µg/kg wet wt Kidney: 100 µg/kg wet wt Muscle: 30 µg/kg wet wt
Monensin-natrium Coxidin; 25 g/100 g (Huvepharma NV Belgium)	$C_{36}H_{61}O_{11}Na$	Chickens: Broilers Layers Turkeys	- 16 weeks 16 weeks	100 100 60	125 125 100	Use prohibited at least 1 day before slaughter Shall not be mixed with other coccidiostats Dangerous for equines MRL: Skin and fat: 25 µg/kg wet wt Liver, kidney and muscle: 8 µg/kg wet wt
Elancoban G100/G200 (Eli Lilly & Co Ltd)		Chickens: Broilers Layers Turkeys		100 100 60	125 120 100	Use prohibited at least 3 days before slaughter Dangerous for equines
Narasin Monteban; 100 g/kg (Eli Lilly & Co Ltd)	$C_{43}H_{72}O_{11}$	Chickens: Broilers		60	70	No withdrawal period Dangerous for equine species, turkeys and rabbits MRL: 50 µg/kg wet wt for all tissues from broilers

Narasin+nicarbazin Maxiban G160; 1:1 ratio, 80 g/kg for both (Eli Lilly & Co Ltd)	$C_{43}H_{72}O_{11} + C_{19}H_{18}N_6O_6$	Chickens: Broilers	-	40 mg Narasin 40 mg Nicarbazin	50 mg Narasin 50 mg Nicarbazin	Use prohibited at least 1 day before slaughter Shall not be mixed with other coccidiostats Dangerous for equines, rabbits and turkeys MRL Narasin: Liver, muscle, kidney, skin/fat: 50 µg/kg wet wt Dinitrocarbanilide (DNC): Liver: 15000 µg/kg wet wt Kidney: 6000 µg/kg wet wt Muscle and skin/fat: 4000 µg/kg wet wt
Nicarbazin 250 g/kg (Phibro Animal Health SA Belgium)	$C_{19}H_{18}N_6O_6$	Chickens: Broilers	-	125	125	Use prohibited at least 1 day before slaughter MRL Liver: 15000 µg/kg wet weight Kidney: 6000 µg/kg wet weight Muscle and skin/fat: 4000 µg/kg wet weight
Robenidine HCl Robenz 66G; 66 g/kg (Alpharma Belgium BVBA)	$C_{15}H_{13}Cl_2N_5-$ HCl	Chickens: Broilers	-	30	36	Use prohibited at least 5 days before slaughter MRL (chickens) Liver: 800 µg/kg wet wt Kidney: 350 µg/kg wet wt Muscle: 200 µg/kg wet wt Skin/fat: 1300 µg/kg wet wt
Robenz 66G; 66 g/kg (Alpharma Belgium BVBA)		Turkeys	-	30	36	Use prohibited at least 5 days before slaughter MRL (turkeys) Liver and skin/fat: 400 µg/kg wet wt Muscle and kidney: 200 µg/kg wet wt
Cycostat 66G; 66		Rabbits		50	66	

g/kg (Alpharma Belgium BVBA)					Use prohibited at least 5 days before slaughter MRL (rabbits) -
Salinomycin natrium	C ₄₂ H ₆₉ O ₁₁ Na	Chickens:			
Kokcisan 120G; 120 g/kg (KRKA, d.d Novo mesto, Slovenia)		Broilers	-	60	70
					Use prohibited at least 3 days before slaughter Dangerous for equines and turkeys MRL: 5 µg/kg wet wt for all animal products
Sacox 120 micro-Granulate; 120 g/kg (Huvepharma NV)		Broilers Layers			
Salinomax 120G; 120 g/kg (Zoetis Belgium SA)		Broilers			
Semduramicin	C ₄₅ H ₇₆ O ₁₆	Chickens:			
Aviax 5 %; 51.3 g/kg (Phibro Animal Health SA)		Broilers	-	20	25
					Use prohibited at least 5 days before slaughter MRL ?

Source: Council Directive (EC) 70/524/EEC ... Amendments to Regulations (EC) Nos. 2430/1999 and/or 1831/2003

Appendix IV

Sampling of poultry faecal material and poultry meat in NORM-VET – as provided by the Norwegian Veterinary Institute



Sampling of poultry faecal material in NORM-VET

Enterococcus spp. has been tested for narasin resistance in the years 2002 - 2013. The sampling procedures for poultry are further described in the text below.

Broiler samples

Broiler faecal material has been sampled in NORM-VET the years; 2002, 2004, 2006 and 2011.

2002, 2004, and 2006

Faecal material was systematically sampled as part of the Norwegian *Salmonella* control program. In the *Salmonella* control program pooled faecal samples were collected from all broiler flocks one to three weeks before slaughter. These were all analysed at four of the Norwegian Veterinary Institutes Laboratories, where the first sample processed on a specific weekday every week at all four laboratories was included in NORM-VET. The number of samples from each laboratory was proportional to the total number of *Salmonella* samples from broilers obtained for each laboratory the previous year. The material for NORM-VET was taken by swabbing the faecal material and sending the swabs for bacterial isolation.

2011

Sample material was systematically sampled as part of the Norwegian *Salmonella* control program. In the *Salmonella* control program one pair of boot swabs were sampled from all flocks 10-19 days before slaughter. From these, a piece of one boot swab from each of the first five flocks processed on a specific weekday during the sampling period (January - November) was collected for NORM-VET and used for bacterial isolation.

Layers

Samples from layers have only been included in NORM-VET once; in 2013.

Faecal sample material was systematically sampled as part of the Norwegian *Salmonella* control program. In the *Salmonella* control program, two pair of boot swabs were sampled from all flocks every 15th week during the egg laying period. From these, a piece of one boot swab from each flock was collected for NORM-VET and used for bacterial isolation.

Turkey samples

Samples from turkey have been included in NORM-VET in 2007 and 2013.

The samples in 2007 were systematically collected through the EU baseline study on the prevalence of *Salmonella* in turkey flocks. In the baseline study, the flocks sampled were randomly selected and sampling was performed with boot swabs. A piece of one boot swab from each flock was collected for NORM-VET and used for bacterial isolation.

In 2013, faecal sample material was systematically sampled as part of the Norwegian *Salmonella* control program. In the *Salmonella* control program one pair of boot swabs were sampled from all flocks 10-19 days before slaughter. From these, a piece of one boot swab from each flock was collected for NORM-VET and used for bacterial isolation.



Sampling of poultry meat in NORM-VET

Enterococcus spp. has been tested for narasin resistance in the years 2002 - 2013. The sampling procedures for poultry meat are further described in the text below.

Broiler meat samples

Broiler meat have been sampled in NORM-VET the years; 2002, 2004, 2006

In 2002, the samples were taken at the slaughterhouses by the Food Safety Authorities. All five slaughterhouses active in Norway at the time was included, and the sampling was distributed throughout the year with a maximum of ten samples from ten different flocks per day. The sample material was part of the wing (~10 g) from which 5 g were used for further bacterial isolation. In total, 212 samples were collected.

In 2004, the broiler meat samples were systematically sampled at retail level according to the *Campylobacter* action plan 2004 where each of four participating local Food Safety Authorities collected samples from 25 fresh cooled broiler products (breast or thighs) every month, making sure that different flocks/batches were included. From these samples, the first five samples taken during the months February, April, June, August and November, were included in NORM-VET (~50 g). From this material, 5 g was investigated for bacterial isolation.

In 2006, three samples of fresh broiler meat (breast) were collected at retail level from at least three different store chains in the Oslo area every week throughout the year, making sure that different production brands and flocks/batches were included. Of the collected sample, 5 g was investigated for bacterial isolation.

Turkey meat samples

Turkey meat has been sampled in NORM-VET in 2007 and 2013.

The turkey meat samples in 2007 were collected at two slaughterhouses participating in a study on *Campylobacter* occurrence in turkey meat (breast) in the period autumn 2006 to autumn 2007. A random sampling was conducted, though taking market share and production brands under consideration. From these samples, collecting samples to NORM-VET took place in 2007, and 5 g was investigated for bacterial isolation.