Executive summary

During the accession negotiations with the European Union, Sweden was granted a derogation from community legislation to maintain national legislation within the area of feed additives of the groups antibiotics, chemotherapeutics, coccidiostats and growth promoters. In Sweden, the use of such substances is restricted to the purpose of curing or preventing diseases, i.e. used as veterinary medicines according to the Feedingstuffs Act of 1985. The Swedish government has appointed a commission to evaluate the hazards and risks associated with use of antimicrobial feed additives in animal production.

Our conclusions can be summarised as follows:

- Antibacterial feed additives have favourable economic effects on livestock production, but from a long term perspective these are questionable, especially regarding animal welfare and animal health. Antibacterial feed additives can, at levels permitted in feedingstuffs, be used for treatment or prevention of animal diseases, which is in violation of directive 70/524/EEC.
- The quinoxalines and the nitroimidazoles are potentially genotoxic and may be regarded as an occupational hazard.
- Halofuginone and the quinoxalines are toxic for target species. This is deleterious for animal well being.
- The risk of increased resistance associated with the general use of antibacterials as feed additives are far from negligible and the potential consequences are serious for both animal and human health. Antibacterials that are presently not used as therapeuticals in human or veterinary medicine are valuable templates for future drugs. As emergence of resistance is considered to be a threat to animal and human health, all AFA should be restricted to medical and veterinary purposes.

It is the conclusion of this commission that the benefits of antibacterial feed additives do not outweigh the risks.

With regard to coccidiostats and other medicinal substances, they are specifically used to prevent disease and should therefore be treated as pharmaceutical specialities, i.e. as medicated feed.

Contents

1   Introduction

1.1   The Swedish derogation

In 1986, the Swedish Parliament imposed a ban on antibacterial growth promoters. During the accession negotiations with the European Union Sweden was granted a derrogation from Community legislation concerning the use of antibiotics, chemotherapeutics, coccidiostats and growth promoters as feed additives. Before December 31, 1998 the Commission shall decide on the applications for adjustment to be submitted by Sweden. These applications are to be accompanied by a detailed scientific report on motives for the applications. The decision shall be taken in accordance with the procedures laid down in article 7 of the directive 70/524/EEC concerning additives in feedingstuffs.

In 1995 the Ministry of Agriculture appointed a Commission (Commission on Antimicrobial Feed Additives) to collect and review scientific data which are relevant for a decision on the above mentioned feed additives. Professor Lars-Erik Edqvist was appointed chairman of the commission and Laboratory Veterinary Officer Christina Greko was appointed scientific secretary. Laboratory Veterinary Officer Susanna Sternberg was appointed assisting scientific secretary. The following experts have been appointed to deal with particular areas of the work of the commission: Professors Sigvard Thomke and Klas Elwinger to conduct a scientific review for Chapter 3; Laboratory Veterinary Officer Desirée Jansson to undertake a similar review for Chapter 7 and Associate Professor Ivar Vågsholm to undertake the economic analysis in Chapter 3.

The appointed Commission has gathered the scientific data which it has found appropriate in two reports, one in English (SOU 1997:132) and one in Swedish (SOU 1997:133). The Swedish report is an abstract the English report.

Among the feed additives dealt with, the coccidiostats hold a specific position since they are used to prevent specific diseases and not as growth promoters. They are according to 705/524/EEC classified as medicinal substances. As the indications for use of these substances are specifically to prevent disease, they are treated separately.

Chapters 3-7 of the report provides scientific documentation on hazards and risks of using antibiotics, chemotherapeuticals, coccidiostats and growth promoters as feed additives. In Annexes A-F is a case by case review of substances which are used as feed additives within the European Union. In chapter 8 the assessment of identified hazards is undertaken.

2   Background

2.1   Introduction

Antimicrobials are probably the single most important discovery in the history of medicine. The antimicrobial era began in the 1930s when the first sulphonamides made their way into clinical use in human medicine. Penicillin, the first antibiotic, was discovered in 1929 but was not introduced for therapy until 1940. Shortly after its introduction into human medicine penicillin was used in animals for the treatment of different bacterial diseases. Since these early discoveries, a large number of antimicrobial substances have been discovered or developed. Although some of these drugs have been developed specifically for animal use, the substantial costs for development are often not covered by profits from the veterinary market alone.

Table 2.I. Some terms used and corresponding definitions

Tabell 2.I. Några använda termer och dessas definition

Term	Abbreviation	Definition
Antibiotic		Substance produced by a microorganism, with an inhibitory effect on other microorganisms
Chemotherapeutical		Chemically synthesised substance with an inhibitory effect on microorganisms
Antibacterial		Antibiotic or chemically synthesised substance with an inhibitory or lethal effect on bacteria
Antiprotozoal		Antibiotic or chemically synthesised substance with an inhibitory or lethal effect on protozoa
Antimicrobial		Substance with an antibacterial and/or antiprotozoal effect
Anticoccidial		Antiprotozoal with inhibitory or lethal effect on coccidia
Coccidiostat		Anticoccidial used specifically for prevention of coccidiosis
Antihistomonal		Antiprotozoal with inhibitory or lethal effect on histomonads
Antibacterial feed additives	AFA	Antibacterials used as feed additives for the purpose of performance enhancement (corresponds to antibiotics and growth promoters in Directive 70/524/EEC)
Medicinal feed additives	MFA	Antimicrobials used as feed additive for the purpose of preventing a specific disease (corresponding to medicinal feed additives according to Directive 70/524/EEC)

An antibiotic is defined as a substance produced by living micro-organisms that inhibits or kills other micro-organisms. Synthetic antimicrobial substances, such as the sulphonamides and the quinolones, are referred to as chemotherapeuticals. The word "antimicrobial" (as a noun) is often used to encompass any substance of natural, semi-synthetic or synthetic origin that kills or inhibits the growth of a microorganism. With respect to spectrum of activity, antimicrobial substances may be divided into antibacterial, antiprotozoal, antifungal and antiviral drugs. In the following, the term antibacterial will be used for antibiotics and chemically synthesised substances that have an inhibitory or lethal effect on bacteria. Terms used in the text are defined as shown in table 2.I.

2.2   Role of antibacterials in animal husbandry

A better understanding of many aspects of animal husbandry have resulted in a development towards improved productivity. Such factors include the combat of epizootic diseases, control of parasitism, improvements in animal nutrition, genetic improvements and the use of antimicrobials.

Antimicrobial agents are used for three major purposes in domestic animals:

1   Therapy, to treat an identified illness
2   Prophylaxis, to prevent illness in advance
3   Performance enhancement, to increase feed conversion, growth rate or yield

2.2.1   Therapeutic and prophylactic use of antibacterials

Therapy usually involves an individual animal or a defined group of diseased animals while prophylactic treatment involves the treatment of a herd or group of animals. The aim of the latter is to prevent diseases that might otherwise occur. A special form of prevention, also called metaphylaxis, is when all animals in a herd or group of animals are medicated, in situations when the proportion of animals diseased during a defined time period reaches a threshold value. In such situations, the probability of most, or all, of the animals getting infected is high. In both therapy and prophylaxis, the drug is administered over a defined, preferably short, period of time and in both instances the drug is used upon prescription by a veterinarian. The dosages used must be high enough so that concentrations that are inhibitory for the infectious agent are reached at the site of infection (e.g. the lung).
   The animal diseases currently requiring the most extensive use of therapeutic or prophylactic drugs are respiratory and enteric diseases of pigs

and calves, and mastitis in dairy cattle. Prophylactic treatment is particularly common during periods when stress is imposed on the animal, e.g.. changes in diet and loss of maternal interaction at weaning, after transport and commingling. Although in recent years much emphasis has been placed on disease prevention through improved management and environmental conditions, intensive animal production systems still depend on antimicrobials, as shown by the continuously growing market for antibacterials.

2.2.2   Chemoprophylaxis of protozoal diseases

In the case of certain protozoal diseases, the probability of clinical outbreaks or production losses due to subclinical disease is so high that chemoprevention has become standard practice. The main diseases in question are coccidiosis of poultry and rabbits and histomoniasis of turkeys and pheasants. The appropriate drugs for prophylaxis are referred to as anticoccidials (coccidiostats) and antihistomonals.

   Anticoccidials and antihistomonals are medicinal substances preventing specific diseases with a high probability of occurrence. In that respect, they are by definition veterinary medicines. On the other hand, in most parts of the world they are used as standard feed additives and are, from a regulatory point of view, treated as such.

2.2.3 Antibacterials as performance enhancers

The growth-promoting properties of antimicrobials for farm animals were discovered in the late 1940s. Trials where fermentation waste from tetracycline production were fed to chicken as a source of vitamin B_{12} revealed that the chickens fed the fermentation waste grew more rapidly than did the controls. It was soon found that this effect was not due to the vitamin content of the feed but to residual tetracycline (Stokestad and Jukes, 1949; Stokestad and Jukes, 1950). This growth promoting effect of tetracyclines was soon confirmed for other antibacterials and other animal species.

Growth enhancement in children

The findings of improved growth as an effect of administration of subtherapeutic doses of antibiotics led to investigations on possible similar effects in humans. In 1952, Snelling and Johnson reported lowered morbidity, increased growth rate and shortened hospital stay of premature infants following daily administrations of 50 mg chlortetracycline. Similar results, from a controlled trial in twins and triplets, were also reported by

Robinson (1952). MacDougall (1957) found that low doses of chlortetracycline given to hospitalised malnourished children resulted in, among other effects, improved weight gain. Positive effects on the weight gain of undernourished school children have also been reported (Mackay *et al.*, 1956; Guzman *et al.*, 1958). MacDougall (1957) concluded that chlortetracyclines could prove a valuable adjunct to dietary therapy in clinical practice under the conditions described. In contrast, both Mackay and co-workers (1956) and Guzmán and co-workers (1958) concluded that there was no justification for considering continued administration of antibacterials to children in underdeveloped areas. Subsequent research with focus on the relative impact of improved nutrition and control of infectious diseases on the growth of children demonstrated, among other things, the absolute necessity of maintaining adequate nutrition during weaning (Taylor, 1967; Behar *et al.*, 1968).

Growth enhancers in animal husbandry

In animal husbandry, the early findings initiated an intensive area of research. The practice of feeding subtherapeutic doses of antibiotics was readily adopted and AFA soon became an integrated part of the systems developed in the animal industry. Apart from increased growth rate and/or increased feed conversion, examples of other observed effects of antimicrobials at low doses are improved egg production in laying hens, increased litter size in sows and increased milk yield of dairy cows. When antimicrobials are used for the latter purposes the term yield promoters has been used. Alternative terms proposed for growth promoters such as "growth permitters" or "digestive enhancers" appear misleading. "Growth permitters" would imply that growth of animals is not permitted without these additives. The term "digestive enhancers" indicates that the effect of AFA is primarily on the digestion of feed. This is not in line with the, admittedly scarce, knowledge on the mode of action of AFA (see chapter 3).

The various claimed benefits of antibacterial performance enhancers have been listed by the European Animal Health Federation (FEDESA) as:

- To improve feed utilisation
- To improve growth rate
- To improve carcass quality
- To improve rate of throughput of livestock
- To improve farm economics
- To improve building utilisation
- To reduce labour
- To permit economical redeployment of resources
- To help indebtedness of farmers
- To permit controlled production from fewer stock to achieve quotas
- To reduce energy consumption
- To conserve natural fuels

- To reduce waste
- To reduce environmental contamination
- To reduce livestock numbers and slurry disposal problems
- To improve the environment

(Source: FEDESA 1994 cit. by Colegrave and Wesley, 1995)

The exhaustive nature of this list is due partly to the fact that some effects have been duplicated using different phrasings, and partly to the listing of various secondary and tertiary effects. If feed utilisation and/or growth rate of the animals is improved, the throughput of animals on a specific premise will increase. The latter is equal to improved building utilisation. From this follows an economic benefit, which, for indebted farmers certainly will mean an improvement. Improved use of buildings and feed would, in certain situations, reduce energy consumption which normally is the same as conservation of natural fuels. Reduced waste, reduced slurry disposal problems, reduced environmental contamination and improvement of the environment also appear synonymous and are sequels to improved feed utilisation. The statement "improved carcass quality" is doubtful. In some cases, a higher fat content of the carcass has been noted (Fiems *et al.*, 1995).

A list encompassing only the primary effects attributed to of AFA would read:

- Prevention of disease
- Improved feed utilisation
- Improved growth rate

2.3   Community legislation

2.3.1 Antibiotics and growth promoters

In the EU, feed additives (AFA and MFA) are regulated separately from veterinary medicines (including medicated feed).

Approval of feed additives is co-ordinated through Directorate General VI of the European Commission. The basic Council Directive concerning AFA is 70/524/EEC, with amendments (especially 84/587/EEC and 96/51/EC). Certain provisions of the latest amendment will be in force on April 1 1998 and the remaining provisions by October 1 1999.

When the "5th amendment" (Council Directive 96/51/EC) takes force, some important changes will follow. Approvals of high technology additives will be brand-specific. The responsibilities of all involved parties have been clearly defined. Further, possibilities of periodical renewal of authorisations are provided.

In article 3 of this Directive (formerly Article 7 of Directive 84/587/EEC) important conditions that must be fulfilled are listed.

It should be noted that, although the phrasing of some of the conditions has been altered (cf. Article 7, 2A of 84/587/EEC), the spirit is essentially the same as in the directive presently in force.

Directive 70/524/EEC as amended in 96/51/EC, Article 3a

Community authorisation of an additive shall be given only if:

a) *when used in animal nutrition it has one of the effects referred to in Article 2 (a)[1];*

b) *taking into account the conditions of use, it <u>does not adversely affect human or animal health or the environment</u>, nor harm the consumer by altering the characteristics of livestock products;*

c) *its presence can be monitored:*
       - as an additive per se
       - in premixtures
       - in feedingstuffs or, where appropriate, in feed materials;

d) *at the level permitted, <u>treatment or prevention of animal disease is excluded</u>; this condition does not apply to additives belonging to the group of coccidiostats and other medicinal substances;*

e) *<u>for serious reasons</u> concerning human or animal health its <u>use must not be restricted to medical or veterinary purposes.</u>*

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[1] *In article 2 of this directive, favourable effects of additives are specified*

According to Council Directive 95/69/EC, establishments where AFA are produced or mixed need specific approval.

Council Directive 94/40/EEC (amending 87/153/EEC), presently under revision, fixes standard data requirements for dossiers used to support approval of additives, so called guidelines. Specified data with relevance for the conditions cited above are to be provided.

After consulting the Member States in the Standing Committee, and evaluation by the Scientific Committee of Animal Nutrition (SCAN) the Commission may, if the substance if found to meet the requirements of the directives, be authorised for use.

If, as the result of new information or a reassessment of existing information, a Member State has specific grounds for establishing that the use of one of the authorised additives (or its use in specified conditions) constitutes a danger to animal or human health or the environment, the Member State may temporarily suspend or restrict the application (so called "safe guard clause", Article 11 of Council Directive 70/524/EEC). The other Member States and the Commission shall immediately be notified and informed of the reasons for the decision. The Commission shall examine the grounds cited by the member state and consult the Member States. The

Commission shall then deliver its opinion and take appropriate measures. If any of the conditions laid down in the article cited above are no longer met, a regulation shall be adopted to withdraw the additive.

Presently authorised AFA (annex I or II of 70/524/EEC) are bacitracin, flavomycin, avoparcin, spiramycin, tylosin, virginiamycin, carbadox, olaquindox, monensin, salinomycin and avilamycin (table 2.II). Ardacin has been approved under Annex II but the authorisation is not expected to be prolonged.

A number of additives have, since the original list in 1970, been withdrawn from the list of authorised additives because of a decision to restrict certain antibiotics to therapeutic use (e.g. penicillin, streptomycin and tetracyclines) .

2.3.2   Coccidiostats and other medicinal feed additives

The anticoccidial agents authorised for use in poultry within the EU (Council Directive 70/524/EC) are listed in Table 2.III. Apart from being used as anticoccidials in poultry some coccidiostats are also approved for the prevention of coccidiosis in rabbits. Some ionophores are also approved for growth promotion (see table 2.II). The other medicinal substances include the antihistomonals which are mainly used in turkeys.

Table 2.II. Antibacterial feed additives authorised for growth promotion, including most of the applications (Council Directive 70/524/EEC)

Tabell 2.II. Antibakteriella fodertillsatser godkända som tillväxtbefrämjande medel, samt flertalet godkända applikationer (rådsdirektiv 70/524/EEG)

Substance	Animal species or category	Maximum age	Min-max content, mg/kg	Examples of other provisions
ANTIBIOTICS				
Avilamycin	piglets	4 months	20-40	
	slaughter pigs	4-6 months	10-20	
	chickens		2.5-10	
Bacitracin	layers		15-100	
(zinc	turkeys	4 weeks	5-50	
bacitracin)	turkeys	26 weeks	5-20	
	other poultry	16 weeks	5-20	certain species excepted
	piglets	4 months	5-50	
	calves	16 weeks	5-50	
	calves	6 months	5-20	
Flavo-	layers		2-5	
phospholipol	turkeys	26 weeks	1-20	
(flavomycin,	other poultry	16 weeks	1-20	certain species excepted
bambermycins)	piglets	3 months	10-25	milkreplacements only
	slaughter pigs	6 months	1-20	
	calves	6 months	6-16	
	beef		2-10	
Spiramycin	turkeys	26 weeks	5-20	
	other poultry	16 weeks	5-20	certain species excepted
	calves	16 weeks	5-50	
		6 months	5-80	milkreplacements only
	piglets	4 months	5-50	
	slaughter pigs	6 months	5-20	
Tylosin	piglets	4 months	10-40	
(tylosin-		6 months	5-20	
phosphate)				
Virginiamycin	layers		20	
	turkeys	26 weeks	5-20	
	other poultry	16 weeks	5-20	certain species excepted
	piglets	4 months	5-50	
	slaughter pigs	6 months	5-20	
	calves	16 weeks	5-50	
	beef		15-40	
Monensin	beef		10-40	
Salinomycin	piglets	4 months	30-60	
	slaughter pigs	6 months	15-30	
GROWTH PROMOTERS				
Carbadox	piglets	4 months	20-50	withdrawal time 28 days
Olaquindox	piglets	4 months	15-50	withdrawal time 28 days
	piglets	4 months	50-100	milkreplacements only

Table 2.III. Coccidiostats and other medicinal substances authorised as feed-additives within the European Union (Council directive 70/524/EEC)

Tabell 2.III. Koccidiostatika och andra medicinska substanser godkända som fodertillsatser inom den Europeiska Unionen (rådsdirektiv 70/524)

Generic name	Species/category	Maximum age	Content in feed (mg/kg)	With-drawal, days
Amprolium	poultry	from laying onwards	62.5-125	3
Amprolium + ethopabate	chickens for laying, turkeys, guinea fowls	from laying onwards	66.5-133	3
Arprinocid	chickens		60	5
	chickens for laying	16 weeks	60	5
Decoquinate	chickens for fattening		20-40	3
Diclazuril	chickens for fattening		1	5
Dinitolmide (DOT)	poultry	from laying onwards	62.5-125	3
Halofuginone	chickens for fattening		2-3	5
	turkeys	12 weeks	2-3	5
Lasalocid	chickens for fattening		75-125	5
	chickens for laying	16 weeks	75-125	
	turkeys	12 weeks	90-125	5
Maduramicin	chickens for fattening		5	5
Meticlorpindol	chickens for fattening	laying and	125	5
	guinea fowl	onwards	125	5
	rabbits		125-200	5
Meticloprindol+ methylbenzoquat	chickens for fattening		110	5
	chickens for laying	16 weeks	110	-
	turkeys	12 weeks	110	5
Monensin	chickens for fattening		100-125	3
	chickens for laying	16 weeks	100-120	
	turkeys	16 weeks	90-100	3
Narasin	chickens for fattening		60-70	5
Narasin + nicarbazin	chickens for fattening		80-100	7
Nicarbazin	chickens for fattening	4 weeks	100-125	9
Robenidine	chickens for fattening		30-36	5
	turkeys		30-36	5
	rabbits		50-66	5
Salinomycin	chickens for fattening		50-70	5
Semduramicin	chickens for fattening		25	5
OTHER MEDICINAL SUBSTANCES				
Dimetridazol	turkeys	from laying on	100-200	6
	guinea fowl	from laying on	125-150	6
Ipronidazol	turkeys	from laying on	50-85	6
Ronidazol	turkeys	from laying on	60-90	6
Nifursol	turkeys		50-75	5

2.4   Current Swedish legislation

2.4.1   Antibiotics and growth promoters

The basic Swedish legislation with relevance for AFA and medicinal feed additives is the Feedingstuffs Act (SFS 1985:295), that came into force in 1986. According to this act, antibiotics and chemotherapeuticals may only be incorporated in animal feed for the purpose of preventing, alleviating or curing disease, i.e. not for growth or yield promoting purposes.

2.4.2   Coccidiostats and other antiprotozoals

In Sweden, as in other countries, the coccidiosis control in broilers relies primarily on coccidiostats. The drugs approved in Sweden for this purpose are listed in table 2.IV.

In accordance with the Feedingstuffs Act, coccidiostats are available as pharmaceutical specialities (veterinary medicinal products), on veterinary prescription only. As a result of the legislative changes in 1986, some coccidiostats earlier approved as feed additives are today instead authorised for use as medicated feed.

Table 2.IV. Coccidiostats approved as pharmaceutical specialities in Sweden

Tabell 2.IV. Koccidiostatika godkända som läkemedel i Sverige

Generic name (ATCvet code)	Species/ category	Maximum age	Content in feed (mg/kg)	With-drawal time, days
Amprolium + ethopabate (QP51A X59)	chickens for laying	14 weeks	125+8 in starter feed, 75+4.8 in grower feed	3
Halofuginone (QP51A X08)	poultry		3	3
Lasalocid (QP51 A H02)	chickens for fattening		75-125	3
Maduramicin (QP51A X10)	chickens for fattening		5	3
Monensin (QP51A H03)	chickens for fattening		100-120	3
Narasin (QP51A H04)	chickens for fattening		70	3

Ronidazole (Ridzola® vet.) and dimetridazole (Emtryl® vet.) were approved as pharmaceutical specialities for use against swine dysentery and, for dimetridazole, also against histomoniasis in poultry, until 1995. Ronidazole and dimetridazole were then withdrawn from the market in accordance with the provisions of the Committee of Veterinary Medicinal Products (CVMP). Ipronidazole has never been approved in Sweden.

2.4.3   Antibacterials for use in therapy or prophylaxis

Antibacterials for use in animals are authorised by the Medical Products Agency. Withdrawal times, based on the maximum residue limits (MRL) fixed by the CVMP, are assigned by the National Food Administration. All antibacterials, including dermatological and other formulations, for use in animals are available on prescription only.

   Veterinary prescriptions are subject to the regulations of the Medical Products Agency. All veterinarians are under the supervision of the Swedish Board of Agriculture, that also issue regulations pertinent to antibacterials. The general veterinary regulations of the Board of Agriculture (LSFS 1982:43) state that "the usage of medicinal products shall be well motivated. When choosing type and dosage of a medicinal product the risk of residues in food of animal origin shall be taken into account". Further, in the regulations for veterinarians with special reference to prescriptions of medicinal products (LSFS 1979:8), it is stated that "prescribing and handing out medicinal products shall be done with great restrictivity and only when the need is apparent...". According to the same regulations, medicinal products may, on each occasion, be prescribed or handed out only after careful examination of the diseased animal or herd, on the premises, except in the case of general prophylactic measures other than antibiotics and chemotherapeuticals, or if other special circumstances are at hand.

   Regarding medicated feed, the provisions of Directive 90/167/EEC are implemented.

   Of the antibacterials authorised as feed additives in the EU, tylosin and virginiamycin are presently available in Sweden for use in medicated feed. Olaquindox was withdrawn from the market in 1997. Spiramycin is authorised for use in food animals for injections only.

2.4.4   Distribution of medicinal products

All prescriptions and deliveries of drugs are administered by the pharmacies which all belong to Apoteksbolaget (The National Corporation of Swedish Pharmacies). This means that all prescriptions of drugs for therapeutic use,

including those mixed by the feed mills, are handled by the Swedish pharmacies. From the above follows that veterinarians are not allowed to sell or dispense medicinal products but may, for practical reasons, hand out the products to the end-user. In such cases, the quantity delivered may only satisfy the need for treatment of the animal or group of animals in question.

2.4.5   Control

The manufacturing of animal feed is supervised by the Board of Agriculture. As part of the supervision, annual statistics on quantities of feed delivered and on specifics such as medicated feed are gathered.

   All veterinarians are, as mentioned, subject to supervision by the Board of Agriculture. On a regional level, the County veterinarian (an official employee) is in charge of supervision of the work of the veterinarians, animal welfare and disease control. Traditionally, most of the veterinarians in food animal practice are employed by the Board of Agriculture. However, private practice in food animals is becoming more common.

   Sales statistics of antibacterials for use in animals is an indirect way of control. As mentioned above, the Board of Agriculture gathers annual statistics on sales of medicated feed. The National Veterinary Institute has, based on data provided by Apoteksbolaget, calculated the total sales of antibacterials for veterinary use in Sweden (see 2.6). Recently, Apoteksbolaget has initiated yearly surveys of all veterinary prescriptions of medicated feed. By combining these three sources of statistics, the surveillance is maximised. Discrepancies or undesired trends may be detected and their causes found and corrected (see 2.6.5).

2.5.   The Swedish ban

2.5.1   The preambles

Similar to the situation in other countries, some Swedish scientists viewed the practice of routine addition of antibacterials to animal feeds with scepticism. The knowledge of the transmissibility of resistance between bacteria through plasmids led to exhortations for a restrictive use of antibacterials in animals (Rutqvist, 1970). Following the recommendations of the Swann Committee in the UK, a broader debate was initiated, which eventually led to a reassessment of the use of antibacterials as feed additives (LBS, 1977).

   A working group of the Board of Agriculture concluded, among other things, that "the use of AFA entails a risk of increased resistance in bacteria but as the substances in use are mainly active against gram-positive bacteria

from which resistance is not transferred, the impact of such development is negligible". On the other hand, a negative attitude to all kinds of additives among consumers was noted by the group. The benefits, in terms of increased production and prevention of certain diseases, were also acknowledged (LBS, 1977). Legislative changes, especially in the requirements for approval, were proposed in order to mitigate possible risks.

At the same time, the farmers were growing increasingly sceptical towards feed antibiotics. They were concerned that the continued use of antibiotics might harm consumer confidence. The Federation of Swedish Farmers (LRF) made a policy statement, declaring that Swedish agriculture aimed towards a more restricted and controlled use of antibiotics. In a letter to the Ministry of Agriculture in 1984, the LRF requested a ban on the use of antibacterials as feed additives.

In response to the above, the Ministry of Agriculture drafted a new Feedingstuffs Act (Government Bill 1984/85:146, Swedish Government). Among other things, the draft proposed that the use of antibacterials in feed should be restricted to treatment, prevention or cure of diseases, i.e. their use for growth promotion should not be allowed. The grounds cited for this amendment was the risk for increased resistance, especially the risk for cross-resistance to other substances and the risk of increased susceptibility of animals to salmonella and other enteric pathogens.

The Feedingstuffs Act was accepted by the parliament in November 1985 and came into force in January 1986 (SFS 1985:295).

2.5.2   Effects on animal health

The effects of the ban on AFA on animal health has recently been reviewed by Wierup (1996). In essence, the following is based on his review.

Calves

In the 60s and 70s different AFA (bacitracin, flavomycin, oleandomycin, spiramycin) were used in concentrate and milk replacers for calves. However, the effect was considered doubtful and documented improvements in animal growth was not always seen in repeated experiments (e.g. Jonson and Jacobsson, 1973; Wierup *et al.*, 1975). Due to this scepticism, the use of AFA in calf rearing had more or less come to an end before the ban in 1986. Negative clinical or other effects as a consequence of the ban have not been reported.

Chickens

The antibacterial feed additive nitrovin came into use in the 70s and through
this, existing problems with necrotic enteritis in broiler chickens were
almost completely eliminated. Later, nitrovin was gradually replaced by
avoparcin. Reports on an effect of avoparcin on salmonella colonisation and
excretion (e.g. Smith and Tucker, 1978; Smith and Tucker, 1980) gave rise to
concern, as the broiler production put considerable efforts into the
salmonellosis control programme. This prompted a change from avoparcin to
virginiamycin in the early 80s. In the autumn of 1985 (before the ban),
outbreaks of necrotic enteritis were reported with increasing frequency. This
was dealt with by increasing the dosage of virginiamycin from 10 to 20 ppm.

   At the time of the ban, the chicken producers identified the occurrence of
clinical or subclinical necrotic enteritis as the main problem to tackle. A
committee with representatives of the producers, the feed industry and
veterinarians from animal health areas as well as from the meat inspection
services was put together. The aim of the committee was to initiate studies
and to suggest appropriate solutions to the problem. It was agreed that a
transition period would be necessary and that virginiamycin would be
prescribed during this period, in the dose of 20 ppm.

   Field trials where no medicated feed was used indicated that a number of
factors needed correction. It was concluded that the construction and climate
of stables, hygiene, management and feed composition all contribute to the
occurrence of necrotic enteritis in broiler production. Further, it was found
that coccidiostats of the ionophore type also prevent necrotic enteritis. Strong
emphasis was placed on improving animal environment because many
diseases, including necrotic enteritis, have a multifactorial background. A
special bonus was given for good animal management and care which also
led to improvements in the total level of quality of the production.

   In 1988, all prophylactic medications were abandoned and, in case of
outbreaks, a two day treatment with phenoxymethyl penicillin in the drinking
water was applied. The amount of active substance of antibiotics which was
used for treatment decreased from about 2 000 kg of virginiamycin in 1987
to 100 kg of phenoxymethyl penicillin in 1988 (expressed as active
substance, Wierup, 1989). Today the need for such treatment of necrotic
enteritis is more or less completely eliminated.

   More formal, scientific studies were also initiated to find alternative ways
to control necrotic enteritis. Studies on animal feed included effects of
enzymes and certain probiotics, the structure of the feed, different levels of
protein and different sources of proteins and coccidiostats (Elwinger and
Teglöf, 1991; Elwinger *et al.*, 1992; Elwinger *et al.*, 1994; Elwinger *et al.*,
1996).

   The results indicated that additions of enzymes acting on carbohydrates
and the addition of certain probiotics to develop an adequate intestinal

bacterial flora reduced the incidence of necrotic enteritis. Dietary levels of protein were reduced and amino acids were added which also resulted in an improved hygiene and health.

In retrospect the most important changes were related to feed, involving a reduction of protein content, a higher fibre content and supplementation with enzymes together with the utilisation of ionophores for prevention of coccidiosis. The feeding strategies employed were developed through close collaboration between the feed industry and the farmers.

As mentioned, the coccidiostats of the ionophore type that are now used do have antibacterial effects and act prophylactically against necrotic enteritis. However, the sanitary situation of broiler chicken rearing in Sweden today would not have been reached without the above mentioned enforcements.

After the ban, coccidiostats were prescribed as medicated feed. Close monitoring of the efficiency of coccidiostats was introduced in Sweden in 1986. Since 1991, the efficiency of the drugs is studied by lesion scoring (according to methods described by Johnson and Reid, 1970) in approximately 5% of the Swedish broiler flocks, representative of all individual feed mills. The results are used for surveillance of the coccidiosis situation and to adapt the coccidiostatic regimen accordingly. In addition, all broiler flocks are under direct veterinary supervision as part of the salmonella control programme.

Egg production

AFA have never been authorised for use in laying hens in Sweden. Consequently, the ban in 1986 had no impact on the egg production. Following the ban, coccidiostats were prescribed as medicated feed to replacement birds. This practice is now being gradually replaced by the use of vaccines.

Turkeys

The situation for turkey producing units was similar to that for production of broiler chickens. Before 1986, AFA were used for prevention of necrotic enteritis. The ban did not result in noticeable clinical problems or reduced growth rate.

Weaner pigs

Before 1986, practically all piglets were given AFA (olaquindox or carbadox), from weaning until delivery to the finishing units at the age of 10-12 weeks. Slaughter pigs were, to a lesser extent, given AFA (avoparcin or virginiamycin) until slaughter.

Production averages from 220 piglet producing herds from the years 1985 and 1986 have been assessed statistically (Robertsson and Lundeheim, 1994). For piglet production, problems emerged as a consequence of the removal of olaquindox which was used for weaning piglets. However, the pre-weaning piglet mortality, and the number of piglets produced per sow and year, did not show significant differences between the two years. In contrast, post-weaning mortality was significantly higher (p<0.001), about 1.5 percentage units, during 1986 than in 1985. The time to reach 25 kg increased by 5-6 days. Total efforts to prevent post-weaning diarrhoea, including advisory and preventive measures and treatments, increased four-fold. Data from units which were used for the above evaluation has now also been evaluated for the 10-year period 1986-1995. A comparison of the average values for 1994-1995 and 1986-1987 reveals post-weaning mortality to have decreased with 0.9 percentage units and the age at 25 kg to have been reduced by 1-2 days (Wierup, 1996).

In connection with the ban of AFA it became obvious that clear guidelines for veterinarians on how to prescribe antibiotics as medicated feed were lacking. Such guidelines were adopted by the Swedish Veterinary Association in 1990 (SVS). The guidelines emphasise that the prescription of feed antibiotics should always be based on a diagnosis together with a thorough evaluation of contributory factors and accompanied by recommendations on hygienic and other prophylactic measures.

In a study from 1994, Holmgren and Lundeheim analysed the results from 55 piglet producing units in the western part of Sweden. They concluded that the medicated feed prescribed to weaning pigs was clinically motivated and in accordance with the guidelines formulated by the SVS. The need for medicated feed differed markedly between herds and this could be ascribed to weaning and management systems. The production results were highly correlated to the rearing systems and to the level of hygiene. In herds rearing post weaning pigs on deep litter bedding the use of medicated feeds was three to four times lower than in herds with pigs in traditional post weaning pens. The study also underlines the problem with preventing weaning diarrhoea in units with limited facilities to arrange satisfactory sectioning and hygiene.

Efforts have also been undertaken to make adjustments in the composition of pig feed. The most prominent changes have been a lowering of the protein content, use of water soluble fibres and supplementation with amino acids (Göransson et al., 1995). An antisecretory factor, preventing liquid penetration to the gut induced by enterotoxin, has also been explored as a preventive measure (Göransson et al., 1993).

A notable effect on weaning diarrhoea through addition of high levels of zinc oxide has been reported (Holm, 1989; Poulsen, 1995). Zinc oxide has a preventive effect on weaning diarrhoea equal to the effect which is reached

when using olaquindox (Holmgren, 1994b). Since 1992, zinc oxide is approved for incorporation into piglet feed, at 2000 ppm of zinc. Not all piglet feed contains high levels of zinc, as many producers do not experience problems with weaning diarrhoea. The intention was that the use of zinc oxide would only be temporary, until other measures to prevent weaning diarrhoea had been developed. Presently, the long term impact of the use of zinc oxide is under debate, and the Board of Agriculture is currently considering a phase-out and subsequent ban on the use of zinc oxide in high doses.

After the ban of AFA numerous measures have been, and are continuously, undertaken to optimise rearing and production systems and to employ available techniques (e.g. sectioning of buildings, age segregation, planned production). The ban also stimulated a development towards new rearing systems. The weaning of piglets on deep litter in large groups is one example and the so called birth-to-slaughter system which is based on production in the same pen from birth to slaughter is another. The adjustment of old buildings and pens to the new production system is expensive and until such adjustments can be done antibiotics are used to combat weaning diarrhoea in some units.

The experiences from the clinical problems which emerged after the ban indicate that single environmental or rearing measures are seldom enough to correct the situation. A combination of efforts aiming towards a health-orientated production system is often necessary. Many problems have been solved while others await solution.

Slaughter pigs

The ban of AFA did not create obvious clinical problems for growing or finishing pigs. The production results from this sector are comparable to those from, for example, the Danish production (source: Advisory Service Optima).

2.6   Sales of antibacterials for veterinary use in Sweden

2.6.1   Materials and methods

Data for 1980-1993 have been compiled from earlier publications (Wierup *et al.*, 1987; Björnerot *et al.*, 1996). For the years 1994-1996, data have been calculated from the same sources as these authors have used (see table 2.V).

All data on pharmaceutical specialities are based on the sales statistics in the Central Statistics System of Apoteksbolaget (National Corporation of Swedish Pharmacies). This system contains registers of all sales from the

wholesalers to the local pharmacies (all belonging to Apoteksbolaget), and to the feed companies. As all pharmaceutical specialities are distributed from these wholesalers to local pharmacies or to authorised feed companies, these figures represent the total consumption in Sweden.

The local pharmacies normally have short storage periods for these products, so it can be assumed that the products sold were also consumed during the respective periods.

With regard to antibacterials used as feed additives before 1986, data has been gathered from the Board of Agriculture. Additionally, for 1980-1993 data on sales of unlicenced pharmaceutical specialities, sold with special permission from the Medical Products Agency were gathered from the respective pharmaceutical companies.

The antibacterials included are pharmaceutical specialities approved for general or local use in veterinary medicine (ATCvet[1] code QJ01, QJ51, QA07A, QG01A, QP51AA and QP51AG). For 1980-1993, preparations for dermatologic use (ATCvet code QD06, QD07C, QS02C) are also included. For 1980-1984, feed additives not approved as pharmaceutical specialities and thus not delivered through pharmacies were also included. Thus, the statistics show the total amount of antibacterials sold by pharmacies or delivered by feed mills during the specified time period. It was assumed that the whole amount sold was used for animals. The figures include antibacterials for all animal species (food animals, fish, pets and horses).

All data are expressed as kilogram of active substance. In formerly published data, the weight of the procaine part of procaine penicillin was included in the presentations. As the procaine part of the molecule is not active in an antibiotic sense, procaine penicillin has been calculated to benzyl penicillin as 0.6 times the weight of procaine penicillin. Further, some corrections have been made of erroneous macrolide figures.

2.6.2   Total consumption

The total usage of antibacterials is presented in table 2.Va. For clarity, table 2.Vb lists examples of pharmaceutical specialities included in the different groups in table 2.Va.

Since 1986, the total usage has decreased from a mean on 45 tonnes/year during 1980-1984 to 20 tonnes in 1996 (a decrease by 55%). The total sales of formulations intended for treatment of groups of animals, i.e. through feed or water, was about 6 tonnes in 1996 (sum of figures in table 2.VI). This means that more than two thirds of the total consumption in 1996 was in the

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[1] ATC = Anatomical Therapeutic Chemical classification system

form of formulations for treatment of individual animals (e.g. parenteral drugs, intramammaries or tablets).

As the different substances in question are not equal in their biological activity per weight unit, total figures might be misleading (i.e. if a substance requiring high dosages for full efficacy is replaced by a more active substance, a false impression of a reduction could be given). Therefore, each substance group should be assessed separately for trends.

Of the pharmaceutical specialities that were authorised at the time of the ban, the sales of G- and V-penicillins, macrolides and trimethoprim-sulphonamides have increased over time. Among the penicillins, only formulations suitable for treatment of single animals (parenteral drugs, tablets) are presently approved. The group consists mainly of formulations for injection and are therefore assumed to be used largely for the treatment of mastitis in dairy cattle (Nilsson and Greko, 1997).

Similarly, the trimethoprim-sulphonamides are only available for treatment of individual animals. About 15 to 20 years ago, the main indication for trimethoprim-sulphonamides in pigs, namely neonatal piglet diarrhoea, was very common. However, due to improved management and housing conditions in combination with efficient vaccines, neonatal piglet diarrhoea is no longer a major clinical problem. The observed increase in consumption is therefore likely to be derived from an increased usage of trimethoprim-sulphonamides in dairy cows and lately, following the introduction of formulations for oral use, in horses.

Although the amount of macrolides sold as formulations for injection increased in the beginning of the 90s (Nilsson and Greko, 1997), the major part of this increase derives from macrolides for oral use (mixed in feed or water). This increase, and that of the pleuromutilins, is believed to reflect an increase in the incidence of swine dysentery (the major indication for those drugs). The increase during 1995 and 1996 is likely to be explained by the fact that the nitroimidazoles, the third category of drugs (formerly) available for this indication, were withdrawn from the market in 1995.

The usage of tetracyclines and aminoglycosides has decreased. The changes in the latter group is partly explained by a decrease in usage of the combination of penicillin and dihydrostreptomycin. The usage of tetracyclines will be further commented on below.

The consumption of substances formerly approved as feed additives and subsequent to the ban approved as therapeuticals (quinoxalines and streptogramins) has decreased substantially in spite of higher doses being given for therapy than for growth promotion.

Table 2.Va. Total animal usage of antibacterials in animals Sweden during 1980-1996 expressed as kg active substance (Data for 1980-1993 from Wierup *et al.*, 1987; Björnerot *et al.*, 1996 and for 1994-1996 Greko, unpublished)

Tabell 2.Va. Total förbrukning av antibiotika för användning till djur uttryckt som kg aktiv substans 1980-1996 (Data från 1980-1993 från Wierup et al., 1987; Björnerot et al., 1996 och från 1994-1996 Greko, opublicerat)

Active substance	Year (kg active substance)												
	1980	**1982**	**1984**	**1986**	**1988**	**1989**	**1990**	**1991**	**1992**	**1993**	**1994**	**1995**	**1996**
G and V penicillins[1]	3232	4153	4788	5934	7144	7007	7414	7423	7446	8301	10374	9082	8555
Aminopenicillins	60	248	714	540	655	681	738	769	837	859	941	928	829
Aminoglycosides	5274	4776	5608	2885	3194	2823	2539	2255	2139	1938	1696	1342	1066
Tetracyclines	9819	10765	12955	6585	4691	4624	4572	5414	8023	8815	7730	4968	2733
Macrolides	603	616	887	1144	1205	1156	1399	1478	1701	1562	1701	1803	1486
Fluoroquinolones						1	84	123	147	173	246	200	173
Sulphonamides	6600	4931	4325	3093	3072	2988	2510	2372	2362	2045	2323	2135	2198
Trimethoprim incl. derivatives	134	142	186	197	250	282	272	257	284	303	352	331	339
Pleuromutilins					124	140	229	236	268	384	465	889	1142
Quinoxalines[2]	6250	7700	9900	1300	7164	7202	5778	5128	4917	3523	1904	1191	1098
Streptogramins[2]	0	0	8800	1610	1088	2388	2413	1350	1275	550	600	575	525
Feed additives[3]	8380	9370	700	870									
Other substances[4]	918	869	1688	1616	1603	1871	2326	2666	1644	1627	1915	1125	163
TOTAL	41270	43570	50551	25774	30190	31164	30274	29274	31043	30080	30247	24569	20307

[1] Procaine penicillin has been calculated to and expressed as bensylpenicinllin as 0.6 times total weight of procaine penicillin

[2] For 1980-1984 used as feed additives, 1986-1996 on veterinary prescription only at therapeutic dosages

[3] Including avoparcin, bacitracin, nitrovin, oleandomycin and spiramycin

[4] For 1980-1995 mainly nitroimidazoles (withdrawn in 1995)

Table 2.Vb. Explanations of groups in main table (table 2.Va)

Tabell 2.Vb. Förklaringar till de grupper som ingår i tabell 2.Va

Active substance	Examples of substances included
G and V penicillins	procaine penicillin, benzyl penicillin, phenoxymethyl penicillin
Aminopenicillins	ampicillin, amoxicillin
Aminoglycosides	dihydrostreptomycin, gentamicin, neomycin
Tetracyclines	oxitetracycline, chlortetracycline
Macrolides	spiramycin, tylosin
Fluoroquinolones	enrofloxacin
Sulphonamides	sulphadiazine, sulphadoxine, formolsulphatiazole
Trimethoprim incl. derivatives	trimethoprim, baquiloprim
Pleuromutilins	tiamulin
Quinoxalines	olaquindox, carbadox
Streptogramins	virginiamycin
Feed additives	avoparcin, bacitracin, nitrovin, oleandomycin and spiramycin as feed additive for growth promotion
Other substances	nitroimidazoles, clindamycin, cephalosporins

2.6.3   Flock treatment

Of special interest is the consumption of antibacterials intended for group or flock medication. When considering the risk for development of resistance, it is unimportant whether these medications take place by feed or water.

Sales data on formulations of antibacterials intended for such use have therefore been extracted from the data on total consumption shown in table 2.Va for the years 1993-1996. In table VI, some trends can be observed. The consumption of tetracyclines and quinoxalines has decreased sharply during the period. The usage of macrolides and streptogramins show only minor fluctuations. The pleuromutilins seem to have replaced the nitroimidazoles following the withdrawal from the market of the latter substances in 1995.

Olaquindox

Olaquindox is exclusively used in pigs. The only indication is diarrhoea around weaning but some off label use against swine dysentery is likely to occur. The usage of olaquindox was reduced by 82% (total quantity of active substance per number of pigs produced) from 1985 to 1987. Thereafter, however, more antibiotics were prescribed and the total amount used during the subsequent years 1988 and 1989 was 5 and 6%, respectively, higher than

in 1985 (Björnerot *et al.*, 1996). Considering that the dosage prescribed in from 1986 and onwards was about three times higher than the AFA dosage employed before, a smaller fraction of the weaning pigs was treated with olaquindox. Calculated this way, the proportion of pigs that were treated was 12% in 1986 and 76% in 1989 and 12% in 1995 (Wierup, 1996). As mentioned, olaquindox is no longer available in Sweden.

Table 2.VI. Sales of formulations of antibacterials intended for treatment of animals through feed or water (flock or group medications) during 1993-1996 expressed as kg active substance

Tabell 2.VI. Försäljning av beredningsformer av antibakteriella medel avsedda för behandling via foder eller vatten (flock- eller gruppmedicinering) under 1993-1996 uttryckt som kg aktiv substans

ATCvet code	Active substance	Year			
		1993	**1994**	**1995**	**1996**
QJ01AA	Tetracyclines	8106	7036	4243	2088
QJ01FA	Macrolides	959	791	1029	975
QJ01XX91	Streptogramins	550	600	575	525
QJ01XX92	Pleuromutilins	344	422	815	1069
QJ01MA	Fluoroquinolones	13	30	31	27
QJ01MB	Quinoxalines	3523	1904	1191	1098
QP51AA	Nitroimidazoles	1490	1764	953	0

2.6.4   Coccidiostats

The combination of amprolium and ethopabate has been the drug of choice for prevention of coccidiosis in chickens intended for laying in Sweden. During the last few years, this product has been largely replaced by vaccination of replacement breeders.

For broilers, a limited number of ionophores have been available to the during the past ten years. Narasin is, by far, the most widely applied product.

The total usage of anticoccidials in feed has been calculated from the same sources as the antibacterials (see 2.6.1). Presently, approximately 13 500 kg active substance of coccidiostats is used annually in Sweden. The quantities in kg active substance sold from 1980-1996 have then been calculated to tonnes medicated feed on basis of recommended dosages. For the year 1986, an apparent error in the reports to the Board of Agriculture was discovered. Therefore, all figures for that year have been excluded. In figure 2, the results are compared with the number of broiler chickens produced in Sweden are shown in figure 2.I.

2.6.5 Some comments on the Swedish consumption statistics

The consumption statistics from Sweden show that the usage of antibacterials in animals can, and has been, reduced substantially. Little data are available on consumption statistics other than from the Nordic countries. Comparisons between countries are difficult, as no generally accepted method of correcting for differing numbers of animals of different species is available. The total numbers of animals in Sweden during 1988-1995 are presented in table 2.VII.

As mentioned, figures on the total usage of antibacterial drugs should be interpreted with caution. Each substance group should be assessed separately, and knowledge on common indications is necessary in order to interpret the figures. In attempts to control resistance to antibacterials and to minimise the environmental impact of these drugs, monitoring systems for both usage of antibacterial drugs per animal species and for resistance to antibacterials in animal microbiota are crucial. Whenever undesirable trends are observed, activities to disclose underlying causes and, if possible, corrected.

During the years 1980-1984, the consumption of tetracyclines in animals in Sweden increased markedly. Thereafter, the usage of these products decreased until 1988. From 1988-1993, an increase was again noted. The latter could not be connected to an altered disease situation, and therefore no obvious explanation was at hand. Further investigations into the precise origins of this were initiated in 1994 by the Board of Agriculture. It was revealed that the increase could almost entirely be explained by the prescriptions of one veterinarian to one herd. The veterinarian was reported, the cause corrected, and the tetracycline consumption is now 50% lower than before 1986.

The comparison of statistics from different sources can also be a valuable asset. Results from the recently introduced system for statistics on prescriptions of medicated feed (Odensvik, 1997), were compared to data on sales from wholesalers and to data on sales of medicated feed reported by feed mills to the Board of Agriculture. An excellent agreement between the different systems for statistics was found in question of antibacterial substances. However, for coccidiostats a major discrepancy was noted. The quantity covered by prescriptions in 1995 was about 65% of the quantities registered in the two other systems. The amounts used, according to the latter, match well the number of chickens produced in that year (figure 2.I). Therefore, it can be assumed that one or more feed-mills have delivered coccidiostats without veterinary prescriptions. One such case has been identified and legal action has been taken.
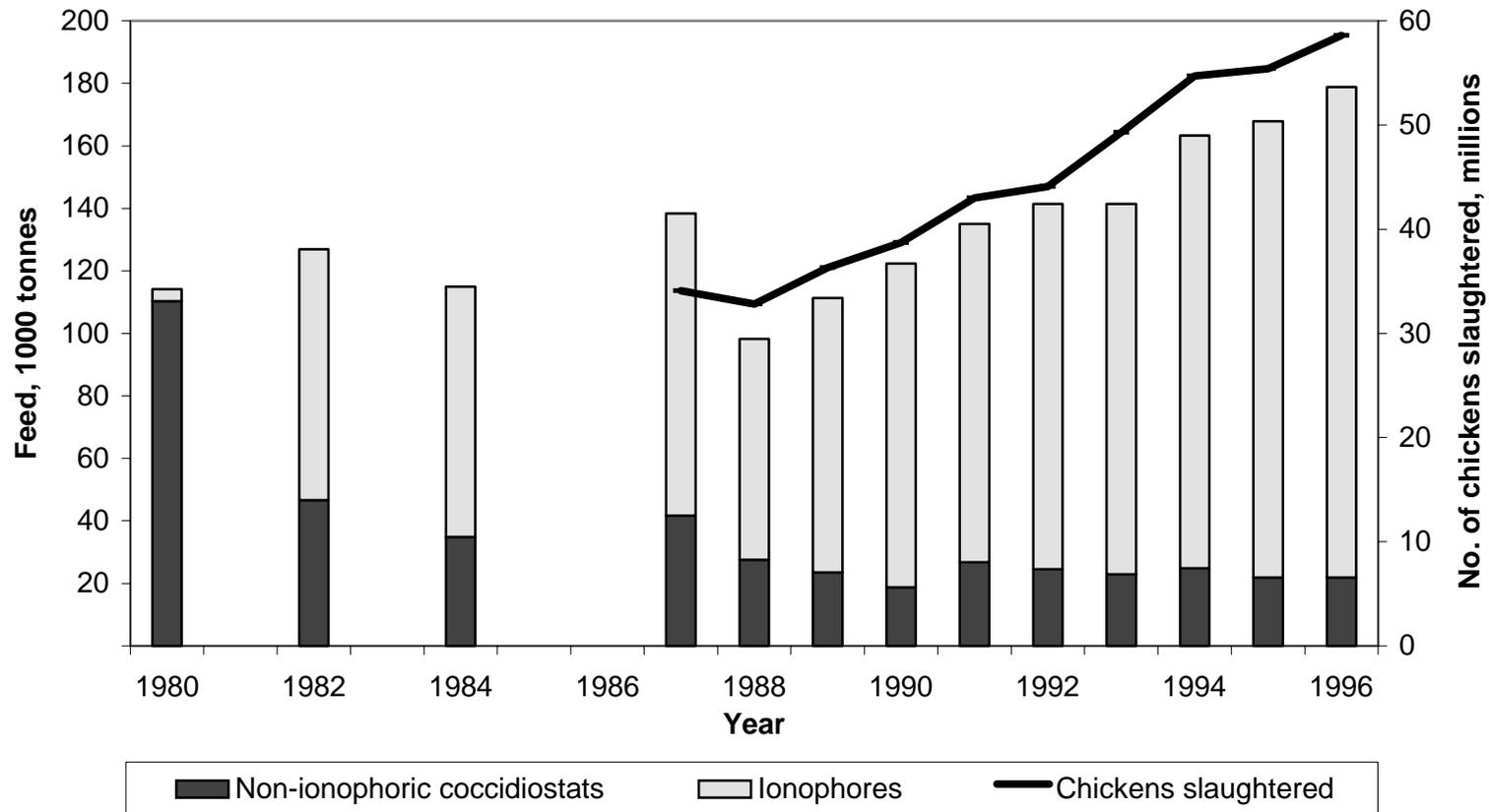
Figure 2.I. Usage of coccidiostats in Sweden expressed as quantity of medicated feed expressed as 1000 tonnes (left axis), and number of broiler chickens slaughtered in millions (right axis)

Figur 2.I. Användning av koccidiostatika i Sverige uttryckt som mängd foder uttryckt som 1000-tal ton med tillsatt läkemedel (vänster axel), och antalet slaktade slaktkycklingar i millioner (höger axel)

Table 2.VII. The number of food- and fur-producing animals and horses in Sweden during 1988 to 1995 (from Björnerot, 1996)

Tabell 2.VII Antal livsmedelsproducerande djur, pälsdjur och hästar i Sverige under 1988-1995 (från Björnerot, 1996)

	Year							
	1988	**1989**	**1990**	**1991**	**1992**	**1993**	**1994**	**1995**
Dairy cows[1]	564 550	568 857	576 409	528 212	525 948	524 520	509 431	482 118
Beef cows[1]	56 412	64 199	74 544	97 653	135 791	153 555	164 578	157 128
Heifers, bulls, bullocks[1]	527 522	533 296	543 458	543 418	565 463	548 620	560 911	595 521
Calves[1]	513 044	521 298	524 032	537 495	548 100	580 671	591 569	542 328
Ewes and rams[1]	159 107	160 647	161 974	168 178	180 067	188 944	195 736	195 439
Lambs[1]	235 606	239 859	243 621	250 605	267 394	281 743	287 692	266 410
Sows and boars[1]	242 872	239 482	229 683	226 839	233 133	249 314	249 171	244 950
Pigs slaughtered[2] (fattening)	3 551 360	3 642 213	3 471 482	3 224 570	3 297 326	3 483 439	3 657 067	3 634 837
Layers[1]	6 411 821	6 366 499	6 391 943	6 145 174	6 063 357	5 764 401	5 918 015	6 100 270
Pullets[1]	2 274 343	2 267 732	2 175 676	2 579 920	2 166 105	1 908 371	2 175 337	1 811 509
Broilers slaughtered[3]	32.8×10^6	36.4×10^6	38.7×10^6	43.0×10^6	44.1×10^6	49.3×10^6	55.8×10^6	60.3×10^6
Turkeys slaughtered[4]	790 387	673 011	660 396	590 733	610 700	719 950	685 727	458 516
Domestic geese slaughtered[4]	22 290	18 512	22 186	20 813	15 771	20 823	22 140	29 962
Ducks slaughtered[4]	97 142	83 516	49 003	30 207	61 508	52 410	32 796	78 839
Horses[2] (estimated figures)	~180 000	~180 000	~180 000	~180 000	~180 000	~180 000	~180 000	~180 000
Minks[5] (breeding females)	490 324	439 271	285 589	255 037	260 919	184 741	~212 450	~244 320
Foxes[5] (breeding females)	19 463	13 028	9 052	4 554	5 239	3 627	~ 3 600	~3 600

Sources: [1]Yearbook of Agricultural Statistics, 1989-1994. The numbers of individuals of different animal species are counted at a certain time (in June) every year, which means that the total numbers are not always included, for example calves born in the autumn that year are missing. Note, only animals on farms with more than 2.1 hectare land or in herds with <u>at least</u> 50 dairy cows or 250 cattle (bovines) or 250 pigs or 500 sheep (including lambs) or 1000 poultry (including chickens) are counted. [2]Swedish Board of Agriculture. [3]Swedish Poultry Meat Association, [4]National Food Administration, [5]Swedish Fur-breeding Association - estimated figures for 1994 and 1995.

2.7   Prevalence of bacterial resistance

Acquired resistance in bacteria to antibacterials is the results of reflects the exposure to the substances in question. Therefore statistics on prevalence of resistance in various bacterial species is an indirect way of assessing the consumption of antibacterials. Trends over time in prevalence of resistance among bacterial populations are likely to reflect quantities and patterns of usage.

2.7.1   Zoonotic pathogens

Salmonella

In accordance with the recommendations of WHO, antibiotic resistance in *Salmonella* spp. isolated from animals in Sweden has been monitored since 1976. Salmonellosis in animals is a notifiable disease in Sweden. All strains from animals isolated at or sent to the National Veterinary Institute are included. This surveillance is mainly motivated by the implications of resistant zoonotic bacteria for human health .

Table 2.VIII. Antibacterial resistance in *Salmonella* Typhimurium from animals in Sweden (Data from Franklin, 1997 unpublished and Franklin and Wierup, 1987; Franklin *et al.*, 1994; Franklin, 1995)

Tabell 2.VIII. Resistens mot antibakteriella medel hos Salmonella *Typhimurium isolerade från djur i Sverige (Data from Franklin, 1997 unpublished and Franklin and Wierup, 1987; Franklin et al., 1994; Franklin, 1995)*

Substance	Breakpoint[1] (µg/ml)	Resistance (%) Year (No. of isolates investigated)		
		1978-86 (n=117)	1988-91 (n=62)	1992-96 (n=108)
Ampicillin	>8	2	2	3
Streptomycin	>32	78	32	25
Tetracycline	>8	14	6	8
Trimethoprim-Sulphonamides	>0.5	0	0	2[3]
Enrofloxacin	>0.5	ND[2]	0	1[4]

[1]Breakpoint = value above which the isolate has been classified as resistant; [2]ND = not determined; [3]Isolated from a pigeon; [4]Isolated from a horse
[1]Brytpunkt = värde över vilken isolatet betecknats som resistent; [2]ND = ej undersökt; [3]Isolaerad från en duva; [4]Isolerad från en häst

The antibiotic resistance in *S.* Typhimurium in different time periods is shown in table 2.VIII. In the period 1978-1986, most strains were isolated from cattle. In 1988-1991 and 1992-1996, 43 and 36%, respectively, of the isolates were of bovine origin. In the last period, 45% were isolated from other sources than production animals, mostly wild birds. As apparent from the table, the number of resistant *S.* Typhimurium has decreased since the surveillance programme was initiated. All investigated *S.* Typhimurium strains and *S.* Dublin strains isolated in 1988-1996 were sensitive to modern quinolones, trimethoprim-sulphonamides, neomycin and gentamicin, except one isolate of *S.* Typhimurium from cattle which was resistant to trimethoprim-sulphonamides and one isolate of *S.* Typhimurium from a horse that was resistant to modern quinolones. The low prevalence of resistant salmonellae can probably be ascribed to the fact that antibacterials are not used to eliminate salmonella infections in animals.

Campylobacter

The frequency of resistance in *Campylobacter jejuni* isolated from chicken was investigated by Berndtsson (1996). Data on obtained minimum inhibitory concentrations (MIC) values are given in table 2.IX. Antibacterials are rarely used in broiler production in Sweden and the low proportion of resistant isolates seems to confirm the absence of a selective pressure. From other countries, quinolone resistance has been reported following the introduction of this drug (Endtz *et al.*, 1991).

Table 2.IX. Minimum inhibitory concentration (MIC) of antibacterials against 200 strains of *Campylobacter jejuni* isolated from chickens in Sweden

Tabell 2.IX Minsta hämmande koncentration av antibakteriella medel för Campylobacter jejuni *isolerade från kycklingflockar i Sverige*

Substance	Minimum inhibitory concentration (µg/ml)													
	< 0.06	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128
Ampicillin						9	28	82	60	14	5	2	0	
Cephalotin										5	11	59	111	14
Doxycycline	15	18	99	40	23	3	1		1					
Enrofloxacin	4	71	91	18	7					9				
Erythromycin				7	39	92	39	21	2					

2.7.2   Animal pathogens

Escherichia coli

Escherichia coli strains with ability to cause piglet diarrhoea and edema disease are among the most important intestinal pathogens in piglets. Weaning diarrhoea in pigs has also been associated with *E. coli* but the role of *E. coli* in that disease is not clear. In order to assess the development of resistance over time, three different investigations regarding antibiotic resistance in porcine *E. coli* were compared. The majority of the included strains originated from pig herds with diarrhoea problems.

   From table 2.X, it is apparent that the frequency of antibiotic resistant strains has not changed dramatically over the last ten years. The number of strains resistant to trimethoprim was unexpectedly high in 1981 to 1982 (9%), taking into account the fact that trimethoprim had only been used for 6 to 7 years in Sweden at that time. A further increase was expected but, as shown, did not take place. This might be explained by the improved health situation in piglets which presumably has been accompanied by a similar decrease in the usage of this particular combination of drugs.

   Resistance to streptomycin and tetracycline is still high, but compared to results from other countries (e.g. Pohl *et al.*, 1991; Morvan and Moisan, 1994) the overall figures indicate a favourable situation.

Table 2.X. Antibiotic resistance in porcine *E. coli* strains isolated in Sweden during different time periods (%). (From Franklin unpublished data and Franklin, 1984; Melin *et al.*, 1996)

Tabell 2.X. Antibiotikaresistens hos E. coli *från svin, isolerade i Sverige under olika tidsperioder (%)(Från Franklin opublicerade data och Franklin, 1984; Melin et al., 1996)*

Substance	Break-point[1] (µg/ml)	Resistance (%) Year (No. of isolates investigated)		
		1981-82 (n=200)[3]	1989-91 (n=248)	1994 (n=464)
Ampicillin	>16	8	6	8
Streptomycin	>32	56	44	41
Tetracycline	>8	40	28	38
Trimethoprim-Sulphonamides	>8[2]	9	15	10
Enrofloxacin	>0.5	ND[4]	<1	<1

[1]Breakpoint = value above which the isolate has been classified as resistant; [2] Denotes the trimethoprim concentration; [3] Denotes number of investigated isolates; [4] ND= not determined

[1]*Brytpunkt = värde över vilken isolatet betecknats som resistent; [2]Avser koncentration trometoprim [3] n = antal undersökta isolat; [4] ND = ej undersökt*

Staphylococcus aureus

Table 2.XI. Proportion of *Staphylococcus aureus* (isolated from clinical mastitis in dairy cows in Sweden during different years) with resistance to different antibacterials (Data from Franklin and Horn af Rantztien, 1983; Robertsson and Franklin, 1987; Nilsson, 1996)

Tabell 2.XI. Andel av S.aureus *isolerade från klinisk mastit hos nöt i Sverige under olika år, vilka uppvisar resistens mot vissa antibakteriella medel (Data från Franklin and Horn af Rantztien, 1983; Robertsson and Franklin, 1987; Nilsson, 1996)*

Substance	Break-point[1] (µg/ml)	Resistance (%) Year (No. of isolates investigated)		
		1982 (n=287)	1985 (n=529)	1995 (n=181)
Penicillin	-[2]	10	10	6
Oxacillin	>2	0	3	0
Erythromycin	>4	1	3	1
Spiramycin	>16	ND[3]	ND	1
Tetracycline	>8	1	3	1

[1]Breakpoint = value above which the isolate has been classified as resistant; [2] Resistant isolates determined on the basis of β-lactamase production; [3] ND = not determined
[1]*Brytpunkt = värde över vilket isolaten bedömts som resistenta; [2] Resitens bestämd som produktion av* β-*laktamas; [3] ND = ej undersökt*

Among the udder pathogens causing bovine mastitis, *S. aureus* is the most important causative agent. It gives rise not only to severe acute inflammation but also to chronic subclinical conditions. In Sweden, about 25% of clinical mastitic cases are caused by this species. Besides *S. aureus*, a number of other staphylococcal species are associated with bovine mastitis. About 5 to 10% of the *S. aureus* isolates from clinical cases of mastitis are resistant to penicillin due to penicillinase production (Table 2.XI). Penicillin resistance is more common in coagulase-negative staphylococci (about 25%, L. Nilsson, personal communication[2])

Serpulina hyodysenteriae

Serpulina hyodysenteriae is the causative agent of swine dysentery. Minimum inhibitory concentrations (MIC) for 67 Swedish strains were determined for seven antibiotics. The strains were isolated from herds with and without dysentery problems in the period 1988 to 1990 (Gunnarsson *et al.*, 1991). The distribution of MIC values are shown in Table 2.XII. The MIC values of tylosin and virginiamycin indicate that these drugs would no longer be effective for treatment of swine dysentery.

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[2] Lolita Nilsson, Laboratory Veterinary Officer, Department of Mastitis, National Veterinary Institute (SVA), Sweden

Table 2.XII. Antibacterial sensitivity of 67 isolates of *Serpulina hyodysenteriae* from Swedish pigs (Data from Gunnarsson *et al.*, 1991)

Tabell 2.XII. Känslighet för antibakteriella medel hos 67 isolat av Serpulina hyodysenteriae *från svenska grisar (Data från Gunnarsson et al., 1991)*

Antibacterial substance	MIC of different antibacterial substances (µg/ml) No. of isolates						
Carbadox	*<0.012*	*0.012*	*0.025*	*0.05*	*0.1*	*0.2*	*>0.2*
	36	2	15	9	2	1	2
Olaquindox	*<0.12*	*0.12*	*0.25*	*0.5*	*1*	*2*	*>2*
	14	2	19	19	2	1	0
Tylosin	*<1*	*1*	*2*	*4*	*8*	*16*	*>16*
	5	1	7	15	11	14	14
Virginiamycin	*<0.5*	*0.5*	*1*	*2*	*4*	*8*	*>8*
	4	1	19	25	11	3	4
Tiamulin	*<0.03*	*0.03*	*0.06*	*0.12*	*0.25*	*0.5*	*>0.5*
	4	4	32	20	3	0	4
Tetracycline	*<2*	*2*	*4*	*8*	*16*	*32*	*>32*
	27	4	19	8	5	4	0
Ronidazol	*<0.06*	*0.06*	*0.12*	*0.25*	*0.5*	*1*	*>2*
	35	1	19	10	2	0	0
Dimetridazol	*<0.06*	*0.12*	*0.25*	*0.5*	*1*	*2*	*>0.2*
	0	1	0	2	4	11	49

2.7.3   Animal commensals

Surveys for antimicrobial resistance in commensals, i.e. non-pathogenic bacteria, have only recently been initiated in Sweden. Preliminary results from these studies are given below.

In a study on poultry bacteria, enterococci and *E.coli* were isolated on selective media from neck skin and hindgut samples representing 52 poultry flocks slaughtered in 9 different slaughterhouses (Greko, 1996). A total of 207 isolates of *Enterococcus* spp. and 194 isolates of *E.coli* were investigated for MIC values for a range of antibacterials. Results from this study are shown in table 2.XIII and 2.XIV.

The figures show an overall low level of resistance for both enterococci and *E.coli*. The one exception is the prevalence of resistance against tetracyclines in enterococci. Tetracyclines are not frequently used in Swedish poultry production, and corresponding figures for other bacteria isolated from chickens (*Campylobacter jejuni*, *E.coli*) do not indicate a high exposure to this drug.

Table 2.XIII Resistance in *Enterococcus* spp. isolated from chicken (neck-skin and faecal samples). No. of isolates = 194

Tabell 2.XIII Resistens hos Enterococcus *spp. isolerade från kyckling (hals-skinn och feces prov), Antal isolat = 194*

Substance	Breakpoint (μg/ml)[1]	Proportion of isolates resistant (%)
Vancomycin	>4	0
Erythromycin	>2	17
Spiramycin	>16	15
Spiramycin	>8	15
Ampicillin	>8	0
Neomycin	>128	0
Streptomycin	>128	2
Chloramphenicol	>8	0
Tetracycline	>4	64
Trimetoprim-Sulphonamide	>0.5	0

[1]Breakpoint = value above which the isolate has been classified as resistant
[1]*Brytpunkt = värde över vilket isolaten bedömts som resistenta*

Table 2.XIV. Resistance in *E.coli* isolated from chicken (neck-skin and faecal samples). No. of isolates = 194

Tabell 2.XIV. Resistens hos E.coli *isolerade från kyckling (hals-skinn och feces prov). Antal isolat = 194*

Substance	Breakpoint (μg/ml)[1]	Proportion of isolates resistant (%)
Ampicillin	>8	5
Neomycin	>64	0
Streptomycin	>64	7
Gentamicin	>8	0
Tetracycline	>4	17
Trimethoprim-Sulphonamides	>0.5	4
Chloramphenicol	>16	1
Enrofloxacin	>0.25	4
Nalidixic acid	>32	6
Olaquindox	>32	<1[2]

[1]Breakpoint = value above which the isolate has been classified as resistant; [2]One isolate, also resistant to enrofloxacin (MIC=1μg/ml)
[1]*Brytpunkt = värde över vilket isolaten bedömts som resistenta;* [2] *Ett isolat, även resistent mot enrofloxacin*

The antibacterial resistance of enterococci and *E.coli* isolated from piglets has recently been investigated in a cohort study (Greko, 1997; Melin *et al.*, 1997). Briefly, in a study aiming to investigate the possible occurrence of resistance to zinc oxide among commensal *E.coli* of piglets in Sweden, a

total of 300 faecal samples were examined. The samples were collected 2-3 weeks after weaning from piglets in a total of 30 herds; 10 herds were using olaquindox as medicated feed (160 ppm), 10 herds were using zinc oxide (2500 ppm) and 10 herds used no medication. No isolates of coliforms with resistance to zinc oxide were found (Melin *et al.*, 1997). All samples were also cultured on selective media containing 50μg/ml of vancomycin. No vancomycin-resistant enterococci were found.

From non-antibiotic containing media, enterococci and coliforms were selected at random from each sample for further investigation. Table 2.XV shows the results of determinations of minimum inhibitory concentration (MIC) of different antibiotics for *Enterococcus.* spp. and table 2.XVI the corresponding results for *E.coli.*

Taken together, the figures are comparatively favourable. The number of isolates investigated from each group is too small to validate any conclusions with regard to differences between groups. Some conflicting observations on differences between groups can be made. For enterococci, the figures on resistance to neomycin and streptomycin in the non-medicated group seem higher than those from the other groups. On the other hand, such differences cannot be observed for *E.coli*. Similar observations can be made for *E.coli* and tetracycline resistance. Resistance to macrolides in enterococci seem to be more frequently of constitutive type in non-medicated groups. A lower prevalence of resistance to chloramphenicol in *E.coli* from the non-medicated group is not reflected in the figures for enterococci.

Table 2.XV. Resistance to different antibacterials in *Enterococcus* spp. isolated from weaned pigs in herds using different regimes

Tabell 2.XV. Resistens mot olika antibacteriella medel hos Enterococcus *spp. isolerade från avvanda grisar i besättningar med olika regim*

Substance	Break-point (μg/ml)	Resistance in isolates from sampling group (%):[1]			
		Olaquin-dox (n=69)[2]	Zinc-oxide (n=57)	No medi-cation (n=92)	Total (n=218)
Erythromycin	>2	29	33	36	33
Spiramycin	>16	6	12	22	14
Spiramycin	>8	14	26	38	27
Ampicillin	>8	0	4	0	0
Neomycin	>128	3	2	10	6
Streptomycin	>128	9	5	18	12
Chloramphenicol	>8	0	2	1	1
Tetracycline	>4	42	54	33	41
Trimethoprim-Sulphonamide	>0.5	0	0	0	0

[1]Breakpoint = value above which the isolate has been classified as resistant; [2] n= number of isolates

[1]*Brytpunkt = värde över vilket isolaten bedömts som resistenta;* [2] *n= antal undersökta isolat*

Table 2.XVI Resistance to different antibacterials in *E. coli* isolated from weaned pigs in herds using different regimes

Tabell 2.XVI Resistens mot olika antibakteriella medel hos E. coli *isolerade från avvanda grisar i besättningar med olika regim*

Substance	Break-point (µg/ml)	Resistance in isolates from sampling group (%):[1]			
		Olaquin-dox (n=60)[2]	Zinc-oxide (n=73)	No medi-cation (n=85)	Total (n=218)
Ampicillin	>8	2	4	2	3
Neomycin	>64	10	12	8	10
Streptomycin	>64	13	26	13	17
Gentamicin	>8	0	4	0	1
Tetracycline	>4	12	4	26	10
Trimethoprim-Sulphonamides	>0.5	2	7	0	3
Chloramphenicol	>16	15	26	7	16
Enrofloxacin	>0.25	0	1	1	1
Enrofloxacin	>0.12	15	11	1	8
Nalidixic acid	>32	2	1	1	1
Olaquindox	>32	35	19	32	28

[1]Breakpoint = value above which the isolate has been classified as resistant; [2] n= number of isolates
[1]Brytpunkt = värde över vilket isolaten bedömts som resistenta; [2] n= antal undersökta isolat

2.7.4 Some comments on the resistance figures

As shown above, available data indicate that antibacterial resistance is comparatively low in most bacteria from Swedish food producing animals. For most substances this reflects the consumption figures. However, other factors than antimicrobial usage may also contribute to keeping resistance to certain substances at a high level (see chapter 4). Continuous monitoring for antimicrobial resistance is necessary for detecting trends in resistance development. It is important to remeber, though, that resistance data only shows the phenotypic expression of resistance traits. Thus, further research on the genes behind the resistance is also necessary.

2.8   Summary comments

Antibacterial substances are valuable therapeutic tools in both veterinary and human medicine. Their use both as therapeuticals and as feed additives have contributed to the development of the contemporary animal industry.

In Sweden, all antimicrobial substances used in animals are classified as pharmaceutical specialities and are only available on veterinary prescription. The ban on antimicrobial feed additives in 1986 was associated with animal health problems in certain production systems. These health problems have to a large extent been overcome, and today's total consumption of antibacterials in veterinary medicine is considerably lower (50%) than what was used before the ban. The lower consumption is reflected in a comparatively favourable resistance situation in most animal bacteria. The Swedish experience shows that changes in production systems are necessary in order to adjust to animal production without antimicrobial feed additives, but also that such adjustments are possible and might pay off in a better situation regarding antibacterial resistance among zoonotic and animal bacteria.

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3   Mode of action and effects of antibacterial feed additives

3.1   Mode of action

The mode of action of antibacterial feed additives (AFA) has been the subject of numerous scientific reports. However, most of these deal with effects, rather than modes of action. The exact mechanisms regulating the growth promoting effect of AFA is not precisely known (for a review see Thomke and Elwinger, 1997c).

3.1.1   Effects

Rosen (1995) has compiled responses in poultry and pigs associated with dietary supplements of AFA (table 3.I). This table illustrates the diversity of the responses to AFA.

   AFA will alter the intestinal bacterial flora and reduce the number of sensitive microorganisms. Most AFA in use within the EU have effects on gram-positive bacteria. An early and fundamental finding was that young germ-free chickens, that grew approximately 20% faster than conventionally-reared chickens, did not respond to dietary inclusion of AFA (Forbes and Park, 1959; Eyssen and De Somer, 1967). Inoculation of germ-free chickens with *Enterococcus faecalis* - a common inhabitant of the intestinal tract - lowered growth performance significantly. Dietary inclusion of AFA, however, restored growth performance of inoculated birds (Lev and Forbes, 1959; Eyssen and De Somer, 1967). Some other micro-organisms that have been associated with growth suppression are: *Enterococcus faecium* (Coates, 1980; Fuller, 1994) and *Clostridium perfringens* (Lev and Forbes, 1959; Stutz *et al.*, 1983a; Stutz *et al.*, 1983b). The mechanism for the proposed growth suppressing effect has not been clarified.

   In animals given AFA, reduced weight of the intestine, thinning of the intestinal wall and shortening of the gut has been recorded (Jukes *et al.*, 1956; Stutz *et al.*, 1983b). These results suggest a lowered number of mucosal cells which, in turn, possibly also reflect the mucosal cell turn-over rate. Research has shown a similar lowered mucosal cell turn-over rate as well as a thinner intestinal wall in germ-free chickens, as compared to conventionally reared chickens (Coates, 1980). This indicates that the dietary inclusion of AFA will alter these intestinal characteristics towards the properties seen in germ free animals.

Table 3.I. Some physiological, nutritional and metabolic effects ascribed to AFA (modified from Rosen, 1995)

Tabell 3.I. Några fysiologiska, nutritionella och metaboliska effekter som tillskrivits AFT (modifierat från Rosen, 1995)

Physiological effects[a]		Nutritional effects		Metabolic effects	
Gut food transit time	↘	Energy retention	↗	Ammonia production	↘
Gut wall diameter	↘	Gut energy loss	↘	Toxic amine production	↘
Gut wall length	↘	Nitrogen retention	↗	Alpha-toxin production	↘
Gut wall weight	↘	Limiting amino acid supply	↗	Fatty acid oxidation	↘
Gut absorptive capacity	↗	Vitamin absorption	↗	Faecal fat excretion	↘
Faecal moisture	↘	Vitamin synthesis	↘	Liver protein synthesis	↗
Mucosal cell turnover	↘	Trace element absorption	↗	Gut alkaline phosphatase	↗
Stress	↘	Fatty acid absorption	↗	Gut urease	↘
Feed intake	↗,↘,→	Glucose absorption	↗		
		Calcium absorption	↗		
		Plasma nutrients	↗		

[a] ↘ denotes a reduction; ↗ denotes an increase; → denotes no change

It seems likely that the change of the intestinal flora and the subsequent changes in the intestine, including a slower intestinal passage rate, associated with AFA usage are linked to the many reported effects on nutrient breakdown, nutrient losses and nutrient absorption. Examples are the lowered breakdown of easily fermentable nutrients and the restriction in breakdown of essential amino acids, leading to a general nutrient saving effect.

3.1.2   Suggested main mode of action

As AFA are antibacterial substances, it is likely that their growth promoting effect is associated with their inhibitory effect on intestinal microbes. As discussed above, at dosages permitted for growth promotion, AFA will alter the intestinal microbial flora. Increased understanding of the complex relationships between the immune system and other functions of the body offer possibilities to better understand the mode of action of AFA.

Infections and growth rate

It is well established that growth rate and feed efficiency are reduced as a sequel to infection. This is why for example specific pathogen free (SPF) animals grow faster than animals in a conventional environment. Similarly animals reared in carefully cleaned and disinfected units grow faster than animals reared under a lower level of sanitary conditions. It has been shown that absence of disease increases the capacity of pigs to grow (Young *et al.*, 1959; Caldwell *et al.*, 1961). In Danish SPF systems, feed conversion is 3.9% lower and growth rate 6.4% higher than for conventionally reared pigs (Jorgensen, 1987). Similar observations have been reported in Swedish studies by Wallgren (1993a) (Figure 3.I). Chickens housed in clean, disinfected quarters also grow faster and more efficiently than chickens housed in less sanitary conditions, even in the absence of any clinical signs of infectious diseases (Roura *et al.*, 1992).

Immune responses and growth rate

An infectious challenge is a common form of stress encountered by growing animals. Infectious challenges may, or may not, result in clinical diseases, depending on the pathogenicity of the challenging microorganism and the immunocompetence of the animal. A stress response is indicated by decreased growth rates and quantitative changes in nutritional requirements (cit. from Klasing *et al.*, 1987). In humans, reduced food intake is considered to play a major role in infection-induced weight loss (Grunfeld *et al.*, 1996). This has also been shown in animals. Studying chickens, Klasing and co-workers (1987), found a growth depressive effect associated with an immune response. This effect was correlated to the immunogenic strength of the agent tested, the duration and the intensity of the immune response. The response to combinations of immunogens was additive. The dominating factor in the reduction of the animals' weight gain was shown to be a decrease in feed intake, while the remainder was the effect of less efficient routes in the intermediary metabolism (Klasing *et al.*, 1987). Roura and co-workers (Roura *et al.*, 1992) showed the influence of the animal environment on weight gain and feed efficiency in chickens (see table 3. IIb.). Studies in pigs

with a low or high level of chronic immune system activation revealed lower feed intake, reduced body weight gain, lower daily protein accretion and more feed required per kg weight gain in pigs with a high level of chronic immune system activation (see table 3.IIa.)(Stahly, 1996).

Table 3.IIa. Responses of pigs with a low or high level of antigen exposure to dietary antibacterial agents (carbadox). Adopted from Stahly (1996)

Tabell 3.IIa. Effekt av den antibakteriella fodertillsatsen carbadox på tillväxt och foderutnyttjande hos grisar med hög eller låg grad av antigen-exponering. Anpassad från Stahly (1996)

Growth and feed utilisation	Antigen exposure	Carbadox		Change (%)
		0-0[a]	55-0[b]	
Daily gain, g	Low	845	858	+1.5
	High	686	735	+7.1
Kg feed/ kg gain	Low	2.75	2.67	-2.9
	High	3.12	2.90	-7.1

[a] Non-medicated control group.
[b] Pigs fed 55 ppm carbadox from 5 to 34 kg body weight and then placed on the control diet without carbadox from 34 to 115 kg body weight.
[a] *Omedicinerad kontrollgrupp*
[b] *Grisar som fått 55 ppm carbadox från 5 till 34 kg kroppsvikt och därefter foder utan carbadox från 34 till 115 kg kroppsvikt*

Table 3.IIb. Responses of chickens with a low or high level of antigen exposure to dietary antibacterial agents (streptomycin and penicillin) Adopted from Rour (1992)

Tabell 3.IIb. Effekt av de antibakteriella substanserna penicillin och streptomycin på tillväxt och foderutnyttjande hos slaktkyckling med hög eller låg grad av antigenexponering. Anpassad från Rour (1992)

Treatment[1]	Weight gain (g/day)[2]	Feed efficiency (g gain/g feed)
Clean	12.65[a]	0.66[a]
Unsanitary	12.10[b]	0.54[b]
Clean+Antibacterial	12.72[a]	0.67[a]
Unsanitary+Antibacterial	12.57[a]	0.63[a]

[1]Sixty-four chickens were raised 14 days in each of the 2 different environments (clean or unsanitary) and fed diets either without antibiotic or with streptomycin (100 ppm) and penicillin (100 ppm); [2]Means in a column with different superscript letters differ significantly ($p < 0.05$).
[1]*Sextiofyra kycklingar vistades 14 dagar i 2 olika miljöer (låg eller hög antigenexponering) och gavs foder antingen utan antibiotika eller innehållande streptomycin (100 ppm) och penicillin (100 ppm); [2]Medelvärden i en kolumn med olika bokstavsmarkeringar skiljer sig signifikant ($p < 0.05$).*

The relationship between cytokines, immunologic stress and growth is complex. Responses in the brain and immune system are expressed as effector

signals in the form of regulatory hormones from the hypothalamus and pituitary and as cytokines from the immune system. Several hormones including corticotrophin releasing hormone, prostaglandins, glucagon, insulin and corticosteroids are induced by cytokines (for ref. see Grunfeld *et al.*, 1996). In this respect the release of corticotrophin releasing hormone and corticosteroids is of special interest since corticosteroids have a catabolic effect, thereby reducing muscle tissue. An activated immune system releases cytokines, that mediate the host response to infection and/or inflammation. Cytokines may also have direct effects on the brain, resulting in, for example, a decreased appetite.

   In summary, cytokines released as a consequence of a stimulated immune system mediate profound host responses including decreased appetite and a general catabolic effect. Many extrinsic stimuli such as bacterial toxins, yeast cell walls, silica particles, bacterial (gram-positive and gram-negative) or viral antigens have the capacity to elicit these responses (Mahé and Oppenheim, 1992; Degré, 1996).

Immune responses and AFA

Two recently published studies report on the effects of AFA on the immune system in relation to performance in clinically healthy animals. In one, avoparcin (having a gram-positive spectrum) was used and in the other carbadox (having a gram negative spectrum).

   In the study on avoparcin, Krinke and Jamroz (1996) compared some immunological parameters in avoparcin-fed chickens with a control group. In birds from the medicated group, most chickens had a suppressed reactive lymphoid tissue of the bursa of Fabricius. This result indicates that there may be a lack of stimulation of the immune system in the antibiotic-treated birds.

   The effects of carbadox in a model using pigs with low or high level of chronic immune system activation was studied by Stahly (1996 see table 3.IIa). The magnitude of the performance enhancement induced by carbadox was greater in the pigs with high level of chronic immune system activation than in pigs with low level of immune system. Similar results were obtained for pigs fed tylosin at 110 ppm (Stahly *et al.*, 1995). In chickens fed therapeutic levels of antibiotics (Table 3.IIb Roura *et al.*, 1992), the effects of antibiotics on immune stressed animals was further confirmed.
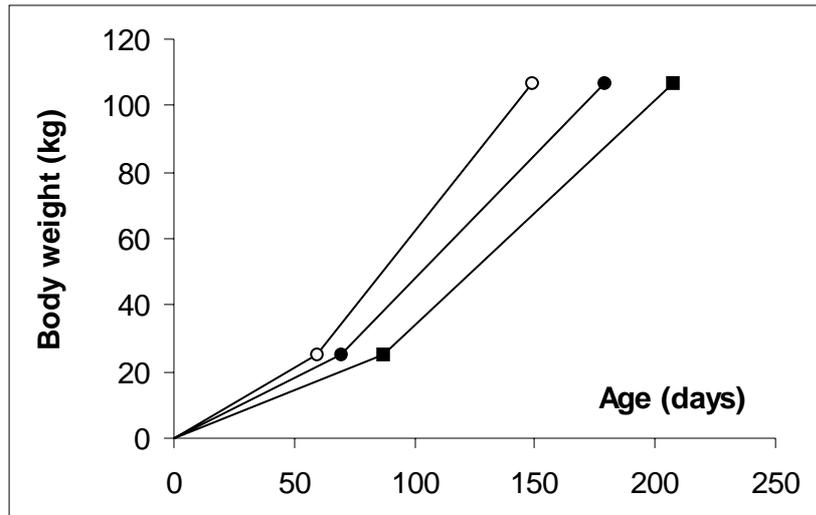
Figure 3.I. Growth rate of pigs in farrow to finish herds with different pathogen loads. ○ denotes specific pathogen free pigs representing the lowest pathogen load, ● denotes conventional pigs reared from farrow to finish but applying an age segregated system representing an intermediate pathogen load; ■ denotes conventional pigs reared from farrow to finish employing a continuos rearing system representing the highest pathogen load (Wallgren, 1993b).

Figur 3.I: Tillväxt hos grisar uppfödda i integrerade besättningar med olika smittämnestryck. ○ visar SPF-grisar (Specific Pathogen Free) representerande det lägsta smittrycket; ● visar konventionella grisar inom en besättning som tillämpar ålderssektionering, representerande ett intermediärt smittryck, ■ visar konventionella grisar som tillämpar ett kontinuerligt uppfödningssystem, representerande det högsta smittrycket (Wallgren, 1993b).

AFA improve growth rate and feed efficiency.

The effects of AFA are inversely related to animal health status. The poorer the health status the better the effect.

Challenge of the immune system will reduce growth rate and feed efficiency. This response is mediated by cytokines. Cytokines have direct effects on the brain, inducing a decrease in appetite, and stimulate the release of catabolic hormones, reducing the mass of muscle tissue.

AFA, through their antimicrobial effects, will alleviate immune system challenges from the intestinal tract on the immune system. This appears to be the major effect of AFA.

3.2   Prophylactic and therapeutic effects of AFA

3.2.1   Introduction

Most substances used as AFA are also used, or have been used, for therapeutic purposes. For some AFA the maximum dosage permitted for growth promotion is the same or nearly the same as what is recommended for prophylaxis and treatment of disease (table 3.III.). It has been pointed out that one expected consequence of abstaining from use of AFA is increased animal health problems due to infectious diseases (McOrist, 1997; Viaene, 1997). Indeed, the Swedish experience from banning AFA in 1986 was the emergence of clinical problems and disturbances of the health status of piglets and broilers which initially created a demand for antibiotic medicated feed at therapeutic dosages. The response to withdrawal of AFA in 1986 on piglet health and performance in 220 Swedish piglet producing herds was statistically evaluated by Robertson and Lundeheim (1994 see chapter 2). Removal of AFA was followed by a doubling of incidence of diarrhoea and number of medical treatments of post-weaning diarrhoea as well as by an increased mortality by about 1.5%. The age at 25 kg increased by 5-6 days. Since then, efforts have been made to improve the rearing and production systems e.g. sectioning between age groups and planned production. Production data of the pig herds from 1986 (Robertsson and Lundeheim, 1994) have now been re-analysed comparing 1986 to 1995 (Wierup, 1996). The comparison revealed a drop in post-weaning mortality from 1.5% in 1986 to 0.9% in 1995 and a reduction of the age at 25 kg by 1-2 days in 1995, as compared to 1986.

   This poses questions as to what degree AFA, at their permitted dosages for growth promotion, also have therapeutic and/or prophylactic effects against certain economically important intestinal diseases in pigs and poultry.

Table 3.III. Recommended dosages of AFA for treatment, prophylaxis and growth promotion in swine and poultry. (Från Allen *et al.*, 1992; Prescott and Baggot, 1993; FASS VET., 1997 and 70/524/EEC). Dosages have been converted to mg/kg and rounded off

Tabell 3.III. Rekommenderade doser av AFT för behandling, profylax och som tillväxtbefrämjare hos svin och fjäderfä. (Från Allen et al., ; Prescott and Baggot, 1993; FASS VET., 1997 och 70/524/EEC). Doseringar har räknats om till mg/kg och avrundats

Substance	Indication	Animal species	Dosage for treatment and prophylaxis mg/kg feed	Permitted dosage as AFA mg/kg feed
Avilamycin	*growth promotion*	*poultry*		*2.5-10*
		swine		*10-40*
Bacitracin	bacterial enteritis	poultry	55-220	
		swine	280	
	growth promotion	*poultry*		*5-50 (100)*
		swine		*5-20*
Carbadox	SD[1], PPE[2], bacterial enteritis	swine	50-55	
	growth promotion	*swine*		*20-50*
Flavomycin	*growth promotion*	*poultry*		*1-20*
		swine		*1-25*
Olaquindox	SD, bacterial enteritis	swine	100-160	
	growth promotion	*swine*		*50-100*
Spiramycin	mycoplasmosis	poultry	400	
	growth promotion	*poultry*		*5-20*
		swine		*5-50*
Tylosin	mycoplasmosis	poultry	900-1100	
	erysipelas, myco-plasmosis, SD, PPE	swine	40-110	
	growth promotion	*swine*		*5-40*
Virginiamycin	necrotic enteritis, respiratory infections,	poultry	20-40	
	SD, other bacterial enteritis	swine	28-150	
	growth promotion	*poultry*		*5-20*
		swine		*5-50*

[1]SD= swine dysentery
[2]PPE= porcine proliferative enteropathy

Below, some studies on therapeutic and/or prophylactic effects of AFA on certain diseases have been reviewed. Studies on this subject where several antimicrobial treatments are administered simultaneously to the same animals have not been included, as they are impossible to evaluate.

3.2.2   Necrotic enteritis in poultry

Necrotic enteritis (NE) is an intestinal disease affecting poultry. It is associated with *Clostridium perfringens.*

Wicker and co-workers (1977) demonstrated a reduced mortality ($p < 0.01$) due to NE in broiler chicks fed bacitracin at 11 ppm. In chickens with experimentally induced disease, a prophylactic effect of bacitracin administered in water at 100 mg/gallon and a therapeutic effect at 200 mg/gallon was demonstrated (Prescott *et al.*, 1978).

In a larger study, Jansson and co-workers (1992) induced NE by feeding chickens a diet high in barley. Virginiamycin given in feed at 20 ppm effectively prevented the disease. Another study by Elwinger and Teglöf (1991) showed similar results; virginiamycin at 20 ppm in feed notably reduced mortality due to necrotic enteritis in chickens ($p < 0.001$) compared to non-medicated groups.

Stutz and co-workers (1983a) showed that bacitracin, carbadox and virginiamycin, all at the level of 55 ppm in feed, reduced the amount of *C. perfringens* in the intestines of chickens compared to non-medicated controls ($p < 0.05$). The numbers of clostridia were inversely correlated with performance data. A similar reduction in numbers of clostridia and correlation with performance data was observed by Elwinger and co-workers in several studies using avilamycin at 10 ppm or avoparcin at 15 ppm (Elwinger *et al.*, 1993; Elwinger *et al.*, 1995; Elwinger *et al.*, 1996).

From the above, it is clear that some AFA are effective against clostridia, both *in vitro* and *in vivo,* at the levels used for growth promotion, resulting in effective disease prevention. Prevention of subclinical NE is associated with performance enhancement.

3.2.3   Swine dysentery

Swine dysentery (SD) is a contagious diarrhoeic disease in swine, caused by the spirochete *Serpulina hyodysenteriae*. Other forms of spirochetal diarrhoea in pigs are associated with other *Serpulina* spp.

Various studies have shown effects on SD by quinoxalines at growth promoting levels. Hunneman (1980) reported a notable decrease in outbreaks of SD in a region when carbadox was introduced as a feed additive for swine. These observations are supported by the results obtained in two clinical trials

(Anonymous, 1980). Molnar and Magyar (1987) reported an attempt to eradicate SD from swine herds by the aid of carbadox. Complete eradication of clinically apparent SD failed but the course and the severity of the disease was altered. The author also noted that the performance of the trial herds had improved markedly. Successful eradication of SD using carbadox in feed has been reported by other authors (Olson, 1986; Wood, 1987).

A swine dysentery model for evaluation of drug prophylaxis has been developed by Raynaud and co-workers (1980a; 1980b). Using this model, carbadox, and to some extent olaquindox, was found to prevent the outbreak of SD. This was substantiated in a large scale trial in commercial pig herds in France, where 50 ppm carbadox was found to be sufficient for both prophylaxis and treatment of swine dysentery (Raynaud and Bretheau, 1973). Prevention of experimentally induced SD with carbadox or olaquindox at levels used for growth promotion has been reported by several other authors (Davis and Libke, 1976; Williams and Babcock, 1978; Williams and Shively, 1978; Rainier *et al.*, 1980a; Rainier *et al.*, 1980b; Taylor and Davey, 1980; Biehl *et al.*, 1984; Jenkins and Froe, 1985; Jacks *et al.*, 1986). In one of these studies, olaquindox-resistant strains were also used as challenge (Williams and Shively, 1978). In those trials, the disease was not prevented, but morbidity was lower than in the non-medicated control group.

There seems to be a good correlation between MIC values of *S. hyodysenteriae* for tylosin and therapeutic effect of this substance. Williams and Shively (1978) found that tylosin at 100-110 ppm completely prevented SD induced by a tylosin-susceptible isolate of *S. hyodysenteriae*, while it was only partly effective against the disease induced by isolates with higher MIC values for tylosin. Jacks and co-workers (Jacks *et al.*, 1986) also prevented swine dysentery in pigs experimentally infected with a tylosin-susceptible strain, by feeding tylosin at 110 ppm. When a tylosin-resistant strain was used the disease was not prevented, although mortality was lower in the tylosin-medicated group than in the non medicated group. A new tylosin compound, 3-acetyl-4"-isovaleryl tylosin was also tested and found to be effective at 50 ppm.

Miller and co-workers (1972) studied the effect of different levels of virginiamycin and tylosin on pigs experimentally infected with *S. hyodysenteriae*. They found a good prophylactic effect of virginiamycin at about 25 ppm but only a moderate effect of tylosin even at about 100 ppm. The antimicrobial resistance pattern of the infecting strain was not presented, but presumably it was resistant to tylosin. Williams and Shively (Williams and Shively, 1978) fed virginiamycin at 50 to 100 ppm to experimentally infected pigs. Virginiamycin could not control the disease, although clinical signs were a little less common in medicated pigs than in non-medicated animals. This was supported by the observations of Rønne and co-workers (1992) who investigated the effect of 20 ppm of virginiamycin.

3.2.4   Porcine proliferative enteritis (PPE)

Porcine proliferative enteritis (proliferative adenomatosis) is an intestinal disease of pigs characterised by thickening of the intestinal mucosa. *Lawsonia intracellularis* (formerly *Ileal symbiont intracellularis*) is the causative agent. For a long time, the cause of the disease was elusive and various causative agents have earlier been pointed out. Therefore, most of the research bearing to prophylaxis or therapy is recent.

McOrist and co-workers (McOrist *et al.*, 1997) evaluated the efficacy of orally administered tylosin for the prevention and treatment of experimentally induced PPE. They found that tylosin at therapeutic levels could prevent PPE in challenge exposed pigs and could also be used for the treatment of previously induced PPE. Even at growth promoting levels, tylosin was effective in preventing the development of PPE. Moore and Zimmermann (1996) reported successful prevention of PPE by tylosin at about 100 ppm. Fleck and Jones (1994) compared 0, 40 and 100 ppm of tylosin for the treatment and prevention of PPE. The higher level of tylosin was effective as treatment of PPE, while 40 ppm did not quite prevent the emergence of clinical signs.

Kyriakis and co-workers (1996a; 1996b) found that in-feed bacitracin had a prophylactic effect against PPE in swine. The dosages used in this study were mostly substantially higher than what is used for growth promotion, and doses were changed over time. Even in the group receiving the lowest levels of bacitracin (50-200 ppm), a notable prophylactic effect could be seen.

3.2.5   Other bacterial enteritis in swine

Among bacterial enteric diseases in swine, weaning diarrhoea is one of the most important, both clinically and economically. *Escherichia coli* has been implicated in the pathogenesis. Bertschinger (Bertschinger, 1976) found that olaquindox at low growth promoting dosages was effective for both prevention and treatment of experimentally induced *E. coli*-diarrhoea in piglets. Kyriakis (1989) studied the effect of avilamycin and olaquindox on stress-induced post-weaning diarrhoea. At 40 ppm avilamycin or 50 ppm olaquindox, mortality was reduced ($p < 0.05$). The diarrhoea score was considerably lower compared to the control, but the difference was not significant. At 80 ppm avilamycin, both diarrhoea and mortality were reduced ($p < 0.05$).

Holmgren (1994) found that prophylactic treatment with 122 ppm or 173 ppm olaquindox in commercial swine herds reduced ($p < 0.01$) the incidence of post-weaning diarrhoea in all but one of the herds investigated. From the herd where olaquindox had no prophylactic effect, olaquindox-resistant *E. coli* were isolated.

Troutt and co-workers (1974) found that carbadox at maximum AFA dosage reduced clinical signs and intestinal lesions in pigs experimentally infected with *Salmonella* Cholerasuis.

Most AFA are, or have been, used for therapeutic purposes and for some AFA the maximum dosage permitted for growth promotion is the same or nearly the same as recommended for prophylaxis and treatment of disease.

Most AFA have prophylactic and/or therapeutic effects on enteric diseases, at permitted dosages.

3.3   Growth and feed efficiency responses to AFA in pigs and poultry

AFA were introduced in the early 1950s and have played a major role in the development of intensive and industrialised livestock production.

There is a great variation in responses of animals to AFA (CEC, 1993 , see table 3.IV). When judging these promoting or enhancing effects one has to consider the age and type of animals, the duration of AFA administration and the performance level of the control group. The major part of the research in this area with different animal species has been performed by the manufacturing and feed industries, whereas a relatively limited part has been performed by independent research bodies (Brenninkmeiyer, 1996).

3.3.1   Growth and feed efficiency responses to AFA in pigs

The response of younger animals, piglets as well as broiler chickens and calves, to AFA is superior compared to responses in older animals. In piglets the growth responses to AFA varies between 9 and 30% and the feed efficiency responses between 6 and 12%, whereas for growing-finishing pigs the level of response is inferior (Thomke and Elwinger, 1997b).

There has been a tendency to increase the level of AFA with time (Rosen, 1995). Some studies indicate a decreasing effect of AFA on daily weight gain and feed efficiency over time (Gruber, 1986). However, Schneider (1992) was unable to find differences in growth rate and feed efficiency for tylosin in growing-finishing pigs comparing the period of 1969/79 versus 1980/90.

A general opinion is that growth and feed efficiency responses of pigs and broiler chickens to AFA is lower under improved environmental conditions than in poorer environments. Rosen (1995) estimated the ratio in response between a very good and a poor environment to 1:2.

Table 3.IV. Percentage responses in growth performance and feed efficiency as a result of AFA usage in livestock production, compared with unsupplemented control diets, as reported by different authors (sources cit. by De Craene and Viaene, 1992; CEC, 1993)

Tabell 3.IV. Sammanställning av procentuell effekt av AFT på tillväxt och foderutnyttjande hos olika husdjur, jämfört med obehandlad kontroll, enligt vad som rapporterats av olika författare (källor cit. av De Craene and Viaene, 1992; CEC, 1993)

Animal species Source	Growth, daily weight gain	Feed efficiency
Piglets CEC (1993)	16	9
Growing pigs (20-50 kg) CEC (1993)	9	5.5
Finishing pigs (50 kg-slaughter) CEC (1993)	0	0
Growing-finishing pigs		
Bickel (1983)	5-10	5-7
Hudd (1983)	4-5	4-5
Mordenti et al. (1979)	6.5	4.1
Weiss (1989)	1.9-8.6	0.7-5.1
Robinson (1969)	10-20	5-10
CEC (1993)	3.5	3
Broiler chickens		
Birzer and Gropp (1991)	4	4
Hudd (1983)	3-4	3-4
Mordenti et al. (1979)	5.0	3.5
Robinson (1969)	5-10	5
CEC (1993)	4	4
Laying hens CEC (1993)	2	1
Veal calves		
Birzer and Gropp (1991)	10	5
CEC (1993)	7	4

Some discrepancy exists between different reports on the effect of AFA on sow performance. Speer (1974) concluded that the farrowing rate was improved by the use of AFA. However, others state AFA had no effects on oestrus, mating behaviour or breeding efficiency (Myers and Speer, 1973). Soma and Speer (1975) reported improved litter weights at birth and at

weaning This could not be corroborated by Frölich and co-workers (1974) under Swedish conditions.

Based on the available literature data, Thomke and Elwinger (1997b) estimated the average response in growth rate to 17% and in feed efficiency to 9%, when piglet diets were supplemented with AFA. Corresponding responses for growing-finishing pigs were inferior, and averaged 3.6% for growth rate and 3.1% for feed efficiency.

Thomke and Elwinger (1997b) also tried to predict the responses on performance in the Swedish pig industry to a re-introduction of APE. They assumed a lower response level on performance as a result of changes in the animal welfare rules, the current rearing and production models and performance levels in Sweden. For the piglet sector they calculated a response to AFA of 4-5% for growth performance and feed efficiency. For growing-finishing pigs a lower level of responses to AFA, of 1.5-2% was estimated.

3.3.2   Growth and feed efficiency responses to AFA in poultry

In broiler production the administration of AFA can yield an effect in comparison with the unsupplemented control of between -1 and 6% (Brenninkmeiyer, 1996).

Swedish and Danish experiments, performed during the period 1967-76 with broiler chickens fed bacitracin have been reviewed by Elwinger (1976). This antibacterial improved growth rate on average by 2.0% and feed efficiency by 1.3%. There were no differences in mortality. Similar to the result for pigs, responses in broiler chickens to AFA are superior in the first growth phase, as compared with the second.

In a comprehensive review, Rosen (1996), arrived at responses in growth rate and feed efficiency of 2 and 3%, respectively. It was also observed that AFA were more effective with respect to live weight gains when used in diseased than in apparently healthy birds. When including anticoccidials, the growth-promoting effect of the AFA themselves was clearly limited, due to the fact that some anticoccidials have antibacterial effects as well.

Based on available literature data, Thomke and Elwinger (1997b) estimated the overall responses of broiler chickens to AFA as regards growth rate and feed efficiency and found it to be on average 3.9 and 2.9%, respectively.

A re-introduction of AFA into today's Swedish broiler chicken industry would induce responses in performance and feed efficiency at a lower level than those reported above. An explanation for this is that the Swedish broiler industry has introduced production models without AFA, with a very high standard of hygiene and by the use of feeding programmes including a mixture of enzyme preparations. A tentative response level for performance and feed efficiency of 1-1.5% may be assumed.

In pigs the responses to AFA in terms of growth performance and feed efficiency are higher in piglets than in growing pigs and for finishing pigs the effects seem to be minute.

A re-introduction of AFA into Swedish pig and broiler meat production would most likely lead to lower levels of responses for growth performance and feed efficiency than what is reported in the literature. This is because these production systems have adjusted to production without AFA usage.

3.4   Alternatives to AFA

Livestock performance and feed efficiency are closely interrelated with the qualitative and quantitative microbial load of the host animal, including the alimentary tract and the animal environment. Appropriate nutrient supply and choice of ingredients and their proper preparation with respect to the animal's digestive capacity will minimise nutrient losses, overloading, disturbances and intestinal overcrowding by harmful microbial flora. Hence, improvements in feed composition and feeding strategies is an alternative to usage of AFA.

   Despite a vast body of information, details on the mode of action of both enzymes and probiotics are still lacking. Obviously, more research is needed to develop more efficient enzymes and to find out efficient combinations of enzyme preparations suitable for individual and compounded feedstuffs

3.4.1   Enzymes

Supplementing feed with enzymatic preparations may improve the digestive capacity, particularly in young animals. Thereby, nutrient utilisation in the anterior part of the gastrointestinal tract may be improved, possibly limiting the incidence of intestinal perturbation.

   Some experiments demonstrate that the beneficial effects achieved by AFA can at least partly be obtained by the inclusion of dietary enzymes affecting the physical properties of the ingested non-starch polysaccharides (Elwinger and Teglöf, 1991; Kronseder, 1993; Miles *et al.*, 1996; Vranjes and Wenk, 1996).

   Thomke and Elwinger (1997a) assessed the effects of various enzyme preparations on the nutrient digestibility and performance in pigs, based on available literature data, and arrived at a value for the average feed efficiency response of approximately 4% in piglets and about 2% in growing-finishing

pigs. For broiler chickens the average improvement of feed efficiency was about 4% (Thomke and Elwinger, 1997a) which is similar to the figure arrived at in the extensive compilation undertaken by Kronseder (1993).

In terms of improved digestibility and feed efficiency a fair estimate of the improvement by enzyme supplementation for young pigs and poultry according to the literature reviewed above would be approximately 3-4%. Calculating with an average digestibility of organic matter in cereal-based diets for these animal species of 80%, one arrives at a decrease in animal nutrient voidings by 15-20%.

3.4.2 Probiotics

Fuller (1992) recently redefined "probiotic" as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance.

The probiotic concept is generally based on a viable micro-organism culture with capacity to adhere to structures in the intestine. In selecting microbial strains with probiotic potentials, their genetic stability and intestinal colonising capacity as well as their stabilising properties are of main concern.

The efficacy of administrating probiotics seems to be dependent on a number of conditions, e.g. the physiological or health status of the animal, environmental factors such as feed regimen and microbial load. Beneficial effects are more often observed in neonatal animals and in weaners than in older animals.

In evaluating the great number of reports on the efficacy of probiotic organisms and substances in poultry, Barrow (1992) points out a number of factors hampering a critical appraisal. The vast majority of results on the efficacy of probiotics in poultry is reported as abstracts with essential information lacking, e.g. on the identity of strains used and on experimental design. The implantation in the gastrointestinal tract of the strains used is rarely assessed. The microbiological results in nutrition-oriented papers are often poorly interpreted. A further comment is that in a number of reports in which performance and health could be improved by probiotics, the general conditions seem to have been poor and non-representative with respect to production level and morbidity. Moreover, Barrow (1992) in his review considers that some of the interpretations of the results are obviously over-optimistic and arise mainly from a naive and uncritical acceptance of the data or speculations by previous workers.

According to the review by Mead (1995), there are a number of commercial preparations available that have been successfully used in the prophylaxis of *Clostridium* and *Salmonella* infections in poultry. Recently, Abu-Ruwaida and co-workers (1995) confirmed the potential of this method

against salmonellosis. The prophylactic treatment of salmonellosis is based on the principle of early establishment of an adult intestinal microflora in the young bird by supplying a suspension of anaerobic cultures of intestinal material, thereby competitively excluding pathogenic organisms. However, criteria on which to select micro-organisms for protective purposes are lacking at present and will remain so until more is known about factors influencing salmonella colonisation at the cellular level and the protective mechanism(s) involved (Mead, 1995).

Swedish experimental results when using the probiotic Broilact® have been promising, with a significantly lowered mortality and lowered caecal counts of *C. perfringens* (Elwinger *et al.*, 1992).

Supplementing feed with enzymatic preparations may improve the digestive capacity, particularly in young animals.

Enzyme additives affect the physical properties of ingested polysaccharides.

Some probiotics have been demonstrated to improve piglet performance and health. The efficacy of probiotics is difficult to evaluate in many instances, since experimental details are poorly presented.

More research is needed both on enzymes and probiotics and their mode of action.

3.5   Addendum: A benefit cost analysis of reintroduction of AFA in Sweden.

3.5.1   Executive Summary

If antibacterial feed additives (AFA) were to be used in the Swedish production of pork, egg and broilers (poultry meat) the expected direct savings in the production costs would be between 0.5-1%. However, these estimates do not regard externalities such as the impact on demand for these animal products, nor the costs of increased antibiotic resistance both in veterinary and human medicine.

The economic impact of AFA on the Community level should be scrutinised, having regard to all relevant factors and externalities including (a) the results of the GATT and CAP reform, (b) the costs of increased antibiotic resistance, (c) the dynamic effects for the industry within the Community, and (d) the smaller distortions of the world market due to subsidised exports from the EU. Previous studies have not accounted for the increased opening of the EU internal market to the world market due to the

GATT/WTO agreement and the increased fickleness of the consumers with regard to the perceived safety of the food of animal origin. The gains from lower export restitutions and increased import levies could be used to compensate farmers if AFA were not to be used as feed additives.

In the final analysis one should, moreover, also consider the danger of losing the consumer confidence in the safety of meat and eggs for which the continued use of AFA could be a risk factor.

3.5.2   Introductory comments

This benefit cost analysis will examine the monetary impact for Swedish farmers, if AFA were to be used in the pork and poultry sector.

The estimates presented were derived from the budget sheets from the Swedish University of Agricultural Science's extension service (SLU-Kontakt, 1996) and the budget sheets were adopted to capture the variation of estimates concerning the effects of AFA under Swedish conditions. The results were presented as estimated savings in production costs in SEK per kg produced meat or eggs, and also as the estimated monetary gain for the whole segment of the industry.

Since Sweden is a member of the EU, the market prices could be assumed to be constant with regard to the putative use of AFA. In consequence, decreased production costs would benefit the Swedish agricultural industry as such. The distribution of benefits between the primary producers, and the industry was not possible to estimate, and was not within the purview of this report.

Another uncertainty was the consumer response if AFA were to be used in pig and poultry production. The assumption both in the CEC report (1993) and the De Crane and Viane report (1992), that the consumer will only respond to price changes, seems to be contradicted by the BSE experience within the EU and also by the scrapie experience in Norway. In both cases considerable drops in the consumption of meat occurred. There is a risk of damaging the Swedish consumer confidence if AFA were to be used in Swedish animal production. This should not be ignored.

This report presents the models based on the budget sheets for the pig industry, for the egg production and finally for the poultry meat production. Moreover, the De Crane and Viane (1992) report is elaborated on to show an alternative analysis of the impact of AFA on the Community level with the egg market as an example. This is because the GATT agreement resulted in a downward slope of prices within the EU and this exogenous downward pressure on prices was ignored by the same report.

3.5.3   Gains if AFA were to be used in Sweden

Jonasson and Andersson (1996) indicated that the costs for AFA were
approximately 0.02 SEK per kg feed for the pig sector, and the same estimate
was also used in the poultry sector. The same authors noted that by using
AFA, one should be able to use cheaper feed ingredients, thus lowering the
net costs of AFA. However, they provided no estimate of this cost reduction.

Simulation of outcomes

All budget sheets were simulated 1000 times using @ Risk (Palisade Corp.)
to capture the uncertainties with regard to the effect on growth and feed
conversions if AFA were to be used in Swedish pig and poultry production.

Results

The results of the study appear in table 3.V, while the more detailed results
for each kind of production appear in tables 3.VI - 3.IX. The detailed budget
sheets used for the estimation of the putative benefits appear in Annex F,
tables 1-4.

   The savings on the supply side is around 1% of the production costs.
However, this benefit must in the final analysis be weighed against the
possibility of disturbances on the demand side due to adverse consumer
reactions and externalities such as the costs of the increased antibiotic
resistance both for animals and humans. Based on the experience from the
last 10 years of not using AFA, the Swedish pig and poultry meat market
could be foreseen to experience disturbances on the demand side if AFA
were to be used.

Table 3.V. Possible gains at the supply side if AFA were to be used in the
Swedish pork and poultry industry

*Tabell 3.V. Möjliga vinster på leverantörssidan om AFT åter började att
användas i svensk fläsk- och slaktkycklingproduktion*

	Reduced production costs (million SEK)	Cost of AFA (million SEK)	Benefit /cost ratio	Industry net gain (million SEK)
Piglet	47	4	11	43
Slaughter pig	47	16	3	31
Egg	11	5	2.3	6
Broiler	9	3	3	6

   If consumers' confidence in the safety of e.g. pig and poultry meat
diminishes, then the consequences tend to result in decreased demand.
Recent experiences suggest that a drop in demand of 10-25% should be

foreseen. Given a price elasticity of -0.5, this could indicate a decrease in the market price of 20 to 50%. Recent similar experiences include the drop in beef prices and increased market management expenses due to BSE and its impact on the demand for beef within the EU during the 90s, and also the diminished demand for mutton due to the fear of scrapie transmission to humans in Norway during 1996.

3.5.4   The pig industry

Piglet production assumptions

The following assumptions concerning the effects of AFA in piglets were made: the number of sows in Sweden amounts to 237 355 in 1995 according to the official yearbook of agricultural statistics (SCB, 1997). However, not all piglets from these sows should be foreseen to receive AFA. Exemptions include e.g. elite breeding herds and organic farming. It was assumed that the offsprings of around 230 000 sows were candidates to receive AFA.

Earlier estimates of an increase in piglet mortality of 1.5% after the prohibition of AFA in 1986 (Robertsson and Lundeheim, 1994) had to be adjusted to the present Swedish situation (see chapter 2). It was assumed that AFA would lead to a decrease in piglet mortality by 0.6%, based on the calculations by Wierup (1996).

To incorporate all relevant information on feed efficacy and piglet growth weighted approaches were used in which 0% was set as the minimum.

Feed efficacy was assumed to improve on average by 4.6%. The estimated effect on feed efficiency (FEFAC according to CEC, 1993) for Danish piglets was 2%, while the average effect on feed efficiency within the EU was 7.3% according CEC. Thomke and Elwinger (1997b) indicated an effect of feed efficiency of 4.5% if AFA were to be used under current Swedish conditions, and the upper estimate given in the same report was 9%. The average of these estimates was used.

Piglet growth was assumed to improve on average by 6.8%. The estimated growth (FEFAC according to CEC, 1993) for Danish piglets was 2% and the average effect within the EU was 8.5%. Thomke and Elwinger (1997b) indicated an effect on growth of around 4.5% if AFA were to be used under the current Swedish conditions, and the upper estimate given in the same report was 16%. The average of these estimates was used.

Veterinary and therapeutic costs was assumed to be reduced by 2 SEK per piglet, if AFA was introduced (Jonasson and Andersson, 1996).

Table 3.VI: The results of 1000 simulations if AFA were to be used in Swedish piglet production, mean value, with the 5th-95th percentile range in parentheses

Tabell 3.VI: Resultatet av 1000 simuleringar av effekten om AFT åter
började användas i svensk svinproduktion; medelvärde, med 5e-95e
percentilen inom parentes

	Saved production costs	Cost of AFA	Net benefit
SEK per piglet produced	10.19 (6.76-13.62)	0.93 (0.86-1.01)	9.23 (5.81-12.67)
SEK per sow	205.01 (134-274)	18.75 (18-20)	186.26 (116-255)
Savings in production costs (%)	1.18 (0.78-1.58)	0.108 (0.10-0.11)	1.07 (0.67-1.47)
Industry benefits (million SEK)	47.2 (31-63)	4.3 (4.1-4.5)	42.8 (27-59)

The expected benefit cost ratio was 11 (mean value of simulations) while the minimum benefit/cost ratio was 5.6, the maximum was 15.9 and the 5th to 95th percentile range was 7.2-14.7. In other words, under these assumptions the use of AFA in piglet production should produce a benefit for the piglet producing part of the pork industry of around 43 million SEK. The distribution of the benefits within the industry was not possible to predict.

Slaughter pig assumptions

It was assumed that around 3.5 million slaughter pigs would receive AFA. According to the official year book of agricultural statistics (SCB, 1997) the number of pigs slaughtered in Sweden was 3.7 million in 1995. However, not all herds would use AFA in their pig production. Moreover, it was assumed that if AFA were to be used in piglet production (production of pigs of 25 kg live weight), this would not interfere with the market for piglets and the piglet price which the pig farmers faced. No effect was foreseen on mortality by using AFA in the slaughter pig production stage.

To incorporate all relevant information on feed efficiency and growth, weighted approaches were used in which 0% was set as the minimum.

Feed efficacy was assumed to improve by, on average, 1.9%. The estimated effect for Danish slaughter pigs was 1.5% and the average effect within the EU was 2.1% according to CEC (1993). Thomke and Elwinger (1997b) indicated an effect of feed efficiency of 1.75%, and an upper estimate of 3.1%. The average of these estimates was used.

Table 3.VII. The results of 1000 simulations of the effect of AFA in the slaughter pig production, the values are represented as mean values (5th-95th percentile)

*Tabell 3.VII: Resultatet av 1000 simuleringar av effekten om AFT åter
började användas i svensk slaktsvinproduktion; medelvärde (5e-95e
percentilen)*

	Saved production costs	Costs of antibiotics	Net benefit
SEK per slaughter pig	13.5 (7.4-19.7)	4.5 (4.3-4.7)	9.0 (2.3-15.3)
SEK per kg pig meat	0.173 (0.094-0.253)	0.058 (0.056-0.060)	0.115 (0.03-0.195)
Savings in production costs (%)	0.99 (0.54-1.4)	0.33 (0.32-0.35)	0.65 (0.16-1.1)
Industry benefits (million SEK)	47.2 (26-69)	15.8 (15-16)	31.4 (8-53)

Growth was assumed to improve by on average 1.6%. The estimate on
growth for Danish slaughter pigs was 1.5% and the average effect within EU
was 2.4% (CEC, 1993). Thomke and Elwinger (1997b) indicated an effect on
growth of 1.75%, with an upper estimate of the effect of 3.6%. The average
of these estimates was used.

The expected benefit/cost ratio was 3 (mean value of simulations) while
the minimum benefit/cost ratio was 0.58 with a maximum of 5.6 and a 5th to
95th percentile range of 1.5-4.4. In other words if examining only the
production stage, the use of AFA would have a net benefit in most cases.
However, in a few scenarios (1%) one would expect a negative benefit in the
production stage. The impact on the demand side and possible externalities
such as the costs for increased resistance to antibiotics were not assessed.

A brief comment on the results

Gains of such magnitude, around 74 million SEK, for piglet and slaughter
pig production could be questioned based on the variations in piglet prices in
Sweden and Denmark. If the only difference were the usage of AFA, then the
Swedish piglet could be foreseen to be 10 SEK more expensive than its
Danish counterpart. However, the market prices have varied significantly and
according to the agricultural paper Land, April 11, 1997, the Swedish piglets
were 37 SEK more expensive than their Danish counterparts, while last
November according to Land, November 29, 1996, the Swedish piglets were
53 SEK less expensive than their Danish counterparts.

3.5.5   Egg production

Assumptions

No effect on the mortality of laying hens due to the use of AFA was
foreseen, and the improvements in feed efficiency and laying performance
were assumed to be in addition to the use of digestive enzymes and
coccidiostats in the feed.

To incorporate all relevant information on feed efficiency and laying
performance weighted approaches were used in which 0% was set as the
minimum.

Feed efficacy was assumed to improve by on average 1.1%. The estimate
by the food industry for Danish layers was 2.2% and the average effect
within EU was 1.2% (CEC, 1993). Thomke and Elwinger (1997b) indicated
an effect on feed efficiency of 1% using Zn-bacitracin. The average of these
estimates was used.

Laying performance was assumed to improve by on average 1.4%. The
estimate by the food industry for Danish laying hens was 3% and the average
effect within EU was 1.3% (CEC, 1993). Thomke and Elwinger (1997b)
indicated an effect on laying performance of 1%, using Zn-bacitracin. The
average of these estimates was used.

Table 3.VIII: The results of 1000 simulations of the effect of AFA in the
consumer egg production, the values are represented as mean values (5th-
95th percentile)

*Tabell 3.VIII: Resultatet av 1000 simuleringar av effekten om AFT åter
började användas i svensk äggproduktion; medelvärde (5e-95e percentilen)*

	Saving of production costs	Costs of antibiotics	Net benefit
SEK/kg egg	0.104 (0.052-0.158)	0.0455 (0.0450-0.0460)	0.0587 (0.006-0.113)
SEK/100 hens	165 (83-255)	72 (70-75)	93 (10-183)
Savings of production costs (%)	1.20 (0.87-1.85)	0.52 (0.51-0.54)	0.68 (0.07-1.32)
Industry benefits (million SEK)	10.9 (5.5-17)	4.75 (4.7-4.8)	6.1 (0.7-12)

Results

The expected benefit/cost ratio was 2.3 (mean value of simulations) while the minimum benefit/cost ratio was 0.37. The maximum was 4.4 and the 5th to 95th percentile range was 1.1-3.5. In other words, if examining only the production stage the use of AFA would have a net benefit in most cases. However, in some scenarios, 4 out of 100 egg producing farms, one would expect a negative benefit in the production stage. The impact on the demand side and possible externalities such as the costs for increased resistance to antibiotics were not assessed in this analysis.

3.5.6   Production of poultry meat (broilers)

Assumptions

No effect of AFA on the mortality of broilers was foreseen. The effect of AFA on feed efficacy was assumed to be in addition to the use of digestive enzymes and coccidiostats.

To incorporate all relevant information on feed efficiency and growth promotion, weighted approaches were used in which 0% were set as the minimum.

Feed efficacy was assumed to improve by on average 1.4%. The estimate by the food industry for Danish layers was 1.85% and the average effect within the EU was 2.0% CEC (1993). Thomke and Elwinger (1997b) indicated an improved feed efficiency of between 1-1.5%, while the upper estimate according to same authors was 2.9%.

Growth performance was assumed to improve by on average 2.1%. The estimate by the food industry for Danish broilers was 2.5% and the average effect within EU was 2.5% (CEC, 1993). Thomke and Elwinger (1997b) indicated an effect on growth performance of 2%, using Zn-bacitracin in 1976, while the usage of AFA in general would indicate an effect of 1-1.5% while the upper estimate for the effect on growth was 3.9% according to the same authors.

Results

The expected benefit/cost ratio was 3.2 (mean value of simulations) while the minimum benefit/cost ratio was negative and the maximum was 9.4, and the 5th to 95th percentile range was 0.4-7.0. In other words, if examining only the production stage the use of AFA would have a net benefit in most cases. However, in some scenarios, 18 out of 100 farms, one would expect a negative result. The impact on the demand side and possible externalities

such as the costs of increased resistance to antibiotics were not assessed in this analysis.

Table 3.IX. The results of 1000 simulations of the effect of AFA in the poultry meat production, the values are represented as mean values (5th-95th percentile)

Tabell 3.IX: Resultatet av 1000 simuleringar av effekten om AFT åter började användas i svensk slakkycklingproduktion; medelvärde (5e-95e percentilen)

	Savings of production costs	Costs of antibiotics	Net benefit
SEK/ kg meat	0.107 (0.0-0.22)	0.034 (0.031-0.038)	0.73 (-0.02-0.19)
SEK/ batch of 80000 chicken	98000 (0-202000)	31000 (28200-33700)	66700 (-28000-173000)
Savings of production costs (%)	1.5 (0.0-3.1)	0.47 (0.44-0.50)	1.0 (-0.4-2.7)
Industry benefits (million SEK)	8.5 (0-18)	2.7 (2.5-3.0)	5.8 (-2.5-15)

3.5.7   A brief comment on an earlier report

In the following, a brief comment on the report by De Crane and Viaene (1992) dealing with the economic effects of performance enhancers in food animal production will be given.

   Two issues are elaborated here i. the impact on the market of the withdrawal of performance enhancers and ii. the impact of the GATT-WTO regime and reform of the common agricultural policy (CAP).

   An alternative point of view is presented, in which the adaptation will fully be on the supply side, since prices are presumed to be constant and unchanged regardless of whether AFA are permitted or not within the EU. This assumption is based on the GATT agreement for agriculture and the CAP reform, where the previous variable import levies and export restitutions are fixed and thereafter shall be reduced annually. Moreover, the market shall be opened for a small, but increasing share of imported animal products including meat and eggs. Hence, there will be a downward pressure on the prices during the next years. The De Crane and Viaene report (1992) mentions these effects in an appendix, but does not account for them in the substantive analysis.

The removal of AFA will result in an upward shift in the supply curve, initially it is here assumed as a parallel shift of the curve. This means that for the same price a smaller quantity will be supplied, or that a higher price is required for the same quantity to be supplied. In the long run, dynamic effects will dominate, and possibly change the slope of the supply curve.

Notice that the producer surplus change is estimated as the increase in production cost times the decrease of the amount supplied, in the case of AFA being prohibited.

The impact on the market of the withdrawal of AFA

The report has examined the impact for each country and we will elaborate this for the egg market within the EU. The price elasticity of demand is assumed to be -0.5 and the price elasticity of supply is assumed to be 0.5.

For eggs the impact would be an increase in production costs of 1.2%, which would result in an increase of market price of 0.6%, which would result in a decrease in supply of 5300 tonnes (0.1% of 4.466 million tonnes) while demand would also decrease 5200 tonnes (0.1% of 4.439 million tonnes). This would increase the need for export by 100 tonnes to 27700 tonnes, and a drop in economic surplus of 28.7 million ECU, i.e., the market seems to be broadly in balance.

Comments: In other words, the impact of the use of AFA in addition to the use of coccidiostats and digestive enzymes would be hard to detect at the market level. Moreover, due to the CAP and GATT reform, an increase in prices is unlikely. The effect of increased production costs would thus be a decrease of supply, i.e., a shift of the supply curve, meaning less surplus export and less subsidies paid (Gardner, 1988). If the market price was kept unchanged, how much would the supply drop due to the cost increase?

If one could use the supply elasticity 0.5, i.e. % change in quantity/ % change in price, which is derived from the aggregate cost function as an estimate for the response to an increase in production costs, the supply would drop by roughly 1.2% * 0.5 which is 0.6%, or approximately 53000 tonnes, i.e., the surplus situation would change to an import situation, with 25300 tonnes of eggs in net import. If one assumed an import levy/export restitution of 10% or 90 ECU/tonne, this would lead to a savings at the community level of 53000 tonnes times 90 ECU/ tonne or 4.8 million ECU. In this case the consumer surplus would be unchanged, while the producer surplus would decrease by 4.4 million tonnes times 10 ECU/tonne or 44 million ECU. A net loss of 39 million ECU could be foreseen, if the prices were assumed unchanged or 0.9% for the producers.

In other words using De Crane and Vianes (1992) assumptions, not using AFA in egg production would have insignificant impact on the economics of egg production within the EU. This does not account for the possible costs of

a loss of consumer confidence, for which the continued use of AFA is a risk factor.

Economic studies of the use of AFA has often arrived at positive conclusions for the use of AFA. Nevertheless the assumptions underlying the studies, in particular about the ignorant consumer, are probably too restrictive and at variance with the experience in the beef market regarding BSE. Hence one could question whether the results are of any significant relevance for future decisions concerning AFA.

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4 Microbiological aspects on antibacterial feed additives

4.1 Introduction

Antibacterial substances (antibiotics and synthetic substances) exert their activity by inhibiting or disturbing vital bacterial processes. The effect is concentration dependent. At levels above the minimum inhibitory concentration (MIC), bacterial growth will cease or the bacteria will be killed. The only way for sensitive bacteria to avoid elimination is to develop resistance. This can be achieved by mutation or by acquisition of genes from other bacteria. Transfer of genes occurs by several mechanisms, namely transduction, transformation, and conjugation mediated by plasmids and/or transposons. The latter mechanism is the most studied and possibly the most important. Horizontal gene transfer between bacteria is a common event and an important factor in microbial evolution.

Antimicrobial resistance, leading to loss of effectiveness of antibacterial drugs, has been targeted by the WHO as one of the major emerging human health problems (WHO, 1994). The consequences of antimicrobial resistance are seen as an increase in morbidity and mortality due to bacterial diseases. Antimicrobial resistance also has a considerable economic impact and is estimated to cost at least US$ 4 billion annually in the United States alone.

The possible influence of antibacterial feed additives (AFA) on resistance in animal and human pathogens has been debated for almost four decades. The development of new techniques, particularly in the field of molecular biology, has provided new insights into these problems.

The antibacterial effect of AFA is also manifested in alterations of the intestinal microflora. The intact microflora is an important barrier against colonisation by enteric pathogens. Disruptions may lead to increased prevalence of zoonotic bacteria in food.

At the end of this chapter, a short glossary of terms used has been provided.

4.2 Antibacterial activity of AFA

The concentrations of AFA used in feed for performance enhancement exceed the susceptibility ranges of naturally susceptible intestinal bacteria (table 4.I). Concentrations reached in various parts of the intestine using the approved dosages will depend on a number of factors. Most AFA are poorly,

if at all, absorbed from the gut. They are therefore likely to be present in concentrations well above the MIC of normally susceptible bacterial species at least in part of the intestine.

The fact that AFA exert an inhibitory effect on the intestinal microflora is evident from various types of experiments. Early studies showed that the growth promoting effect could be associated with changes in the microflora, especially the enterococci and the clostridia, of the animals (see chapter 3.1). This is further substantiated by the preventive effect of AFA against certain intestinal diseases (see chapter 3.2). For therapy, higher doses are generally needed. By definition, therapy will be instituted once the disease is overt. When the infectious agent to be combated is already established in tissues, higher doses will be needed in order to have a satisfactory effect. If the infectious process is located in an internal organ other than the intestine, the administered substance must reach this organ at concentrations well above MIC. As the amount of substance given will then have to be absorbed in adequate amounts and be distributed in a larger volume (the whole body as opposed to only the intestine), higher doses are needed.

Table 4.I. Normal susceptibility ranges of clostridia and enterococci for some AFA compared to permitted levels in feed for performance enhancement (modified from Dutta and Devriese, 1984; Devriese *et al.*, 1993)

Tabell 4.I. Känslighet hos klostridier och enterokocker för några AFT jämfört med tillåtna halter i foder för avkastningsökning (modifierat från Dutta and Devriese, 1984; Devriese et al., 1993)

Antibacterial substance	Range of minimum inhibitory concentration (ppm) for:		Dosages used for performance enhancement (ppm)
	Clostridia	Enterococci	
Avilamycin	<0.25-0.5	NA[1]	2.5-40
Avoparcin	0.5-2	1-2	5-40
Bacitracin	<1-4[2]	<0.5-16[2]	5-100
Flavomycin	<1-8	0.25-4	1-25
Monensin	0.5-4	1-2	10-40
Spiramycin	0.25-8	0.5-4	5-80
Tylosin	<1	1-4	4-40
Virginiamycin	0.25-1	0.25-8	5-80

[1] NA = no information available; [2] Given in IU/ml
[1] NA= information saknas; [2] Uttryckt i IE/ml

The mechanisms of action of the different types of substances used as AFA have been studied in detail, with the exception of the quinoxalines. Currently approved AFA exert their effect on bacteria by one of four mechanisms (table 4.II):

- inhibition of protein synthesis
- inhibition of cell wall synthesis
- inhibition of DNA synthesis
- alterations of the cytoplasmic membrane

Concerning information on resistance mechanisms and their genetic background, practically all published studies on resistance mechanisms have focused on substances, or classes, also used in human therapy and mostly on resistance in human pathogens. The characterised resistance genes and mechanisms are probably only "the tip of the iceberg".

It is often stated that the AFA used in animal production are not used as therapeuticals in humans. This is true only for avilamycin, flavomycin and the quinoxalines. Spiramycin is used in human therapy. So is bacitracin, although mainly for local application. Small chemical differences between substances within the same class of antibacterials may have profound effects on their pharmacokinetics but their mode of action on the bacterium will usually be the same. Therefore, the bacterial defence in the form of resistance will often confer cross-resistance to all or most substances of the same class. Tylosin, avoparcin, ardacin and virginiamycin all belong to classes of antibacterials that include valuable therapeuticals.

Old, presently not used substances may become valuable therapeuticals in the future. Such is the case with everninomycins, a substance of the orthosomycin class. This substance is presently undergoing trials as a new drug candidate. Another member of the orthosomycin class is avilamycin.

Most AFA are similar to, or identical with, antibacterials used for therapy.

AFA are, in concentrations given for performance enhancement, inhibitory to inherently susceptible intestinal bacteria.

4.3   AFA and development of resistance

It has been argued that the concentrations of antibacterials used for performance enhancement are too low for any development of resistance to occur. As noted in 4.2, they are high enough to inhibit the growth of susceptible microorganisms. The usage of AFA would therefore be expected to favour the occurrence of resistant strains in exposed populations.

Table 4.II. Mode of action of substances used as AFA and known mechanisms of resistance (modified from Witte, 1996)

Tabell 4.II. Verkningsmekanismer hos substanser som används som AFT och kända resistensmekanismer (modifierad från Witte, 1996)

Class	Substance	Examples of other substances in the class	Mode of action	Known resistance mechanisms	Known genetic background of resistance	Cross-resistance (to substances of other classes)
Glycopeptides	avoparcin ardacin	**vancomycin, teicoplanin, daptomycin**	inhibition of cell wall synthesis by preventing transglycosylation	modification of binding site (peptidoglycan precursor)	*van* genes	
Ionophores	monensin salinomycin	other ionophores	disaggregation of cytoplasmic membrane			
Macrolides	**tylosin spiramycin**	**erythromycin, azithromycin, clarithromycin**	inhibition of protein synthesis by stalling the ribosome	modification of binding site (methylation of 23S rRNA) drug inactivation, efflux	*erm* genes, point mutation	**lincosamides, streptogramins**
Orthosomycins	avilamycin	everninomycins	inhibition of protein synthesis by preventing elongation			
Phospho-glycolipids	flavomycin		inhibition of cell wall synthesis by preventing transglycosylation			no information available
Polypeptides	**bacitracin**		inhibition of cell wall synthesis by preventing transpeptidation	active efflux, alteration of binding site (IPP), reduced membrane permeability	*bcr* gene, *bac*A gene	no information available
Quinoxalines	**olaquindox carbadox**	cyadox	inhibition of DNA synthesis			no information available
Streptogramins	**virginia-mycin**	**pristinamycin, quinupristin-dalfopristin**	inhibition of protein synthesis by stalling the ribosome	modification of binding site (methylation of 23S rRNA), drug inactivation, active efflux	*erm*, *sat*, *vat*, *vga*, *sbh* genes	**macrolides, lincosamides**

Substances with well known therapeutic use in human and/or veterinary medicine are in bold characters.

Substanser med välkända terapeutiska applikationer inom human- och/eller veterinärmedicinen är skrivna med fetstil

4.3.1   Prospective studies

Experimental studies

Several studies by the research group of Linton (Hinton *et al.*, 1986b; Kaukas *et al.*, 1987; Kaukas *et al.*, 1988) illustrate the influence of AFA on development of resistance in enterococci.

In two of these studies (Hinton *et al.*, 1986b; Kaukas *et al.*, 1987), the control groups were given a diet containing growth promoters (bacitracin or virginiamycin) and/or a coccidiostat with antibacterial activity. The confounding effect of growth promoters and anticoccidials in the control groups of these latter studies cannot be ignored. Consequently, only comparisons between the groups in the study from 1988 are deemed to be valid and will, in the following, be discussed in detail.

In this study (Kaukas *et al.*, 1988), small groups of chickens were given feed containing either avoparcin, nitrovin, virginiamycin or bacitracin or a diet without AFA during the first three weeks of life. The resistance to virginiamycin, bacitracin or nitrovin in *E. faecium* increased in the groups receiving the respective substance. Moreover, the incidence of resistance to therapeutic antibiotics, expressed as Antibiotic Resistance Index (ARI), was higher (p=0.003) in all groups receiving antibacterials. This increase could be associated with an increase in the proportion of *E. faecium* in the enterococcal population of the treated birds.

An experimental study on germ-free mice fed carbadox, olaquindox, flavomycin and chlortetracycline was reported by Corpet (1984). The mice were inoculated with intestinal microflora from 4 piglets, and the drugs were given at dosages corresponding to those used for growth promotion in livestock. Little effect on coliform resistance against olaquindox and carbadox was observed, while resistance against tetracycline increased in the flora of mice fed this drug. In the group given flavomycin, resistance to tetracycline was lower than in the control group. Resistance against flavomycin was not tested.

Dealy and Moeller (1977a) investigated the effect of in-feed flavomycin on the antibacterial susceptibility patterns of faecal *E. coli* from calves. They found an increase in resistance to flavomycin, but a decrease in resistance against streptomycin and tetracycline in the medicated group.

Observations on an apparent suppression of coliforms carrying resistance plasmids has been suggested in reports for both flavomycin, carbadox and bacitracin (Pohl *et al.*, 1975; Walton, 1978; Sepulchre, 1979; Gedek, 1981; Walton, 1984; Walton and Wheeler, 1987; Brophy, 1988). Some of these reports, however, merely studied the synergistic effects of the AFA and other

antibacterials and in others, the information provided is not enough to fully evaluate the issue.

Only for flavomycin have the possible mechanisms for a direct effect on numbers of plasmid carrying strains been addressed. George and co-workers (1984) investigated several possible mechanisms for the effects of flavomycin in concentrations from 1-4 ppm on *E. coli* strains harbouring different types of plasmids. The growth of some, but not all, of the plasmid carrying strains was inhibited in the presence of flavomycin. The plasmid transfer rates were mostly reduced, but also in some cases increased, depending on the type of plasmid. The authors suggest, among other explanations, that the differences observed could be due to flavomycin interacting with transfer-related cell wall structures, such as pili, coded for by certain plasmids. Publications concerning possible effects on gram-positive plasmid-carrying bacteria have not been found. The transfer proteins implicated in the mechanism have not been demonstrated in gram-positives and therefore, such effects would not be expected.

Field studies

Linton and co-workers (1985b) studied antibiotic resistance in the enterococci of commercial broiler flocks fed virginiamycin, bacitracin and avoparcin, and of pigs from commercial farms using in-feed tylosin and virginiamycin. In short, feeding of virginiamycin and/or tylosin resulted in increased resistance to tylosin and variable effects on virginiamycin resistance in both pigs and poultry. Feeding of bacitracin led to an increased resistance to bacitracin. Resistance to avoparcin or other glycopeptides was not tested. Unfortunately, only the pig study included a non-medicated farm as control group, outcome was somewhat variable, and the enterococci were not identified to the species level.

The influence of virginiamycin on the prevalence of resistant enterococci within flocks of turkeys has recently been reported by Thal and co-workers (1996). Different turkeys from the same flock were found to share the same type of streptogramin-resistant *E. faecium*, and the prevalence of resistance to both streptogramins and ampicillin increased over time. All flocks received in-feed virginiamycin, plus other AFA and coccidiostats.

A study to monitor the development of olaquindox resistance in coliforms following the introduction of olaquindox as a feed additive was conducted in commercial farms in Suffolk by Linton and co-workers (1988). The results concerning olaquindox resistance in coliforms in farms with and without use of the antibacterial are shown in table 4.III. As is often the case in field studies, there were problems with sampling variability and difficulties to control the management on the farms. In spite of this, the overall results were consistent and showed an increasing level of resistance to olaquindox in

coliforms from farms using olaquindox. Incidence and level of resistance increased on neighbouring farms not using olaquindox as well, but to a lesser extent. The latter finding is not surprising as the herds were not isolated from the environment.

Table 4.III. The average percentage of coliforms resistant to olaquindox in each year of the survey (from Linton *et al.*, 1988)

Tabell 4.III. Genomsnittlig procentandel av koliformer resistenta mot olaquindox under respektive studieår (från Linton et al., 1988)

Year	Resistance (%)	
	Control farms	**Test farms**
1981[1]	0.00	-
1982	0.03	0.04
1983	0.12	5.63
1984	0.68	6.14

[1] Sampled before olaquindox was used in the UK

[1] *Provtagna innan olaquindox användes i Storbritannien*

4.3.2   Retrospective studies

Devriese and co-workers (1993) studied the antibacterial resistance patterns for some AFA in *C. perfringens* isolated from various animal sources and found no notable increase in resistance between 1979 and 1992.

Ohmae and co-workers (1981; 1983) reported the incidence of carbadox resistance in *E. coli* from cattle, pigs and chickens, collected during 1976-1980. Carbadox resistance was only found in isolates from pigs. All carbadox-resistant isolates originated from 6 farms, where carbadox was used for preventing swine dysentery or promoting piglet growth. All isolates from one farm harboured a conjugative plasmid, carrying carbadox, spectinomycin, streptomycin and ampicillin resistance.

An increase in carbadox resistance in salmonellae over time was noted in a study by Mills and Kelly (1986). The study included clinical isolates from necropsied swine in Kansas during 1980-1983. A steady increase in carbadox resistance, from 37% 1980 to 61 % 1983 was noticed. The authors note that in-feed carbadox in Kansas is labelled for prevention of swine dysentery and for treatment of salmonella infections.

A strong association between the use of avoparcin and prevalence of vancomycin resistant enterococci (VRE) in animals has been reported. Bager and co-workers (1997) investigated poultry and pig farms in a retrospective cohort study. The relative risk for occurrence of vancomycin resistant *Enterococcus faecium* was 3.3 (0.9-12.3) for pig herds exposed to avoparcin. The corresponding figure for poultry flocks was 2.9 (1.4-5.9). These findings are supported by observations from countries where avoparcin is not used, no

VRE have yet been isolated from animals (Coque *et al.*, 1996; Greko and Lindblad, 1996).

4.3.3   Point-prevalence studies

Shortly after the introduction of avoparcin, no glycopeptide resistance was found among 15 strains of *E. faecium* isolated on vancomycin-free media (Dutta and Devriese, 1982), nor was resistance found in other enterococcal species. In table 4.IV, recent results concerning glycopeptide resistance from the Danish surveillance system are presented. As in the above mentioned study, the isolates investigated were obtained without the use of antibiotic containing media in the course of a monitoring programme.

Studies using media favouring resistant isolates indicate that enterococci with high level resistance to glycopeptides (VRE) are widespread among animals including pets and horses (Bates *et al.*, 1994; Klare *et al.*, 1995b; Devriese *et al.*, 1996). The lack of earlier data on VRE in animals precludes conclusions on whether the resistance trait was present in animal populations at the time of introduction of avoparcin in animal husbandry.

As mentioned in 4.3.2, in studies from USA where avoparcin has never been used and from Sweden where avoparcin has not been used for 10 years no VRE were found in samples from animals using selective techniques (Coque *et al.*, 1996; Greko, 1996). Thus, in the absence of avoparcin, the prevalence of VRE in animals is, at most, very low.

Table 4.IV. Frequency of resistance to glycopeptides (vancomycin) in *E. faecium* from different sources[1]

Tabell 4.IV. Frekvens av resistens mot glykopeptider (vancomycin) hos Enterococcus faecium *från olika provtyper*[1]

Animal source	No. of isolates	Resistance in %
Cattle	13	0
Beef	40	0
Swine	58	20
Pork	23	0
Poultry	54	59
Poultry meat	71	18

[1] Data from DANMAP (1997). In samples from food, only high-level resistance was reported whereas in animals samples, all resistant isolates are included

[1] *Data från DANMAP (1997). För prover från livsmedel har endast höggradig resistens rapporterats, från djurprover inkluderades däremot alla resistenta isolat*

It has often been assumed that resistance observed in animal bacteria is entirely a result of the use of therapeuticals. A comparison between the situation in countries where macrolides are used both for therapy and as

AFA, and where they are only used for therapy is therefore of interest. In table 4.V, results from investigations in Denmark, Finland and Sweden are presented. Spiramycin, being a 16-membered macrolide was chosen in the example as indicator of constitutively expressed *erm*-genes, encoding for MLS_B type resistance (see annex E). A lower prevalence of resistance is reported from Finland and Sweden, using only therapeutical macrolides compared to Denmark where they are also used as AFA.

The tables above illustrate phenotypic expression of resistance. However, information on phenotype at best only permits inferential conclusions about the genes conferring these resistance traits. All the resistant isolates from Sweden and Finland were cross-resistant to erythromycin (a 14-membered macrolide). The enterococci are inherently resistant to moderate concentrations of lincosamides (e.g. clindamycin) but high level resistance can be acquired (Murray, 1990). For all the Swedish isolates that were resistant to 16-membered macrolides, MIC values higher than for the remaining group of isolates were noted. This leads to the assumption that the resistance shown is mediated by constitutively expressed *erm*-genes. MICs for clindamycin were not given for the Finnish isolates.

Table 4.V. Resistance to MLS_B antibacterials (indicated by spiramycin resistance) in enterococci from various species and countries (From Greko, 1996; DANMAP, 1997; Greko, 1997; MAF, 1997)

Tabell 4.V. Resistens mot MLS_B antibiotika (indikerat av spiramycinresistens) hos olika djurslag (Från Greko, 1996; DANMAP, 1997; Greko, 1997; MAF, 1997)

Bacterial species	Animal source	No. of isolates	Year	Resistance in %	Country
E. faecium	poultry	54	1995-96	*54*	Denmark
E.faecium	poultry	234	1996	*10*	Finland
E.faecalis	poultry	299	1996	*9*	Finland
Enterococcus spp.	poultry	207	1996	*15*	Sweden
E. faecalis	swine	123	1995-96	*89*	Denmark
E. faecium	swine	58	1995-96	*88*	Denmark
E.faecium	swine	89	1996	*12*	Finland
E.faecalis	swine	85	1996	*15*	Finland
Enterococcus spp.	swine	92	1996	*14*	Sweden

Further information on frequency of resistance to AFA can be found in annexes A-F.

4.3.4   Some comments on study design

Studies performed to date are extremely difficult to compare. Selection criteria, methods used and definition of resistance vary. Further, a serious limitation of most studies on antibacterial resistance is the restricted range of microbes surveyed (Salyers, 1995). This is particularly true if the question asked is how the use of a substance affects the evolution and spread of resistance genes in a certain environment. Inclusion of numerically predominant organisms, such as certain anaerobic genera, would give a more complete picture. For practical reasons, most studies focus on easily cultured bacteria. Until further knowledge about the role of the predominant part of the flora is obtained, we have to accept that much information concerning gene transfer in natural settings is missing (Salyers, 1995).

Some species are inherently (naturally) resistant to certain antibacterial substances. For example, *C. perfringens* and *E. faecium* have been reported to be inherently resistant to flavomycin and enterococci, apart from *E. faecium,* are inherently resistant to streptogramin A. Such species will be favoured if a selective pressure is applied. Other species, that are inherently susceptible to the substance in question, may become resistant either by mutation or by the acquisition of already existing resistance genes. When the aim is to assess the influence of antibacterial usage on the prevalence of antibacterial resistance, bacterial populations with a potential for acquiring resistance should be targeted. Enterococci are inherently susceptible to most AFA and are known to easily acquire resistance genes. In view of this, and bearing the above mentioned limitations in mind, the choice of enterococci as one of the indicators of the development of resistance seems acceptable for most AFA.

The study time in both experimental and field exposure studies is generally short. Dissemination of antibacterial resistance will be governed by two main factors; presence of the antibacterial in concentrations high enough to inhibit susceptible bacteria and presence of resistance traits (intrinsic or acquired) in bacteria. If either is continually absent, a resistance problem will not emerge (Levy, 1996). Acquisition of the relevant gene and its subsequent adaptation to a new host bacterium may take time (years). As closed experimental herds are often used, resistance may in fact never appear, even if it would with time in a field situation where herds are expected to have numerous direct and indirect contacts with potential gene reservoirs.

Given the presence of both a selective pressure and resistance, the resistance trait will be selected for and propagated. The spread of resistant bacteria themselves, as well as cell to cell spread of genes conveying resistance, further adds to the equation. Longer study periods (years) would be expected to give more applicable results, but may be impractical for other reasons.

In view of the long standing debate on usage of AFA and resistance, there are surprisingly few studies including data on prevalence of resistance against the substances in question. Even less data is available on development over time. Well-planned screening studies, conducted in several countries and combined with data on consumption of antibacterial feed additives in these countries (amount given to different animal species), as well as therapeutic usage might provide at least some of the information on exposure necessary for an accurate analysis of potential hazards and associated risks. Programmes, such as the Danish monitoring (DANMAP, 1997), as well as the one lately initiated by the European Commission, are therefore to be commended.

> Various types of studies show that exposure of bacterial populations to AFA in approved dosages favours resistant clones or subpopulations.

4.4 Acquisition of resistance in bacteria

Acquired resistance can arise in a bacterium through mutation(s) in existing genes or through the uptake of a pre-existent gene. In the first case, the resistance trait will be confined to the mutant clone. Spread of resistance will depend on the ability of that clone to multiply (vertical transmission) and infect new hosts. In the second case, the resistance trait can also spread to other bacterial clones, to other bacterial species and genera (horizontal transmission).

Following uptake, acquired resistance genes may be modified, or optimised, by means of mutation. Once the optimal configuration has been accomplished, little change would be expected within the gene, although transfer to a new host species or genus may require some adaptation for maximum expression. "Silent" resistance genes, i.e. non-expressed genes, may be "activated" by mutations.

4.4.1   Transfer of resistance genes

Pre-existent resistance genes are mostly associated with transfer elements such as plasmids or transposons, residing in plasmids or on the chromosome. Detailed studies concerning transfer elements associated with relevant genes have only been published for antibacterials in clinical use. As acquired resistance has been reported for other AFA as well, there is no reason to believe that resistance against these substances would be any different in this respect. In table 4.VI, described genes conveying resistance to macrolides and/or streptogramins and examples of their localisation have been compiled.

The plasmids and transposons mentioned are often of a conjugative type, meaning that in addition to the resistance genes, they carry the information necessary to initiate and complete their own transfer to new hosts.

Transfer of resistance determinants with relevance for AFA has been shown *in vitro* and, for some, *in vivo*.

Resistance to the A component of streptogramins is mediated by various genes carried on plasmids. The *vat*B gene has been shown to transfer between coagulase-negative staphylococci and *S. aureus* (Allignet *et al.*, 1996).

Resistance to streptogramin B and 16-membered macrolides (spiramycin, tylosin) is mostly mediated by constitutively expressed *erm*-genes of different classes (Leclercq and Courvalin, 1991b). The product of the gene, a ribosomal methylase, alters the ribosome slightly with the result that macrolides, lincosamides and streptogramin B cannot bind (MLS_B resistance). The *erm*-genes in staphylococci are often inducible only by 14-membered macrolides (erythromycin), meaning that the methylase will only be produced in presence of erythomycin. The gene can convert to constitutive expression by a single or two point mutation rendering the bacterium phenotypically resistant to all macrolides, to lincosamides and to streptogramin B (see annex E).

Poyart-Salmeron and co-workers (1990) showed by way of *in vitro* mating experiments that a self-transferable plasmid designated pIP811 carrying an *erm*B gene (also carrying resistance determinants for chloramphenicol, tetracycline and streptomycin) could transfer from *Listeria monocytogenes* to, among others, *E. faecalis* and vice versa. The donor efficiency[3] depended on the combination of donor-recipient tested and varied from 10^{-3}-10^{-9} with enterococci and streptococci being the most and staphylococci the least efficient. The plasmid, originally identified in *Listeria monocytogenes*, was suggested to have originated from streptococci or enterococci as it is similar to pAMβ1, the prototype for broad-host range plasmids in those genera.

Acquisition of macrolide resistance through transfer of the chromosomally carried transposon Tn*1545,* harbouring MLS_B (*erm*B), kanamycin (*aphA*3') and tetracycline (*tet*M) resistance determinants from *E. faecalis* to *L. monocytogenes* has been demonstrated both in *vitro* by mating experiments and *in vivo* in gnotobiotic mice (Doucet-Populaire *et al.*, 1991). The *in vitro* and *in vivo* transfer frequencies were around 10^{-7} and 10^{-8}, respectively. In both cases, subinhibitory concentrations of tetracycline increased the transfer frequency around 10 times.

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[1]The donor efficiency expresses the number of successful transmissions from donors to recipients per total number of recipients. The donor:recipient ratio in experiments is often 1:1

Table 4.VI. Examples of resistance to macrolides and streptogramins by different mechanisms and genes[1]

Tabell 4.VI. Exempel på resistens mot makrolider och streptograminer, via olika mekanismer och gener[1]

Phenotype[2]	Mechanism	Gene	Described localisation	Examples of bacterial hosts
MLS$_B$	target modification	*erm*A	Tn*554*	*S. aureus,* coagulase negative staphylococci
		*erm*B, *erm*AM	Tn*551*, pAMβ1, Tn*917*, Tn*1545*, plus various other plasmids and transposons	*S. aureus, S. intermedius, S. hyicus, Streptococcus* spp, *S. pneumoniae, E. faecalis, Lactobacillus* spp.
		*erm*C	pE194, plM13, pE5, pNE131	*S. aureus*, coagulase negative staphylococci, *S. hyicus, Bacillus subtilis*
		*emr*CX	Tn*5432*	*Corynebacterium xerosis*
		*erm*D	chromosome	*B. licheniformis*
		*erm*BC	pIP1527	*E. coli*
		*erm*F	pBF4	*Bacteroides* spp.
		*erm*E	chromosome	*Streptomyces erythreus*
		*erm*P, *erm*Q		*Cl. perfringens*
		*erm*Z	Tn*5398*	*Cl. difficile*
		*erm*J		*Bacillus anthracis*
		*erm*M		*S. epidermidis*
S$_B$	drug inactivation	*sbh*	pIP524	*S. aureus*
		vgb	pIP680	*S. aureus*
S$_A$		*satA*	pAT424	*E. faecium*
		vat	pIP680, IS257	*S. aureus*
		vatB	pIP1156	*S. aureus*
S$_A$	active efflux	*vga*	plasmids	*S. aureus, S. epidermidis*

[1]Data compiled from Leclercq and Courvalin, 1991a; 1991b; Brisson Noel *et al*, 1988; Arthur *et al*, 1987; Eady *et al*, 1993; Weisblum, 1995; Mullany *et al*, 1995; Allignet *et al*, 1996; Tauch *et al*, 1995

[2] Resistance phenotype; MLS$_B$ = macrolide-lincosamide-streptogramin B; S$_B$ = streptogramin B; S$_A$ = streptogramin A

[1]Data sammanställda från Leclercq and Courvalin, 1991a;1991b; Brisson Noel et al, 1988; Arthur et al, 1987; Eady et al, 1993; Weisblum, 1995; Mullany et al, 1995; Allignet et al, 1996; Tauch et al, 1995

[2]Resistensfenotyp; MLS$_B$ = makrolid-linkosamid-streptogramin B; S$_B$= streptogramin B, S$_A$ = streptogramin A

In the above cited experiment, germ-free mice were used. McConnell and co-workers (1991) used mice with a complex microflora functionally equivalent to that of conventional mice. They investigated transfer of the plasmid pAMβ1, carrying *erm*B. Transfer of the plasmid from *Lactobacillus reuteri* to *E. faecalis* was detected within days after birth in the offspring of the mice inoculated with the donor strain. The parent animals were given lincomycin at subtherapeutic concentrations. No transfer was detected in offspring of mice not given lincomycin.

The fact that conjugal transfer was easily detectable within a relatively short time period in both *in vivo* experiments cited above confirms that the intestinal environment is highly conductive to conjugal transfer, even when the donor and recipient are of different genera. Further, they show that *in vitro* results on transfer and transfer rates can be predictive of *in vivo* results.

Transferable glycopeptide resistance is usually mediated by the gene clusters *van*A or *van*B. These are generally located on plasmids and/or transposons (Arthur and Courvalin, 1993). High level resistance to glycopeptides mediated by the *van*A-gene cluster has been detected in *E. faecium*, other enterococcal species (Arthur and Courvalin, 1993), *Oerskovia turbata* and *Archanobacterium haemolyticum* (Power *et al.*, 1995). The gene cluster is mostly associated with the conjugative transposon Tn*1546* and/or self-transferable plasmids (Arthur *et al.*, 1996). Transfer of the *van*A-gene cluster has been shown *in vitro* from *E. faecium* to *Listeria monocytogenes*, *Staphylococcus aureus*, and various streptococci (Leclercq *et al.*, 1989). Transfer frequencies were 10^{-4} for *E. faecium* to *E. faecium* and 10^{-6} - 10^{-9} for transfer to other species. When resistance to MLS antibiotics was also present, the two traits were transferred *en bloc* (Leclercq *et al.*, 1989). Conjugal co-transfer of resistance to high levels of glycopeptides, erythromycin and chloramphenicol, from *E. faecalis* to *S. aureus* on the skin of hairless obese mice was demonstrated in an experiment by Noble and co-workers (1992). The mice used cannot be regarded as "normal" mice. Nonetheless, they are a better model for the *in vivo* situation than a petri dish.

Resistance to glycopeptides mediated by the *van*B gene cluster has, in relation to AFA, attracted less attention. The *van*B-gene cluster is transferable either directly from the chromosome by a transposon (Tn*1547*) or through plasmids (Quintiliani and Courvalin, 1994; Woodford *et al.*, 1995; Quintiliani and Courvalin, 1996) at a low frequency. The *van*B-gene cluster has been found in *E. faecalis*, *E. faecium* and, recently, in *S. bovis* (Arthur and Courvalin, 1993; Poyart *et al.*, 1997). The gene usually confers inducible resistance to glycopeptides by a mechanism similar to that of the *van*A gene-cluster. The *van*B-gene cluster is induced by vancomycin but not by teicoplanin, meaning that when strains carrying the gene cluster are exposed to teicoplanin, the gene will not be activated and the strain phenotype will remain susceptible (Arthur and Courvalin, 1993). According to available

information, the *van*B gene cluster does not seem to be inducible by avoparcin. No information is available on the capacity of ardacin to induce *van*B.

In vitro as well as during therapy, mutants expressing the *van*B gene constitutively have been reported (Hayden *et al.*, 1993; Green *et al.*, 1995). Recently, transfer experiments with a strain expressing *van*B constitutively were reported (Hayden *et al.*, 1997). The resulting transconjugants were either of constitutive or of inducible type. The use of a non-inducing antibacterial such as avoparcin, and possibly ardacin, could favour strains harbouring *van*B-gene clusters with the mutation required for the gene to be constitutively expressed should the gene be present in animal populations or their environments. Further information is needed on this topic.

4.4.2   Bacterial interspecies transfer of resistance genes

As evident from 4.4.1, similar or identical resistance genes can be found in various bacterial species, indicating that transfer of resistance genes between different bacterial species is not uncommon in nature. Conjugal transfer systems are able to cross genus, phylogenetic, and even kingdom lines (Amabile-Cuevas and Chicurel, 1992). There are broad host range genes that can be expressed in a variety of species as well as broad host range gene transfer elements.

As an example, high level resistance to MLS_B antibacterials in a clinical isolate of *E. coli* was characterised by Brisson-Noël and co-workers (1988). The resistance trait was found to be due to the presence of an *erm*-gene highly homologous to *erm*B, previously described in *E. faecalis* and *S. sanguis.* The occurrence of *erm*B-like genes in enterobacteria was confirmed in other *E. coli* strains and in *Klebsiella*. The findings indicate that the gene pool of gram-positive cocci has extended to gram-negative bacteria, that such transfer can occur in nature and that the extension is recent. Further inferential evidence of such trans-gram promiscuity exists for other resistance genes (for a review see Courvalin, 1994).

4.4.3   Co-transfer of genes and multiresistance

A plasmid or transposon may gradually acquire one gene after another. Integrons and transposons appear to be largely responsible for this phenomenon. When they are located on the same transferable element, the transmission of different genes is linked and transfer leads to co-resistance. This means that a bacterium concurrently becomes resistant to two or more different antibacterials, mediated by different resistance mechanisms,

governed by different genes. Co-transfer, linked transfer or transfer *en bloc* are different expressions for this phenomenon.

The transmission of resistance genes with relevance for AFA can be linked to the transfer of other genes. The *E. coli* plasmid pNV13 conveys resistance to carbadox, streptomycin, spectinomycin and ampicillin (Ohmae *et al.*, 1981; Ohmae *et al.*, 1983). Conjugal transfer of resistance to streptogramin A, between *S. aureus* strains, linked to lincosamide, trimethoprim and penicillin resistance has been reported (Allignet and El Solh, 1995; Allignet *et al.*, 1996). Enterococcal plasmids from several host species have been shown to harbour and transfer MLS_B, kanamycin, streptomycin and, sometimes, tetracycline resistance (Rollins *et al.*, 1985; LeBlanc *et al.*, 1986; Christie *et al.*, 1987). The transposon Tn*1545* has been shown to carry and transfer resistance genes to kanamycin, macrolides and tetracycline (Doucet-Populaire *et al.*, 1991). Genes conveying resistance to glycopeptides and macrolides have been found to co-reside on plasmid pIP819 (Leclercq *et al.*, 1989). Other examples of co-transfer, have been given in 4.4.1.

A similar linkage between different genes has been shown between some resistance and virulence genes, such as haemolysin- or toxin-encoding genes. Co-transfer of virulence and resistance genes has been well studied in enteric pathogens (among others Franklin and Möllby, 1983). Less information is available concerning gram-positive bacteria. Christie and co-workers (1987) demonstrated *in vitro* co-transfer of genes encoding for macrolide and tetracycline resistance and haemolysin production from *E. faecalis* to *Bacillus subtilis*. *In vivo* transfer of pheromone-responsive plasmids carrying genes conferring cytolytic properties and macrolide resistance (*erm*B) between strains of *E. faecalis* has been studied in a model using Syrian hamsters (Huycke *et al.*, 1992). Transfer occurred in high frequencies (10^{-1} - 10^{-2} per donor). Even more worrying is the transfer *in vitro* of macrolide resistance (*erm*BZ) and toxin A, apparently carried on a transposon (Tn*5398*) from *C. difficile* to non-toxigenic *C. difficile* strains and to *B. subtilis,* reported by Mullaney (1995).

Consequently, a selection for antibacterial resistance may, in some cases, also select for virulence. The danger of transfer elements carrying multiple genes is evident; a single transfer event conveys not only resistance to the selector but also other properties to the recipient bacterium. The recipient may acquire multiresistance and/or increase its virulence. Any advantage given by one of the transferred genes helps to maintain all of the transferred genes in the new host.

4.4.4   Resistance genes and animal hosts

Highly homologous resistance genes and their associated transfer elements have been found in natural isolates not only of different bacterial species but also in bacteria from a variety of host species. The macrolide resistance gene

*erm*B has been demonstrated in bacteria of different species originating from pigs, chickens, cattle, dogs and humans (Rollins *et al.*, 1985; Arthur *et al.*, 1987; Stuart *et al.*, 1992; Eady *et al.*, 1993; Roberts and Brown, 1994; Wasteson *et al.*, 1994).

As an example, the results of Eady and co-workers (1993) who screened for resistance determinants in macrolide resistant isolates of staphylococci (excluding *S.aureus*) from humans, pigs and dogs are shown in table 4.VII.

Table 4.VII. Distribution of macrolide resistance genes in staphylococci from various hosts given as percent of total number of genes identified in isolates from respective animal host[1] (modified from Eady *et al.*, 1993)

Tabell 4.VII. Fördelningen av makrolidresistensgener hos stafylokocker från olika värddjur uttryckt som procent av totalantalet identifierade gener[1] (modifierat från Eady et al., 1993)

Gene[1]	Swine	Dogs	Humans	
			group 1[2]	group 2[2]
*erm*A	0	6	12	6
*erm*B	20	65	0	0
*erm*C	63	6	41	54
*msr*A	6	6	38	39
erm untypable	11	18	9	2

[1]No. of genes identified and No. of investigated isolates: swine 35 and 33, dogs 17 and 16, humans group 1 58 and 55, humans group 2 121 and 117, respectively.
[2]Group 1 includes isolates from patients undergoing ambulatory peritoneal dialysis (n=27) and from blood cultures (n=28); group 2 includes patients with acne (n=117).
[1] *Antal identifierade gener respektive antal undersökta isolat: svin 35 och 33, hundar 17 och 16, människor grupp 1 58 och 55, människor grupp 2: 121 och 117.*
[2] *Grupp 1 inkluderar isolat från patienter i ambulatorisk peritonealdialys (n=27) samt blododlingar (n=28); grupp 2 inkluderar patienter med akne (n=117).*

The suggested spread of vancomycin resistance between enterococci of various origin is another example of the spread of resistance among gram-positive bacteria. VRE harbouring the *van*A gene cluster have been isolated from humans, both in hospitals and community, from pig, rabbit, dog, cat, horse chicken, turkey, pheasant, duck, food of animal origin and sewage (Bates *et al.*, 1994; Torres *et al.*, 1994; Klare *et al.*, 1995b; Chadwick *et al.*, 1996; Devriese *et al.*, 1996; DANMAP, 1997). A polyclonal nature of the VRE strains has been demonstrated (Klare *et al.*, 1995b). The *van*A gene cluster consists of 7 gene components and two transposition gene sequences. It is extremely unlikely that such a complicated gene should have developed separately in so many different host populations. Its occurrence therefore strongly suggests a spread between bacteria of different host species.

4.4.5   The encumbrance of resistance for bacteria

In an environment that contains antibacterials, possession of a corresponding resistance gene is clearly beneficial for the bacterium. The resistance traits have, however, been considered to impose a burden on their bacterial hosts. Synthesis of plasmids require energy and metabolites. The products encoded for by resistance genes may interfere with the bacterium's physiology, alterations of the cell wall may lead to loss of adhesive properties and alternative metabolic pathways may be less efficient. Constitutive expression of resistance genes encoding for such traits would be a handicap in the absence of antibacterials. For such reasons, resistant clones have been thought to suffer a competitive disadvantage in the absence of a selective pressure. Even so, this might not always be the case. Much evidence of the cost for the bacterium of harbouring the resistance trait has been obtained in laboratory experiments with bacteria that have recently acquired the resistance gene.

Other experiments show that, given time, bacteria can eliminate the cost of resistance by adapting their system in order to counteract the harmful side-effects of the resistance genes. This will result in increased fitness of resistant strains with consequent persistence of the resistance trait in the microbial population even after the selective pressure is removed. Such adaptation has been shown for resistance acquired through mutations as well as through plasmids (for reviews see Lenski, 1996; Gillespie and McHugh, 1997). As an example, McConnell and co-workers (1991) demonstrated the need of a selective pressure for the establishment of a resistance plasmid in the microflora of mice. However, once established, the strain originally carrying the plasmid adapted and competed well with susceptible strains in the absence of a selective pressure.

4.4.6   Some remarks on horizontal transmission of genes

Transmission of genes between bacteria occurs readily *in vitro*. Experimental transfer of a conjugative element may require little manipulation besides the mixing of two cultures. The rates, or frequencies, of such transmissions will depend on the transfer element involved and the donor-recipient combination as well as on the conditions of the transfer experiments. In this respect it should be noted that failure to demonstrate transfer does not mean it cannot take place, simply that it did not occur under the conditions of the experiment. On the other hand, demonstration of transfer indicates that the event can take place.

Bacteria are equipped with very efficient gene transfer systems. Similar, or identical, resistance genes and associated transfer elements are present in field isolates from different host species as well as in different bacterial

species. A multitude of transfer elements are found in bacteria isolated from natural settings. Resistance traits arise within species that were formerly considered uniformly susceptible.

The experiments demonstrating *in vivo* transfer in laboratory animals have been conducted to test a hypothesis based on firm observations from "real life" of emerging resistance. An example of this is the studies on macrolide and tetracycline resistance in *Listeria monocytogenes* cited in 4.4.1. The settings are, albeit artificial, certainly closer to the real situation than the test tube.

The opinion that the transfer rate in nature would be significantly lower than what is obtained *in vitro* is based on the assumption that laboratory settings provide optimal conditions, and that such conditions are rare in real life. This is not necessarily true. The laboratory conditions normally used to test DNA transfer employ rapid growth, aerobic conditions and nutrient excess. Such conditions might enhance transfer rates, but it might also repress gene transfer in some bacteria (Salyers, 1995). Further, the event does not have to be frequent in order to have an effect. Once the resistance gene is present in a recipient, given a selective pressure the resistant clone will multiply.

Taken together, it is evident that transfer of genes does occur in nature (in/on living hosts or in their environments). The question that remains is how often it occurs.

Resistance mechanisms and resistance genes have only been investigated for AFA belonging to classes containing therapeuticals.

Described resistance genes are generally associated with transfer elements. Transfer of resistance has been shown experimentally both *in vitro* and *in vivo.*

Transfer of resistance has been shown between bacteria of different genera.

The same resistance genes are found in bacteria from different host species.

Co-transfer of multiple genes is common.

Resistant bacteria are not necessarily less competitive in the absence of a selective pressure.

4.5   Transfer of resistance between various host species

There are two theoretically possible ways for transmission of antibiotic resistance from bacteria of one host species to those of another; firstly, direct

transmission of resistant bacteria, and secondly, transmission of resistance genes between bacteria colonising different host species. The debate has mostly been concerned with transmission of resistant bacteria from animals (taken as a group) to man. It has been questioned whether such transmission can occur. Logically, if transfer did not occur between bacteria from a specific animal species to man, then transfer between different animal species would not be expected to occur either. After all, humans are the only species in constant contact with all the other species.

4.5.1   Transmission of bacteria

Studies concerning transmission of resistant bacteria from animal to man have mainly focused on gram-negative enteric pathogens such as *Salmonella* spp. and *Campylobacter* spp. For antibiotic resistant salmonellae, the pathways from animals to man and subsequent infections have been clearly demonstrated (Bezanson *et al.*, 1983; Holmberg *et al.*, 1984; Spika *et al.*, 1987; Mishu *et al.*, 1991). Several reports suggest the transfer of other antibiotic-resistant enteropathogens such as quinolone-resistant campylobacter (Endtz *et al.*, 1991) and chloramphenicol-resistant *Yersinia enterocolitica* (Perez Trallero *et al.*, 1988).

Transfer of *E. coli* between various animals and humans has been demonstrated in various studies. The spread of a labelled multiresistant *E. coli* from cow to pig, mouse, fowl, fly, water and human, and from pig to fly, mouse, fowl, water and human was reported by Marshall and co-workers (1990). Levy (Levy *et al.*, 1976) reported the transfer of a labelled multiresistant *E. coli* from chicken to a farm worker. Linton and co-workers (1977) demonstrated the transfer of sulphonamide-resistant *E. coli* from a chicken carcass to the faecal flora of five human volunteers handling the chicken.

Although the above cited studies concern resistance to antibacterials not used as AFA in the EU, they demonstrate that bacteria can be transmitted from animals to man directly, via food, or by other routes and in the case the bacterium carries resistance this trait will not disappear along the line. Transfer of gram-positive bacteria has been less studied. Hummel and Witte (1981) found macrolide-resistant *S. aureus* of human biovariant in farm workers and in occasional pigs on farms using in-feed tylosin and tetracycline. This seems to indicate a human to animal transmission. As staphylococci are known to be relatively host specific, it is likely that the finding was merely the result of a transient colonisation. Interestingly, no macrolide-resistant strains were found among the family members of the farm workers, indicating the need for a selective pressure for transferred bacteria to persist in the new host. In the case of the farm-workers, they were likely to be exposed to tylosin through dust.

Direct transmission from chicken to man has been implicated in a recent report on a case of wound infection with a vancomycin resistant *E. faecium* in a worker at a chicken packaging plant (Das *et al.*, 1997). Available information strongly suggests that the infection was occupationally derived (contracted from chicken carcasses).

Human *E. faecium* strains have been widely used as probiotics and do, at least transiently, colonise animal intestines. Further, human VRE have successfully been used to colonise mice experimentally (Whitman *et al.*, 1996). This indicates that at least certain enterococcal strains can colonise, or transiently inhabit, a variety of hosts. Another report on occupational exposure provides further evidence (van den Bogaard *et al.*, 1997). The prevalence of VRE in turkeys, turkey farmers, turkey slaughterers and urban residents was found to be 50%, 39%, 20% and 14%, respectively. Further investigations showed that VRE isolated from one of the farmers and his turkeys could not be differentiated by phenotypic or genotypic (pulsed-field gel electrophoresis) methods. Investigations of the *van*A-gene cluster by polymerase chain reaction (PCR) and hybridisation showed the two strains to be identical in the tested areas, having an insertion in a previously nondescribed position, between the *van*X and *van*Y gene, and a deletion in the right end of the cluster.

4.5.2   Transfer of genes

The majority of the bacteria in the animal intestinal microflora are relatively host specific and would not be expected to colonise other animal species or man. Similar restrictions do not apply to the bacterial host range of resistance genes. Therefore, transfer of genes between animal and human bacteria is more likely to have an impact on interspecies exchange of resistance. Transfer of resistance genes between animal and human microflora has been shown in several studies. Again, many of those deal with resistance genes related to therapeutical antibacterials.

However, in the light of the close relatedness between resistance genes in human and animal bacteria and the current knowledge of gene transfer mechanisms and genetic drift, it would be naïve to presume that there is no exchange of resistance genes between animal and human microflora. On the contrary, this is the most, if not the only, plausible explanation for the spread of antibacterial resistance against both therapeuticals and AFA in various populations. The majority of the studies addressing this topic concern resistance in gram-negative bacteria against therapeutic antibacterials. Nonetheless, they illustrate the fact that transmission of genes occurs between animal and human bacteria.

Resistance genes identified in both animal and human bacteria

Levy and co-workers (Levy *et al.*, 1976) reported spread of an *E. coli* multi-resistance plasmid between chickens and from chicken to man.

The spread of streptothricin resistance from animal to human bacteria has been documented in former Eastern Germany. A streptothricin antibiotic, nourseothricin, was commonly used in pig feed from the beginning of the 80s. A novel transposon-encoded resistance mechanism was identified in *E. coli* from pigs in 1983. Subsequently, this transposon, Tn*1825*, has been found in the normal microflora of pig farmers and their families and of healthy unrelated adults in the community, and in urinary tract infections in humans. Finally, the resistance determinant has been detected in salmonella and shigella from human cases of diarrhoea (Hummel 1986, Tschäpe 1994). Streptothricin antibiotics are not used in humans. In areas where the antibiotic was not used in animals, the resistance determinant was not found.

Another example is the spread of aminoglycoside-acetyl-transferase IV (apramycin resistance) and hygromycin B phosphotransferase (hygromycin B resistance). The corresponding genes, *aac*C4 and *hph*B, form part of one resistance gene operon associated with an insertion sequence IS*140,* probably part of a transposon, that is found on plasmids. The organisation of *aac*C4 and *hph*B is such that they are always co-transferred. Apramycin and hygromycin B are used exclusively in animals. The gene *aac*C4, conferring cross-resistance to gentamicin, was first identified in *E. coli* and *Salmonella typhimurium* from animals in France and the United Kingdom (Chaslus-Dancla *et al.*, 1986; Wray *et al.*, 1986). It has since been demonstrated in various enterobacteria, including salmonella, from human as well as environmental sources in different countries (Threlfall *et al.*, 1986; Chaslus-Dancla *et al.*, 1989; Hunter *et al.*, 1993; Hunter *et al.*, 1994). A high degree of genetic homology between plasmids harbouring *aac*C4 and *hph*B of human and animal origin has been demonstrated (Salauze *et al.*, 1990; Chaslus-Dancla *et al.*, 1991). In a prospective study on a pig farm, Hunter (1994) demonstrated a widespread dissemination of plasmids harbouring *aac*C4 in *E. coli* from pigs, calves, the stockman and a variety of environmental sources including rainwater puddles and water from a stream nearby. *Klebsiella pneumoniae* with a slightly smaller conjugative plasmid and similar resistance pattern was isolated from the stockman's wife.

As mentioned in 4.3.4, investigations concerning antimicrobial resistance tend to focus on a limited range of microbes. Few investigations concerning transfer of genes between the predominant, anaerobic, part of the microflora of man and animals are available. Nikolich and co-workers (1994) examined the possible gene transfer between microflora of ruminants and man, by using the *tet*Q gene, conveying tetracycline resistance, as indicator. By DNA sequencing of the gene from *Bacteroides* spp. and *Prevotella intermedia* of human origin and *Prevotella ruminicola* of bovine origin, they showed

identical, or nearly identical, gene sequences in bacteria of the two hosts from different geographic origin. The findings indicate that extensive transmission of the *tet*Q gene has occurred in nature between bacteria normally colonising different hosts and that the transfer is recent.

No similar investigations with direct relevance for AFA used in Europe have been found, nor studies concerning dominant microflora of other animal species. *In vitro* co-transfer of macrolide and tetracycline resistance between *Bacteroides* spp. and *Prevotella ruminicola* has been shown (Shoemaker *et al.*, 1992), indicating a strong possibility of similar *in vivo* transfer events related to macrolides and streptogramins. Further, successful transfer of *erm*F genes from *Treponema denticola* to *Enterococcus faecalis* has been demonstrated (Roberts *et al.*, 1996a)

The question of "identity" of genes has been a matter of debate in relation to the discussion on the use of avoparcin in animal husbandry. It is important to point out that the bacterial genome is subject to changes over time. Mobile gene-sequences may insert into or close to non-essential parts of a gene, and deletions or point-mutations may take place. Consequently, the sequence of a specific gene may differ from one point in time to another.

The *van*A gene cluster contains 9 genes (7 *van* and two transposition genes). Between those genes are intergenic, non-coding regions. The coding regions would be expected to be highly conserved once their sequences are optimal for function. As the intergenic regions are not essential for the function of the gene cluster, they are more likely to vary. Several recent studies have addressed the matter by amplification by polymerase chain reaction (PCR) and sequencing of the genes and/or their intergenic regions (Jensen, 1996; Haaheim *et al.*, 1997; Kirk *et al.*, 1997).

In a study from Norway (Haaheim *et al.*, 1997), PCR for the *van*A and *van*B genes combined with restriction fragment analysis of a long PCR covering the entire gene cluster and sequencing of the intergenic *van*S-*van*H region were used to analyse the vanA gene cluster from VRE of Norwegian poultry and humans of various nationality (Swedish, Norwegian and American). In 9/12 human isolates and 7/10 poultry the results were identical, indicating horizontal transfer of the gene cluster.

Kirk and co-workers (1997), investigated 37 VRE isolates from one UK hospital and 36 VRE isolates from poultry meat bought in national supermarkets. By PCR, three intergenic regions and three genes of the *van*A-cluster were investigated (*van*S-*van*H, *van*X-*van*Y, *van*Y-*van*Z, *van*X, *van*Y and *van*Z) In the chicken isolates, all three investigated genes were amplified as well as the three intergenic regions. Evidence of a not previously described insertion sequence in various locations of the intergenic region between *van*X and *van*Y was found in some of the chicken isolates. The gene sequences of the strains from humans in this study were clearly atypical.

The focus of a Danish study, reported by Jensen (1996), was slightly different. In order to investigate the degree of variation within the *van*A gene cluster, isolates from different animals and humans from a wider geographic range were investigated. Similar to Kirk and co-workers (1997), Jensen found evidence of an insertion sequence in the *van*X-*van*Y region in 7 of 12 British human isolates. Based on sequencing of coding and non-coding regions, the remaining isolates could be divided into 3 groups, each containing isolates both from man and animals from different countries. The designation of one of the groups was based on the presence of a point mutation in *van*X and an insertion sequence (IS*1216V*) in a specific position in the transposon (Tn*1546*). The group contained isolates from humans (Denmark and USA) and pigs (Denmark and UK). Mutations within the coding regions appear to be rare. Insertion sequences are highly mobile, and frequently vary in their location. The occurrence of an insertion sequence in the same location in strains of different origin could either be interpreted as evidence of an epidemiological relationship, or as the site being a "hot spot" for insertion of the specific sequence. However, the likelihood of both a point mutation and an insertion in a specific location occurring independently in different strains is extremely low. Therefore, the genes present in those isolates must be very closely related and their presence the result of horizontal transfer.

Experimental transfer studies

Lacey (1980) examined the possibility of transfer of macrolide resistance between animal and human isolates of *Staphylococcus aureus*. Transfer by phage conjugation from human to animal isolates was shown at low frequencies (10^{-8}-10^{-9}) for some of the isolates (see comments on transfer rates under 4.4.6 and 4.5.4). No transfer from animal to human isolates could be demonstrated. The genetic nature of the resistance traits for which transfer was attempted is not known. The same author also investigated transfer of resistance between animal and human streptococci, including *Streptococcus* group D (now *Enterococcus*) (Lacey, 1984). In about 10% of the animal strains investigated, transfer to human recipients was demonstrated. Transfer of resistance occurred more readily from the enterococci. No transfer to group A or group C streptococci was demonstrated. The most common transfer frequencies were between 10^{-6} and 10^{-8}. However, the experiments were conducted in broth, filter matings being used only for isolates that had failed to transfer initially. Under optimal conditions, the transfer rates would be expected to be considerably higher (by up to 3 logarithms). Further, the experiments also failed to demonstrate conjugative transfer from human enterococci to human group A or group C streptococci. Such transfer has, contrary to the statement of Lacey, been demonstrated by other authors

(Malke, 1979; Horodniceanu *et al.*, 1981; Ravdonikas, 1983; Horaud *et al.*, 1985). This indicates that the conditions of the experiment may not have allowed optimal transfer rates.

4.5.3   Routes of transmission - food as a vehicle

Direct contact with animals carrying resistance genes provides excellent opportunity for uptake of these genes. Examples of farmers carrying the same resistance genes in their microflora as do their animals have been given in 4.5.1 and 4.5.2. Logically, this transfer works both ways, resistance genes may also spread from humans to animals. However, as animals are in many ways part of the human food chain, transfer from animals to humans would be expected to occur more easily.

Enteric pathogens are readily transmitted through foods as are antibiotic resistant pathogens and commensals. It has been suggested that, in the normal human population, most resistant enterobacteria in faeces come from contaminated food (Corpet, 1988). In an experiment (Corpet, 1988), 7 healthy volunteers were given a control diet for 3 weeks followed by a sterile diet for 2.5 weeks. During both periods, total and antibiotic resistant *Enterobacteriaceae* in stools were counted. A drastic drop in faecal concentrations of antibiotic resistant enterobacteria was observed during the sterile diet period ($p<0.05$).

Antibiotic resistant gram-negative bacteria are present in animal products as well as on vegetables and fruits (Levy, 1983). Antibiotic-resistant gram-positive bacteria have also been isolated from various foods (Roosen *et al.*, 1989; Franco Abuin *et al.*, 1994; Perreten and Teuber, 1995; DANMAP, 1997; Perreten *et al.*, 1997; Wegener *et al.*, 1997). Moreover, various transferable resistance genes, including *van*A-genes in enterococci and *erm*-genes in *Listeria monocytogenes*, have been found in gram-positive bacteria isolated from food (Bates *et al.*, 1994; Aarestrup, 1995; Klare *et al.*, 1995a; Perreten and Teuber, 1995; Roberts *et al.*, 1996b; Perreten *et al.*, 1997).

Most foods are heat treated before consumption and hence, no viable resistant bacteria would be expected to be present in the final product. However, food-borne infections with infectious doses as high as 10^6-10^9 (as for salmonellosis) are relatively common. This proves that recontamination is common and viable bacteria can be present in relatively large numbers in food when consumed. Certain bacteria, such as *E. faecium* may also have an increased heat tolerance (Panagea and Chadwick, 1996).

The origin of these resistant bacteria is difficult to determine but there can be no doubt that they at least partly originate from contamination of meat and vegetables with animal bacteria. Exchange of resistance genes between bacterial species has been demonstrated in water, soil, on kitchen towels, on cutting boards, and on the surface of food (Kruse and Sørum, 1994).

Epidemiological studies comparing the prevalence of antibiotic resistant bacteria in vegetarians and meat-eaters are of limited value for quantifying the spread of bacteria from animals to man via the food chain. Vegetables are likely to become contaminated by animal microbes through manure. The higher prevalence of resistance in vegetarians found in some studies is therefore not surprising as vegetables are less often heat treated, as compared to meat.

4.5.4   Likelihood of resistance being transferred from animals to man

It has been proposed that both types of transfer discussed above are extremely rare events (Knothe, 1977; Lacey, 1980; Lacey, 1984; Lacey, 1988; Shah *et al.*, 1993). The basis for this opinion is the rarity of isolation of the same bacteria from animals and man (Lacey, 1988; Shah *et al.*, 1993), the rarity of gene transfer *in vitro* between human and animal isolates of some bacterial species (Lacey, 1980; Lacey, 1984) and lack of association between drug consumption in veterinary medicine and resistance patterns in human pathogens (Knothe, 1977). Acknowledging the logic of these arguments, they are mainly based on observations on staphylococci and to a lesser extent streptococci and enterococci. Staphylococci and streptococci are indeed rather host specific, and transfer frequencies between staphylococci are, compared to other genera, rather low irrespective of host origin. Nevertheless, it is doubtful whether the finding of a low number of animal isolates capable of transferring resistance to human isolates, and vice versa, in the laboratory at a frequency in the order of 10^{-6}-10^{-8} (Lacey, 1980; Lacey, 1984) under suboptimal conditions should be regarded as proof of a rare event. Considering the vast amount of bacterial subpopulations present in the intestinal flora, even a comparatively low transfer frequency may result in a large number of successful transfer events. The rarity of macrolide resistance in *Campylobacter* spp. isolated from humans has also been pointed out (Lacey, 1988). The species distribution of the bacterial isolates investigated was not given, but as the vast majority of human isolates are *C. jejuni,* this species can be assumed to be the predominant in the material. It was stated that in animal husbandry, macrolides are mainly used as AFA in pigs. As the main animal host of *C. jejuni* is poultry, prevalence of macrolide resistance in this bacterial species provides little information about the effects of macrolide use in pigs. In *C. coli*, which is mainly found in pigs, a high prevalence of macrolide resistance has been reported (Wang *et al.*, 1984; Burridge *et al.*, 1986; Jacobs-Reitma *et al.*, 1994; DANMAP, 1997). This has also been shown in *C. coli* isolated from humans (Reina *et al.*, 1992).

   As stated by Lacey (1988), it is known that animal bacteria can colonise man, at least transiently, and that resistance genes can be transferred between animal and human bacteria, but the frequency of these events in nature is unknown.

Antibiotic-resistant bacteria and transferable resistance genes in food are ingested by humans.

Transmission of resistant bacteria between host species occurs, although depending on bacterial species the resulting colonisation may be transient.

Transmission of resistance genes can and does occur in a variety of surroundings.

The question that remains is how often successful transfers cause clinical problems.

4.6 Epidemiology of antibacterial resistance

The causal relationship between use of antibacterials and resistance has been shown both for human and animal bacteria (among others Richmond, 1972; Levy *et al.*, 1976; Seppälä *et al.*, 1997). In evolutionary terms, exposure to antibacterials exert a selective pressure on bacterial populations, giving bacteria with advantageous traits a competitive advantage (survival of the fit).

All factors influencing the usage of antibacterials and/or the spread of infectious agents will also affect the emergence of resistance. In human medicine, such factors are; changes in human demographics and behaviour, changes in technology and industry, economic development and land use, international travel and commerce, microbial adaptation and change and breakdown of public health measures (Cohen, 1996). Corresponding factors are known to influence epidemiology of contagious diseases in animals. Apart from the two latter, these factors all influence the degree of contact between individuals, either by affecting the population density or by providing new contact routes. This emphasises the similarity between spread of resistance and spread of infectious diseases.

4.6.1 Reservoirs - the gene pool

As noted in 4.3.4, dissemination of resistance requires both presence of the antibacterial in concentrations high enough to inhibit growth of normally susceptible bacteria and presence of resistance genes (Levy, 1996). This spread involves a variety of commensals and environmental bacteria (see 4.3, 4.4 and 6.4) which act as reservoirs.

In order to create a problem, or even to be noticed, the resistance genes have to pass from the reservoirs into clinically relevant bacterial hosts. The size and accessibility of the reservoir available will determine the likelihood of such events. The prevalence of resistance genes (i.e. the size of the

reservoir) will primarily depend on the selective pressure applied in different microbial habitats. In most surveys on antibacterial resistance, only potential pathogens are included and thus, the bulk of the resistance gene pool will go unnoticed.

4.6.2   Risk factors for spread of resistance

The kinetics of resistance spread in bacterial populations will be dependent on the total time and degree of exposure to risk factors, and on the sizes and numbers of populations exposed (Levy, 1996).

The selective pressure

The main risk factor for increased resistance is, as mentioned, exposure of bacteria to the specific antimicrobial under study. The selective pressure can be defined as the product of exposure dose and exposure time. The importance of time is illustrated by the investigations by Garber (1989) on the association between antibacterial use and the nosocomial spread of infections with resistant gram-negative bacteria. By using a mathematical model, he found that the risk of an individual acquiring an infection with a resistant strain was directly proportional to the length of time the patient was treated with the drug in question. Further, for some antibacterials, the risk for such infections also increased for non-treated individuals in the same ward.

   The number of bacteria exposed is important, but even more so the number of separate bacterial subpopulations. Orally administered antibacterials will exert a larger selective pressure than for instance if locally applied on the skin, as the intestine contains a larger number of various bacterial species (Salyers, 1995).

   The proportion of individuals exposed in a subpopulation, as well as the number of subpopulations exposed, is of importance for the spread of resistance between the microflora of individuals.

   When antibacterials are used as AFA, they are administered for a long period of time, and usually to all individuals in a group or even herd. As discussed in 4.2, the doses given are certainly inhibitory enough to disrupt the initial balance between resistant and susceptible strains in the normal flora. With time, resistance will and does evolve (see 4.3).

Population factors

The spread of resistance genes between the microflora of different individuals depends on the number of direct or indirect contacts between the bacteria of the individuals. As for infectious diseases, the incidence of resistance genes will depend on size, and structure of the host population.

The degree of contact between individuals and subpopulations, population density, is also crucial (Schwabe *et al.*, 1977). As an example, studies on human skin flora show that sequential therapy for acne promotes the carriage of resistant staphylococci on the skin of close contacts (Miller *et al.*, 1996).

In animal husbandry, high stocking densities, frequent indirect contacts with other herds, frequent trading of animals, and a low level of hygiene will favour the spread and maintenance of resistance traits. Other factors such as feeding practices, management, temperature, humidity and light intensity may influence the composition of the bacterial population and thus, to some extent, influence the incidence of resistance.

Individual factors

Transfer of resistance and/ or colonisation with resistant strains appears to occur more readily in the intestine of young animals as compared to adults (Langlois, 1988). McConnell and co-workers (1991) found, in previously cited experiments (see 4.4.1), that transfer of a resistance plasmid could only be detected in the offspring of the mice originally inoculated. The authors suggested that this might be correlated to the differences in the composition of the intestinal flora between suckling and adult mice. This was thought to affect the donor-recipient ratio. Other explanations might be that colonisation by resistant bacteria is favoured in young animals. This may be ascribed to factors favouring adhesion, including less competition from established microflora (Corpet, 1986).

Individual features of the combination of bacterial species and specific antibacterial involved also play a role. For example, penicillin resistance developed rapidly in human *Staphylococcus aureus* following the introduction of penicillin, whilst in group A streptococci, such resistance has not yet been demonstrated in spite of 4 decades of use of the drug. On the other hand, group A streptococci seem to readily acquire resistance to macrolides (Hamilton-Miller, 1990; Seppälä *et al.*, 1997). Certain genera, such as enterococci, seem to have a high capacity for both acquisition and transfer of resistance (Murray, 1990).

4.6.3   Persistence of resistance

Field observations indicate that the relation between antibacterial use and resistance is not as clear-cut as it may seem (Leak *et al.*, 1986; Langlois, 1988). Whether resistance would regress in the absence of antibacterials depends, among other things, on the availability of susceptible strains to replace the resistant ones. The importance of the environmental reservoir is illustrated by some experiments by Levy (1976) in which chickens excreting multiply resistant *E. coli* were studied. Despite repeated cleaning of the

cages, over several months, the resistance level did not decline. However, when the cages were relocated to different sites, the flora slowly returned to more susceptible levels. This was interpreted as the first environment lacking susceptible strains to replace the resistant ones.

The concept that susceptible strains will replace the resistant ones once the selective event has passed relies on the idea that resistant strains will be at a disadvantage once the antibacterial is withdrawn. This might not always be the case, as a long-term selective pressure can lead to bacterial adaptation (see 4.4.5).

Another factor to be considered is co-selection (see 4.4.3). Due to this phenomenon, exposure to factors other than the substance in question might help sustain antibacterial resistance within a bacterial population.

4.6.4 Some comments on epidemiology

While each single use of an antibiotic has only a small effect on the probability of a resistant strain developing, the consequences add up across all other users in the community. Logically, the selective pressure within a dense population, such as patients in a hospital or animals in a herd, is the major factor contributing to amplification of resistance genes within that particular population. However, very few populations of either animals and humans are isolated from others and transmission of resistance genes can easily occur between groups. This is illustrated in the findings of Linton (1988 see 4.3.1). A resistance gene originating in an animal population may spread to a population of humans and persist there, under the selective pressure from antimicrobials used for human therapy (for example apramycin resistance, see 4.5.2), and vice versa.

Disproportions in the usage of antibacterials between different populations of humans or animals may cause "spill-over" of resistance genes to other populations Hypothetically, if a gene from animal bacteria, conferring resistance to virginiamycin, were to enter a hospital where regular streptogramin therapy had recently been introduced, the gene would be likely to spread within the hospital and soon become a problem. In this case, the animal population would have served as a reservoir for the resistance gene

subsequently maintained within the hospital population. This illustrates the fact that although the antimicrobial usage within a certain population contributes to most of the antimicrobial resistance within that population, other populations serve as reservoirs for resistance genes.

Commensals and environmental bacteria act as reservoirs for resistance genes.

In the presence of antimicrobials, resistant bacteria will have a competitive advantage.

The effects of the selective pressure on bacterial populations will depend on the exposure dose and exposure time, and on the sizes and numbers of populations exposed.

If susceptible strains are available in the environment, these may replace the resistant ones when the selective pressure is removed.

Bacterial adaptation may result in increased fitness of resistant strains.

Factors influencing the usage of antibacterials and/or the spread of infectious agents will also affect the emergence of resistance.

Microbiota of animals serve as resistance gene reservoirs for human bacteria and vice versa.

4.7   Effects of an increase in resistance

Presence of resistance genes in the human or animal microflora is not in itself a problem. The problem arises when bacteria causing a disease withstand antibiotic therapy.

For substances that are used both as AFA and for therapy or prevention (tylosin, spiramycin, virginiamycin, olaquindox, carbadox) the consequences for animals of resistance in animal pathogens are obvious. As an example, for important swine pathogens such as *Serpulina hyodysenteriae* and *Lawsonia intracellularis* tylosin or tiamulin are the drugs of choice (Fellström, 1996; McOrist *et al.*, 1996; McOrist *et al.*, 1997). Tylosin resistance in *S. hyodysenteriae* is already widespread, leaving tiamulin as the only available alternative (Gunnarsson *et al.*, 1991; Buller and Hampson, 1994; Molnar, 1996). In table 3.III (chapter 3) some indications for treatment and prophylaxis with substances also used as AFA are listed.

In human medicine, macrolides, streptogramins and glycopeptides all have important indications such as respiratory infections, mycoplasmas, chlamydiae (macrolides only) and other infections with staphylococci, streptococci enterococci and clostridia, (see annexes D and E). For some of

these indications, other drugs might be the first choice but substances in the groups listed constitute valuable second- or even last-resort choices. For example, in infections such as streptococcal infections (common in children), penicillins are generally considered first choice. When this is not an option, for instance due to penicillin allergy, macrolides are often used (Huovinen *et al.*, 1996). Macrolide resistance in streptococci is currently regarded as an emerging problem (Coranaglia *et al.*, 1996).

An increase in resistance against these drugs may lead to therapeutic failures and will substantially diminish the available therapeutic arsenal. As long as there are alternative drugs available, the situation can still be managed, although possibly at a higher cost.

The relative importance of the different therapeutical substances is subject to change over time. Vancomycin became a last resort antibiotic as a consequence of the spread of multiresistant staphylococci in hospitals. Emergence of resistance is one explanation, but also that new pathogens emerge due to a combination of technological and societal changes (Cohen, 1992).

Some potential alternatives have not yet been introduced into human medicine. Old, underexploited antibiotic classes such as orthosomycins, phosphoglycolipids and elfamycins as well as antimicrobial peptides all have modes of action that make them interesting as templates for therapeutic substances (van den Bogaard and Stobberingh, 1996; Chopra *et al.*, 1997). Cross-resistance between old and new substances of the same class is to be expected. The most recent examples of this are quinpristin-dalfopristin and everninomycins. Quinpristin-dalfopristin is a streptogramin now launched in human medicine for the treatment of infections with vancomycin resistant bacteria, and everninomycins, an orthosomycin compound, is a promising candidate for the same area (Nicas *et al.*, 1997). Unfortunately, as these agents are widely used as AFA, their lifespan as therapeuticals may be shortened by rapidly emerging resistance problems.

Although safe and effective new antibacterial substances may still be found, the efficacy of the older drugs is imperative in keeping fatal infections at bay.

> Resistance becomes a problem when bacteria causing a disease withstand antibiotic therapy.
>
> Some substances used as AFA are also used for therapy in animals and humans. Increased resistance to AFA may therefore cause clinical problems
>
> Other substances used as AFA may serve as templates for future drugs. An increase in resistance may shorten the life-span of the drugs.

4.8   Other effects on the microflora

The normal intestinal flora of healthy animals and humans does not cause the host any harm. On the contrary, it protects against colonisation with enteric pathogens. Intensive livestock production systems can, however, induce imbalance in this normal flora, thereby making way for enteric disease (McOrist, 1997). All exposure of the normal flora to antibacterials, for growth promotion or for therapy, will disturb its balance (Corpet, 1996). Depending on the antibacterial spectrum, different microbes will be suppressed. This may lead to a loss of the protective role of the normal flora, impairing resistance to colonisation. As many of the enteric pathogens are of zoonotic character and are known to cause foodborne infections, this might present a hazard for human health.

   If the antibacterial feed additive is effective against certain pathogenic bacteria, it would be expected to prevent intestinal colonisation by these organisms and perhaps even remove already established infections. In this case, however, feeding the antibacterial substance must be regarded as prophylaxis or therapy and cannot be categorised as growth promotion.

4.8.1   Salmonella

Concern over potential salmonella contamination of human food has led to the instigation of various measures for its control. Reports in the medical literature that antibiotics may prolong the course of salmonella colonisation has led to the scrutiny of the effects of AFA on salmonella in animals.

   The protective effect of the normal microflora against salmonella infection was demonstrated by Nurmi and Rantala in the early 70s (Nurmi and Rantala, 1973). As most AFA, apart from quinoxalines and perhaps flavomycin, are not active against salmonella, their presence in the intestine would be expected to favour rather than disfavour salmonella colonisation.

   The main concern regarding human health is contamination of the animal products. There are numerous ways by which salmonellae can contaminate animal products. Stress during transportation may lead to translocation of enteric bacteria with subsequent contamination of internal organs at slaughter. Faecal material on skin or from the intestine can spread to the meat of several carcasses along the processing line (Lillard, 1990). This problem is particularly important when chickens are slaughtered. Rigorous measures following Hazard Analysis Critical Control Points (HACCP) may reduce the problem.

   The important factor when assessing the overall risk of contamination of food is the total amount of salmonella organisms brought into the processing plant by animals (Jordon, 1990). Thus, the epidemiological unit of concern is the flock or herd.

Theoretical effects of AFA on salmonella colonisation could be a lowering of the infectious dose necessary to achieve colonisation, an increase in the amount of organisms shed by colonised animals or a prolonged duration of shedding by colonised animals.

A lowered infectious dose will increase the probability of the flock becoming infected by exposure to low doses of salmonella. Moreover, a lowered infectious dose will facilitate the subsequent spread within the flock.

Likewise, an increased amount of organisms shed will contribute to the spread of the infection within the flock. More importantly, animals shedding a large amount of salmonella organisms can have a substantial impact on the cross-contamination of carcasses at slaughter and on the spread to subsequent flocks through contamination of the environment.

A prolonged duration of shedding also contributes to the spread within the flock, as well as the proportion of animals shedding salmonella at slaughter.

A substantial amount of studies have been performed on the effects of AFA on salmonella colonisation. Most of these studies are, however, inconclusive and while some are excellent, many have considerable shortcomings regarding study design and conclusions drawn. As the study designs used are very different, it is almost impossible to compare the results of one study to those of another. Further comments on the different studies can be found in annexes A-F. In table 4.VIII an attempt has been made to summarise the results of some studies in this area. One must remember, though, that do not allow for comparisons.

Table 4.VIII. Results from various studies on the effects of AFA on No. of animals shedding salmonella.

Tabell 4.VIII. Resultat från olika studies avseende effekten av antibakteriella fodertillsatser på antal djur som utsöndrar salmonella

Study	Animal species	Serovar	AFA		Conclusions drawn[1]	Comment
Abou-Youssef *et al*, 1979	swine	*S.* Typhimurium	virginiamycin	➔	No statistical difference between medicated and controls	
Abou Youssef *et al*, 1983	chickens	*S.* Typhimurium	virginiamycin	➔	No statistical difference between medicated and controls	
Barrow, 1989	chickens	*S.* Typhimurium, *S.* Pullorum, *S.* Choleraesuis, *S.* Dublin, *S.* Arizonae	avoparcin	↗	Increased No. of birds shedding salmonella in medicated group	Dose-response relationship established for avoparcin
Dealy and Moeller, 1976	swine	*S.* Typhimurium	flavomycin	↘	Reduced duration and prevalence of salmonella shedding by medicated animals	Infecting organism susceptible to flavomycin
Dealy and Moeller, 1977	calves	*S.* Typhimurium	flavomycin	↘	Reduced duration and prevalence of salmonella shedding by medicated animals	Infecting organism susceptible to flavomycin
George *et al*, 1982	chickens	*S.* Typhimurium	flavomycin	➔	No statistical difference between medicated and controls	
Gustafson *et al*, 1981	chickens	*S.* Typhimurium	avoparcin, virginiamycin	➔ ➔	No statistical difference between medicated and controls	All birds also received monensin
Gustafson *et al*, 1983	chickens	*S.* Typhimurium	avoparcin	➔	No statistical difference between medicated and controls	Also tested effect of clean versus used litter

Table VIII. Continued

Tabell VIII. Fortsättning

Study	Animal species	Serovar	AFA	Conclusions drawn[1]		Comment
Hinton *et al*, 1986	chickens	*S.* Bredeney, *S.* Cubana, natural infection via feed	avoparcin, virginiamycin	?	Results inconclusive	All birds also received monensin
Hinton, 1988	chickens	*S.* Kedougo	avilamycin	→	No statistical difference between medicated and controls	
Humbert *et al*, 1991	chickens	*S.* Typhimurium	avoparcin, bacitracin, flavomycin, virginiamycin	→ → → →	No significant effect of AFA alone, interaction with CE treatment	All birds shed salmonellae, comparisons based on amount of organisms in faeces
Jacks et al, 1988	swine	*S.* Typhimurium	efrotomycin	→	No statistical difference between medicated and controls	Both quantity, duration and prevalence of shedding evaluated
Latour and Barnum, 1981	ducks	*S.* Typhimurium	bacitracin	↗ →	Increased prevalence in medicated group in 2 experiments, no statistical difference in 2 experiments	
Leuchtenberger, 1981	chickens	*S.* Typhimurium	avoparcin, tylosin, virginiamycin	↗ ↗ ↗	Increased prevalence and duration of shedding in medicated groups	
Linton *et al*, 1985	chickens	Various, natural infection via contaminated feed	avoparcin	→	No statistical difference between medicated and controls	All groups except one of the controls also received monensin
Manning *et al*, 1994	chickens	*S.* Enteritidis	bacitracin	↗	Increased prevalence of salmonella shedding in medicated group	All birds kept on used litter

Table 4.VIII. Continued

Tabell 4.VIII. Fortsättning

Study	Animal species	Serovar	AFA	Conclusions drawn[1]		Comment
Matthes et al, 1981	chickens	not given	avoparcin, tylosin, virginiamycin	↗ ↗ ↗	Prolonged salmonella shedding in medicated groups	Possibly identical to one of the experiments reported by Leuchtenberger
Nurmi and Rantala, 1974	chickens	*S*. Infantis	bacitracin	↘	Reduced prevalence and amount of salmonella shedding in medicated group	
Smith and Tucker, 1975	chickens	*S*. Typhimurium	bacitracin, flavomycin, tylosin, virginiamycin	→ ↗ ↗ →	Small or no increase in prevalence of shedding in groups fed virginiamycin or bacitracin. Larger increase in most groups fed flavomycin and tylosin	
Smith and Tucker, 1978	chickens	*S*. Typhimurium	avoparcin, tylosin	↗ ↗	Increased prevalence of salmonella shedding in medicated groups	
Smith and Tucker, 1980	chickens	*S*. Typhimurium, *S*. Heidelberg, *S*. Oranienburg, *S*. Infantis, *S*. Senftenberg	avoparcin, bacitracin	↗ →	Increased prevalence and duration of shedding in groups fed avoparcin. Little or no increase in groups fed bacitracin	Effect of infectious dose, poultry breed and feed type also investigated
Troutt *et al*, 1974	swine	*S*. Choleraesuis	carbadox	↘	Diminished clinical signs in medicated group	

[1] Arrows indicate conclusions as drawn by the authors regarding effect of AFA on colonisation; → denotes no difference, ↘ denotes a decrease, ↗ indicate an increase in shedding time and/or prevalence when medicated groups were compared to control groups

The interest in these studies has mainly been focused on prevalence and duration of salmonella shedding in experimentally infected animals. Most studies have been conducted for 40-50 days and evaluated at the end of the study period. Roughly, 18 experiments show non-significant results, 14 experiments indicate an increase in prevalence/duration of salmonella shedding among medicated animals, and 4 trials indicate a decrease in shedding by medicated animals (see table 4.VIII). Only one study (Barrow, 1989) established a dose-dependent response, to in-feed avoparcin.

Three studies (Nurmi and Rantala, 1974; Barrow, 1989; Humbert *et al.*, 1991) evaluate the amount of organisms shed. Two indicate a possible decrease in numbers shed by bacitracin-fed animals and two an increase in the amount shed by animals receiving avoparcin. However, none of these studies investigate the amount of organisms shed over time.

Regarding changes in the infectious dose, or increased susceptibility of infected animals, the prevalence of salmonella in the different experimental groups shortly after infection may give a hint. One study, by Smith and Tucker (1980) clearly showed, by dose-titration, a decrease (from 10^4 to 10^3) in the infectious dose necessary for colonising avoparcin-fed birds as compared to non-medicated birds.

Some substances do appear to affect the course of salmonella infection within an animal herd. Gustafson and co-workers (1981) used an inoculation dose optimised to achieve colonisation of about 50 % of the animals, which resulted in a faster spread of the infection, with an earlier peak of the epidemic curve, in birds receiving either avoparcin or virginiamycin, compared to the control group. At day 47, the prevalence of salmonella in samples from live birds was larger in the control group than in the medicated groups, but the prevalence of salmonellae in caecal contents after slaughter was larger in the medicated groups. This illustrates that there is sometimes a difference between the epidemic curves of the control group and the medicated group.

Comments on study design

One problem with many of the studies in the table above is that the groups of animals are too small. Population size and density are important factors influencing the spread and persistence of an infection within a herd (Schwabe *et al.*, 1977). Studies performed on small groups of chickens are not necessarily applicable to real poultry flocks with tens of thousands of birds.

The need for a study design as realistic as possible must be balanced against the need for a controlled environment. Practices vary in animal husbandry and what is a realistic study design in one part of the world may

be unrealistic in another area. For example, Gustafson (1983) conducted a series of studies on the effect on salmonella shedding if chickens were raised on clean or used litter and found a higher prevalence of shedding among birds raised on clean litter.

As evident from table 4.VIII, most investigators use *S.*Typhimurium for the experimental inoculation, and most strains are laboratory mutants resistant to nalidixic acid. It is unclear whether the results obtained with such strains are applicable to all salmonella serovars. Further, the properties of an old laboratory strain are not necessarily comparable to wild strains. *S.* Enteritidis is currently the main cause of poultry-derived salmonellosis in humans. As this serovar has a distinctly different behaviour compared to *S.* Typhimurium in the host animals in natural infections (Barrow and Lovell, 1991), separate studies should be conducted. Similarly, for swine, other serovars may be of interest.

Titration of infectious dose and establishment of dose-response relationships is an essential part of risk assessment (see chapter 8). This type of investigation is crucial when determining causal relationships and also for explaining the seemingly conflicting results obtained in different studies. If the doses used are close to the threshold value of response/no response, different studies are bound to show equivocal results. In all dose-response experiments, Barrow (1989) found significant increases in salmonella shedding by chickens in all groups receiving concentrations of avoparcin from 15 ppm. For lower concentrations, variable results were obtained. This explains the contradictory results obtained in studies where 10 ppm has been used.

Doses used for inoculation are often large enough to cause colonisation of almost every animal in the control groups. While this allows for a maximum number of animals to monitor the shedding time, differences in susceptibility or epidemic curves are impossible to detect.

From a statistical point of view, these studies were designed to demonstrate differences between experimental groups, not to prove the absence of differences. In the former type of experiments, the null hypothesis is that there is no difference. In such cases, most statistical analyses use figures for significance and power that accept a 5% risk of falsely rejecting the null hypothesis (type I error) and a 20% risk of falsely maintaining the null hypothesis (type II error). Consequently, results calculated under these assumptions cannot simply be reversed to argue that the null hypothesis is true. When trying to prove safety, the allowances for type I and type II errors should be reversed. Alternatively, other statistical methods can be used, such as the detection level (a parameter indicating the minimum effect that could have been detected) suggested by Hansson (1995).

4.8.2   Other pathogens

In addition to various *Salmonella* spp., colonisation of the animals with other zoonotic and animal pathogens could also be affected. Little is known of such effects in relation to pathogens such as *Campylobacter* spp., *Yersinia* spp., verotoxin-producing *Escherichia coli*, *Clostridium perfringens*, *Serpulina hyodysenteriae* and *Lawsonia intracellularis.* Increased presence in the animal intestine of these bacteria could have serious consequences for either human or animal health. No studies specifically addressing this topic have been found.

4.8.3   Other alterations of the microflora

Resistant bacteria may carry other traits that are disadvantageous for the host, such as other resistance genes, or virulence genes. An increase of the population of naturally resistant bacteria following exposure to antibacterial substances can be expected. Moreover, the prevalence of species with a high capacity for acquisition of resistance genes can be expected to increase. Although this may indirectly lead to an overall increase in resistance, this issue has not attracted much attention.

  In an earlier cited study by Kaukas and co-workers (1988 , see 4.3.1), the feeding of AFA increased the proportion of *E. faecium*. As this species was generally more resistant than the other enterococcal species, the ARI increased in groups fed AFA. Resistance to the antibiotics studied is mainly conferred by acquired resistance genes in *E. faecium.*

AFA cause disruption of the intestinal microflora which may increase the susceptibility to colonisation by enteric pathogens.

Avoparcin enhances intestinal colonisation with salmonella.

Some other AFA also affect salmonella colonisation.

Information on enteric pathogens other than salmonella is scarce.

Published studies do not directly address the influence of AFA on contamination of food of animal origin with zoonotic pathogens.

Well-designed studies, and use of appropriate statistical methods, are needed in this area.

4.9   Summary comments

Use of AFA results in dissemination of resistance to AFA and related substances. Resistance against AFA can and does transfer between different bacteria and between different human and animal hosts. The use of AFA will thereby contribute to the increase of the environmental pool of resistance genes. The relative importance of this contribution for the increased resistance in animal and human pathogens cannot be determined. In a situation where the substance in question is not used for therapy, the bulk of the resistance genes will be directly attributable to AFA usage. At least two of the classes formerly primarily used as AFA are today options for new therapeutical drugs.

The possible effects of AFA on prevalence of food-borne pathogens in animal products have not been fully addressed. For salmonella, the effects would be expected to be more important in areas where the prevalence is low, or where control programs are in force. Regarding other pathogens, such as *Campylobacter* spp. and *Yersinia* spp., no information has been found.

Glossary

active efflux - active transportation of a substance out of the bacterial cell, mostly by a membrane "pump"

chromosome - circular DNA structure, the major part of the bacterial genome

co-resistance - simultaneous resistance to several different antibacterials by different mechanisms, encoded by different genes

co-transfer - simultaneous transfer of different resistance genes, located on the same mobile element

conjugation, or conjugal transfer - direct transfer of plasmids or transposons between different bacteria via cell-cell contact

conjugative plasmids and transposons - mobile DNA elements that carry genes encoding the mechanisms for their own conjugal transfer

constitutive expression - the gene product is synthesised regardless of the presence of an inducer

cross-resistance - resistance to different antimicrobials by the same resistance mechanism

deletion - disappearance of DNA within or between genes

gene - DNA sequence that encodes a single protein; promoter plus open reading frame

gene cassette - gene lacking promoter, that encodes a specific mechanism, and can be inserted into integrons

gene cluster - several genes located together, forming a functional gene with products acting together to exert an effect

inducible resistance - the product of the resistance gene is only synthesised in the presence of the inducing substance, usually the antimicrobial that the resistance mechanism counteracts

insertion - addition of DNA within or between genes

integron - small mobile element carrying gene cassettes and genes encoding its own transfer and insertion into the bacterial genome, as well as genes encoding rearrangement, expression and uptake of gene cassettes

intergenic sequence - DNA sequence separating functional genes

multiresistance - resistance to a large number of different antimicrobials by various resistance mechanisms encoded by different genes

mutation -random change in DNA sequence, can change the amino acid of an encoded protein

open reading frame (ORF) - DNA sequence that encodes a single protein

operon - gene plus the operator controlling its expression

phage - virus that infects bacteria and inserts its own DNA into the bacterial genome

plasmid - extrachromosomal small, covalently closed, usually circular DNA element that can replicate autonomously and be transferred between bacteria

ribosome - cytoplasmic structure where protein synthesis takes place through translation of mRNA

transduction - phage mediated gene transfer

transformation - gene transfer via bacterial uptake of free, extracellular DNA

transposon - small, mobile DNA element that carry one or several genes, plus genes encoding for its on transposition between various locations in the bacterial genome

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5   Toxicological and related effects of antibacterial feed additives

5.1   Introduction

As animals are fed AFA for long periods of time, knowledge on bioaccumulation, chronic toxicity and problems associated with residues is imperative. AFA are defined chemical substances and might as such have toxic or allergenic properties. According to directive 70/524/EEC and the guidelines provided in 94/40/EC, satisfactory information relating to these topics has to be presented before approval.

Toxic effects, if any, could be seen either in target animal species (or non-target animals as a result of accidental intake) or in humans.

Animals and humans may be exposed via residues in animal products and humans also when handling products containing the substances. If the product has unwanted properties, such as organ toxicity, mutagenicity or allergenicity, both ways of exposure could be harmful.

Some AFA are poorly absorbed from the gut. Residues are therefore normally not an issue for these substances. Other AFA such as ardacin and avilamycin are absorbed to some extent, and tylosin, spiramycin, olaquindox and carbadox are well absorbed after oral administration (FAO/WHO, 1991; Magnussen *et al.*, 1991; FAO/WHO, 1994). Further, the possibility of accumulation in the animal must also be addressed.

In the following, some information with relevance to the topic will be discussed. Toxicological aspects of coccidiostats, nitroimidazoles and ionophoric AFA are discussed in chapter 7.

5.2   Toxicity for target species

With a few exceptions, AFA are not expected to cause toxic reactions in target species at the levels permitted.

Bacitracin is nephrotoxic (Prescott and Baggot, 1993), but is not absorbed from the gut. Toxic effects are therefore not to be expected. Flavomycin is reported to have very low toxicity (Huber, 1979) and is only absorbed in small quantities (Sambeth *et al.*, 1974).

Some macrolides have been reported to cause gastrointestinal disorders, mainly diarrhoea, at therapeutic levels (Prescott and Baggot, 1993). This is not likely to pose a clinical problem as the concentrations used for growth promotion are comparatively low. Moreover, this should probably be

regarded as a disturbance of the intestinal microflora and not as a toxic effect on the animal.

Carbadox and olaquindox are known to cause adrenal damage at growth promoting levels (van der Molen, 1988; Nabuurs *et al.*, 1990). Lowered aldosterone production is found in porcine adrenal glands exposed to carbadox *in vitro* (Spierenburg *et al.*, 1988b; Spierenburg *et al.*, 1988a). Van Der Molen (1988) demonstrated a dose-response as well as a time-response relationship between in-feed carbadox and adrenal damage in pigs. After 10 weeks of carbadox at 25 ppm or more, damage to adrenal glomerular cells could be observed histologically at post-mortem examination. Adrenal damage leads to profound hormonal disturbances. This means that the use of carbadox as a feed additive probably results in, at least to some degree, Addisons disease (a syndrome caused by impaired function of the adrenal glands) in pigs.

The clinical signs observed are dose-related, ranging from mild to severe. At 50 ppm, mild effects of increased faecal dryness may be observed. Other signs include symptoms indicating salt imbalance (urine drinking), decreased abdominal volume and lowered haematocrit values. Further, changes in hair quality, with hair becoming longer and withered, irritable behaviour and a decrease in feed intake and weight gain may be observed.

Olaquindox has the same toxic effects, although less pronounced, at dosages of 100 ppm or more (Nabuurs *et al.*, 1990). Also in other respects, there is a difference in activity between the substances. Carbadox is permitted in feed at a concentration of 50 ppm, whereas olaquindox is used at 100 ppm (see table 3.III). Accidental overdosing of olaquindox (Köfer *et al.*, 1990; Stockhofe-Zurwieden *et al.*, 1991) and carbadox (Power *et al.*, 1989) has been reported to cause death and severe adrenal damage in piglets.

As the main early sign of intoxication with quinoxalines, i.e. dry faeces, may be mistaken for recovery from enteric disease, mild intoxications are expected to be overlooked by farmers and farm workers.

Most AFA are not expected to cause toxic reactions in target species at the levels used.

Quinoxalines and carbadox in particular, cause adrenal damage in pigs at doses used for growth promotion. This poses a serious risk for the well-being of the animals.

5.3   Toxicity for non-target species

There are various possibilities for ingestion of AFA by non-target species, like mix-ups or contamination of feed at the feed mill, inadvertent feeding or inclusion of poultry litter in animal feed. Problems associated with residues

in animal products in pet food could occur if the raw material contains
residues. The latter is a direct parallel to residue problems in products
intended for human consumption.

The susceptibility to accidental intoxication varies between different non-
target species. Even AFA that are innocuous for the target species may cause
adverse reactions in non-target species. For example, ruminants and horses
are generally very sensitive to disturbance of the intestinal microflora by
antimicrobial substances. Such disturbances may, in severe cases, cause
fatalities.

Accidental feeding of low doses of tylosin to cows has been reported to
cause ruminal stasis, inappetence, decreased milk production and
hypersensitivity (Crossman and Poyser, 1981; Prescott and Baggot, 1993).
Contamination of concentrate feed for dairy cows with bacitracin has also
been reported. This may cause sudden drops in milk production (Woodger,
1979). Feeding of olaquindox at a concentration of 400 ppm to turkeys and
broiler chickens caused adrenal damage in the birds (Reetz *et al.*, 1991).

The severity of the consequences of accidental feeding of some AFA to
non-target species underlines the importance that maximum care should be
taken to avoid such events.

Accidental intake of AFA by non-target species can result in serious
consequences. Adequate procedures for risk management, such as adherence
to Good Manufacturing Procedures, have to be applied.

5.4   Adverse effects in humans

If the substance used has unwanted properties, ingestion of residues in meat
or occupational exposure could produce toxic effects of AFA in humans.

Substances with a potential for causing allergic reactions may be harmful
at extremely low concentrations. Ingestion of a very small dose of any of
these substances might trigger a reaction in an already sensitised person
(Rico, 1985).

5.4.1   Exposure to residues

Regulations such as withdrawal times are meant to ensure that no harmful
residues remain in animal products after slaughter. With the exception of the
quinoxalines, AFA do not have regulated withdrawal times but the use is
usually restricted to a maximum age (see table 5.I.). The maximum age limits
are often equal to, or perhaps in some regions even longer than, the life span
of the animals. In reality, this means that some animals, particularly broilers
and slaughter pigs, will be fed AFA right up to the time of slaughter.

Table 5.I. Examples of MRLs and withdrawal times for some AFA within the EU. Data compiled from Council directive 70/524/EEC annex I and Commission regulation EC 1442/95 unless otherwise indicated

Tabell 5.I. Exempel på MRL-värden (maximal restkoncentration) och karenstider för några AFT inom EU. Data sammanställda från Rådsdirektiv 70/524/EEG annex I och Kommissionens reglering EG 1442/95 om inte annat anges

Substance	Animal species	Maximum age	Withdrawal time	MRL (µg/kg)	Tissue	Comments
Avilamycin	swine	6 months		ND[1]		
Bacitracin	slaughter hogs	6 months		ND		
Bacitracin	broilers	16 weeks		ND		
Carbadox	piglets	4 months,	28 days	5[2] 30	muscle liver	MRL based on sensitivity of analytical method available
Flavomycin	broilers	16 weeks				
Flavomycin	slaughter hogs	6 months		ND		
Olaquindox	piglets	4 months,	28 days	ND		temporary acceptance, no ADI
Spiramycin	broilers	16 weeks		400 300 200	liver fat, skin muscle	
Spiramycin	slaughter pigs	6 months		(600) (200) (300)	liver fat kidney, muscle	provisional MRL, expired July 1997. Presently no MRLs for pigs
Tylosin	slaughter pigs	6 months		100	muscle, liver, kidney, skin ,fat	
Virginiamycin	broilers	16 weeks		ND		
Virginiamycin	slaughter pigs	6 months		ND		

[1] ND = not determined
[2] According to JECFA (FAO/WHO, 1991)

Residue problems are addressed in reports from the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee for Veterinary Medicinal Products (CVMP) are responsible for fixing acceptable daily intake (ADI) and maximum residue levels (MRL) for veterinary drugs in the European Union. For AFA that are at present only used for growth promoting purposes, MRLs have not yet been set.

For substances that are poorly absorbed from the gut, no residues in meat would be expected. Little is known about the possibility of residues due to faecal contamination. If the pharmacokinetics of these substances are similar in humans, ingested residues would not be absorbed to any large extent and would thus not be expected to cause toxic reactions.

Spiramycin

Spiramycin, when fed to pigs at 16 mg/kg per day for 7 days, resulted in residues of around 6000 µg/kg in liver at 0.5 days withdrawal time (FAO/WHO, 1991). The dosage used corresponds to 400 mg to a pig weighing 25 kg, roughly equivalent to 400 ppm in feed. For poultry receiving 300 ppm for 10 days, at zero-withdrawal liver residues were 3800 µg/kg. Neospiramycin is a major active metabolite which is included in the MRL. Maximum approved doses for growth promotion are 8 (swine) and 15 (poultry) times lower than the dosages used above. Assuming a linear relationship, MRLs are not likely to be reached in poultry even at zero-withdrawal. For pigs, the assumed residues at zero withdrawal are above MRL. ADI (50µg/kg bodyweight, based on microbiological data) may be exceeded for pig liver and possibly also for poultry liver. Further investigations into residues of spiramycin, including neospiramycin, should be considered.

Tylosin

When tylosin was fed to pigs at 200 ppm for 17 days, residues of 30 µg/kg were detected in liver at zero withdrawal time (FAO/WHO, 1991). At dosages approved for growth promotion, MRLs are not expected to be reached. However, tylosin is extensively metabolised in the animal and there is still some uncertainty as to the appropriate marker residue(s).

Carbadox

During storage, carbadox is rapidly decomposed to desoxycarbadox in kidney and liver samples, but is stable in eggs and muscle (Keukens et al., 1990; Binnendijk et al., 1991). Carbadox has shown dose-related increases of benign and malignant liver tumours in long-term feeding studies in rats, and

gave positive results in 14 out of 15 mammalian and non-mammalian genotoxicity studies (FAO/WHO, 1990).

Quinoxaline-2-carboxylic acid (QCA) is another major residual metabolite of carbadox. Studies on QCA elimination from liver after feeding of 50 ppm carbadox yielded residues above 30 µg/kg in liver and kidney for 4 to 5 weeks (Baars *et al.*, 1990; Baars *et al.*, 1991). The authors' opinion was that a withdrawal time of 8 weeks should be recommended. In an experiment by Rutalj (1996), QCA was still present at around 10 µg/kg at 62 days after cessation of feeding of carbadox at 50 ppm. Detection of QCA is dependent on the extraction method used, and it has been suggested that this is due to other intermediate metabolites in the pathway of carbadox to QCA (Baars *et al.*, 1991).

In the 36th report of JECFA (FAO/WHO, 1990) it was concluded that an ADI could not be established, due to the carcinogenic and genotoxic nature of carbadox and some of its metabolites. MRLs set in 1990 (FAO/WHO, 1990) for QCA as marker substance were apparently based on the detection limit of the analytical method.

Olaquindox

Olaquindox is extensively metabolised in the animal. The metabolites found vary between tissues and between animal species (FAO/WHO, 1995). One of the metabolites, 3-methylquinoxaline-2-carboxylic acid (MQCA), is known to be responsible for the *in vitro* mutagenicity of other quinoxaline derivatives and has therefore been chosen as a marker compound (FAO/WHO, 1995). The drug is rapidly absorbed from the gut and mainly excreted via urine. No bound residues appear to be present in tissue (FAO/WHO, 1995). In pigs given 60 ppm in-feed olaquindox up to 16 weeks of age, and with a withdrawal period of 28 days, olaquindox residues were below 0.005 ppm in muscle and below 0.01 ppm in kidney (FAO/WHO, 1995). An ADI could not be allocated by JECFA 1995, because of the genotoxic potential of the parent compound and the absence of specific toxicity studies on the metabolites. No MRL has been set.

Some comments on the quinoxalines

Carbadox has, and olaquindox is suspected of having carcinogenic and genotoxic properties (Cihák and Srb, 1983; Nunoshiba and Nishioka, 1989; FAO/WHO, 1990; FAO/WHO, 1994; FAO/WHO, 1995). Carcinogenic and genotoxic effects in consumers could be possible at very low intake levels, especially if the substance in question is ingested regularly over a number of years. Farm and feedmill workers are a special risk group, frequently exposed to AFA when handling animal feed. If appropriate protection cannot

be ensured, the handling of animal feed containing quinoxalines and other AFA with potentially toxic effects must be regarded as an occupational hazard. A conservative approach is often recommended for genotoxic substances in order to prevent underestimation of the risks.

Avilamycin

Experimental feeding of 60 ppm radiolabelled avilamycin to swine for 14 days resulted in residues of 140 µg/kg in muscle, 660 µg/kg in liver, 340 µg/kg in kidney and 550µg/kg in fat at zero withdrawal time (Magnussen *et al.*, 1991). The majority of the parent compound is metabolised or degraded and most of the residue is derived from the oligosaccharide portion of avilamycin (Magnussen *et al.*, 1991). MRL has not been determined.

Ardacin

Experimental feeding of broiler chickens with 15 ppm ardacin for 30 days resulted in liver residues of up to 50 µg/kg after a 7 day withdrawal period (Gottschall *et al.*, 1995). Ardacin is not biotransformed to any large extent (Gottschall *et al.*, 1995). No MRL has been established.

5.4.2   Allergy

Humans may be exposed to AFA substances during production and mixing in feed. Allergic reactions due to macrolides (including spiramycin and tylosin) are reported to be frequent in farmers and other people who handle these substances daily (Veien *et al.*, 1980; Gollins, 1989; Lee *et al.*, 1989; Caraffini *et al.*, 1994; Danese *et al.*, 1994). Allergic reactions to streptogramins (Pillette *et al.*, 1990) and bacitracin (Katz and Fisher, 1987; Grandinetti and Fowler, 1990) have been reported in association with clinical therapy. Photoallergic reactions due to exposure to olaquindox are well known (de Vries *et al.*, 1990; Schauder *et al.*, 1996).

   Although the population at risk for these reactions must be regarded as comparatively small, the consequences of an allergy can be debilitating. This occupational hazard for farmers handling AFA cannot be ignored.

Most AFA, if present in food, would be expected to be at concentrations too low for toxic effects.

Quinoxalines have a genotoxic potential which may be harmful even at extremely low concentrations.

> Many AFA have allergenic properties. Such properties are clearly unwanted in substances of common use. The consequences of an allergy can be debilitating
>
> As some AFA can cause allergic reactions and are potentially genotoxic, the risks of long term continuous exposure to low dosages of genotoxic substances (e.g. workers at feed mills and farmers) need to be evaluated.

5.5   Interactions

As AFA are fed continuously, other substances such as therapeuticals are likely to be administered simultaneously at some time. Therefore, there is a need for information on possible interactions. Some interactions that could be envisaged are intoxication due to amplification of toxic effects, loss of therapeutic effect due to antagonism between the two drugs, or other side effects. Intoxication by ionophores induced by simultaneously administered tiamulin is well known (see chapter 7). No reports have been found on interactions between other AFA and therapeutic substances. As AFA are a constant part of the feed, it is likely that their use would not be connected with disturbances during therapy. Interactions between AFA and other substances might therefore easily be overlooked.

> Little information is available on possible interactions.

5.6   Summary comments

For humans, the use of AFA is an occupational hazard. The risks involved are allergy and, for quinoxalines, genotoxicity. The population at risk includes farmers, farm workers, feed mill workers and other persons handling products containing the substances.

   In target animals, the use of quinoxalines results in adrenal damage (Addisons disease). This is deleterious for animal well being.

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6 Environmental aspects on antibacterial feed additives

6.1 Introduction

Several factors are of interest for the assessment of environmental effects of AFA. The environmental burden, i.e. the amount of the substance that enters and is deposited in the environment, as well as the distribution and transport between different environmental compartments, defines the exposure. Effects to be considered include: effects on soil microbes, earthworms, algae and aquatic organisms. Furthermore, safety for wildlife and other unintended recipients must also be considered.

The environmental deposition of AFA is related to the number of animals fed these substances, duration of use, dosage, metabolism, excretion pattern and method of waste disposal. As most of these substances are poorly, or not at all, absorbed in the gut, the amount excreted in faeces is almost as large as the total amount fed to various animal species. The impact on the environment from the use of AFA is therefore through excretion in faeces. Some AFA may also be excreted in urine. Although the concentrations in urine would be expected to be far lower than in faeces, evaporation and precipitation during storage could lead to higher concentrations, especially at the bottom of the storage tank.

According to directive 70/525/EEC and the guidelines in 94/40/EC, studies on excreted residues and an environmental assessment should be performed on all feed additives.

An overall assessment of environmental effects must include thorough estimations of secondary effects and evaluations of different production systems as a whole. For example, waste from the manufacture of AFA has a high nitrogen content and may also include other potentially harmful components, excess AFA among others. The disposal of this waste must be taken into account. The need for long-term assessments when it comes to environmental effects cannot be overemphasised.

In the following, some information available on environmental aspects of AFA will be discussed. Corresponding information on coccidiostats (and ionophore AFA) is found in chapter 7.

6.2   Exposure of the environment to AFA

Many of the AFA are derived from soil microorganisms and are therefore likely to be, eventually, degraded in soil. Nonetheless, degradability and degradation rates must be determined for each substance. This testing must be standardised as far as possible. Many variables need to be considered in this standardisation (Bouwman and Reus, 1994). As the compounds will be excreted in faeces, degradation studies should be conducted in soil mixed with manure. The type of manure used is important in this regard. Animal species from which the manure originates and dry matter content is of prime importance, but feed composition, management factors and geographic area may also influence the composition of the manure.

Other factors that may affect the outcome of degradation studies are temperature, light, oxygen concentration, microbial composition in soil and manure, and sampling procedures. The methods for analysing the substance in question, as well as its major metabolites, must also be standardised. When all these requirements are fulfilled, it will still be very difficult to apply the results of experimental studies to the environment as a whole. Antimicrobial substances that are released into the environment via faeces will disperse through a number of transport mechanisms. A model for this was presented by Addison (1984), in which volatilisation, degradation, diffusion, adsorption to sediment, losses to ground water and streams etc. is calculated. This model clearly illustrates the complexity of the matter and how difficult it is to predict the exact fate of antimicrobials that are released into the environment.

Various studies have been reported on aerobic degradation in soil of substances used as AFA including bacitracin, tylosin spiramycin and flavomycin (Bewick, 1978; Jagnow, 1978; Bewick and Tribe, 1980; Gavalchin and Katz, 1994). Most study designs are fairly simple; the substance under investigation is mixed with soil or soil/manure mixes and the decline in concentration under different conditions is measured over time. Most substances appear to have a half life in soil of about 2-3 weeks at 20°C, while lower temperatures generally cause a slower degradation. In sterile soil, no degradation occurs, indicating that microbes are essential for the process (Gavalchin and Katz, 1994). Two studies have been found that demonstrate no uptake of tylosin (Bewick, 1979b) or bacitracin (Vogtmann *et al.*, 1978) by vegetables from soil fertilised with AFA-containing manure.

For the quinoxalines, no information has been found. Being synthetic substances, they are of special interest as they might not be as easily degradable by existing soil microflora.

Some losses to surface or ground water are to be expected. Bewick (1979a) demonstrated a notable difference in adsorptive capacity of different clays. When release from soils was studied, the concentration in leakage was related to the amount of tylosin applied. Under practical conditions, the

degree of leaching will depend, among other things, on fertilising practices, weather conditions and type of soil. In view of the large variation of such factors within the European Union, experimental data are especially difficult to interpret. No data on degradation in water has been found.

Larger standardised studies would be necessary to draw firm conclusions on the environmental burden (Bouwman and Reus, 1994). As light and temperature are important variables regarding degradation times, it may be necessary to conduct separate studies for application to different geographic areas.

Most antibiotic AFA appear to have a half life in soil of about 2-3 weeks at 20°C. Information is lacking on the synthetic substances (quinoxalines).

Due to the complexity of the matter, it is difficult to predict the exact fate of antimicrobials that are released into the environment.

6.3   Effects of AFA on the environment

6.3.1   Disruption of microecology

Many ecosystems depend on complex microbial interactions. Soil is a heterogeneous system with a mixed biota and fluctuating local conditions. The microbiota is essential for supplying nutrients to crops, stimulating plant growth, control or inhibition of the activity of plant pathogens and improvement of soil structure. Microbes are also important in the degradation of pollutants. Disruption of these microbial populations is detrimental for agriculture as well as for the society as a whole.

Antibacterial substances such as AFA, if present in animal wastes in inhibitory concentrations, are expected to affect the environmental microflora. Bewick (1978), in one of a series of experiments on waste from tylosin production, investigated the effects of added tylosin on various parameters for soil activity. At concentrations corresponding to 37 ppm or more, nitrogen mineralisation was reduced. A decrease in microbial respiration could be noted for 5 to 7 weeks after the addition of the antibiotic. Degradation of most AFA would be expected during composting (Vogtmann *et al.*, 1978).

Other microbial systems could be affected. Treatment of wastewater from animal production facilities and slaughterhouses, as well as sewage, often includes a microbial process. This may be impaired by low concentrations of antimicrobial substances in the wastewater. No information has been found on this subject.

Source separated municipal solid waste and agricultural waste can be utilised for biogas production. Substances with an antimicrobial effect against anaerobic bacteria could disturb this process. Thaveesri (1994) found that monensin negatively affected the performance of UASB (upflow anaerobic sludge blanket) reactors. Contrary to this, in studies on manure from pigs and poultry fed avilamycin, Sutton (1989) reported efficient operation of experimental and large mesophilic digesters. The presence of avilamycin appeared to alter the metabolism of the microflora, increasing the efficiency to degrade volatile solids.

Considerable research and investments are currently spent on optimising biogas plants in order to meet with the requirements for more sustainable systems. In such highly efficient, modern anaerobic digestion plants, the processes are strictly controlled. The effects of AFA on such sensitive systems must therefore be carefully evaluated.

Albeit transient, the deleterious effects of antibiotics on environmental microflora cannot be ignored.

6.3.2   Decreased nutrient losses

The beneficial effect of AFA on nitrogen excretion and total amount of animal manure has been widely discussed (Roth and Kirchgessner, 1993; Lindermayer and Propstmeier, 1994; Roth et al., 1994; Verbeke and Viaene, 1996). This effect is a consequence of the improved feed efficiency associated with AFA use. The amounts of nutrients excreted in faeces and urine are lowered in proportion to the decreased amount of feed consumed by the animals, i.e. by approximately 3-4 % (Thomke and Elwinger, 1997).

In combination with AFA, high protein diets are often used. Dietary manipulations can also reduce nitrogen excretion while maximum growth is maintained (Williams, 1995; Cromwell et al., 1996; Henry, 1996). Multiphase feeding, where the daily supply of nutrients is adjusted as closely as possible to the requirements of the animals, will substantially reduce nitrogen excretion while supporting maximum growth (Henry, 1996). Appropriate feeding strategies will not only affect nitrogen excretion, but also reduce pollution by other substances, such as phosphorus and trace elements. Investments in feed storage, feed preparation and feed distribution with computerised automated systems may be costly but adjustment to the physiological requirements of the animals may still be a beneficial alternative to costly waste treatments and the risk of future environmental damage.

It appears logical that the amount of nitrogen in the feed will affect the amount of nitrogen excreted in faeces and urine. A pig feed with a protein content of 14% with added amino acids will result in similar or smaller nitrogen excretion per animal than feed with 18% protein content supplemented with AFA (Roth and Kirchgessner, 1993; Williams, 1995;

Verbeke and Viaene, 1996). Supplementations of feed with enzymes and probiotics also aim at a situation in which a greater share of the nutrients supplied in the feed is made available for absorption by the animal. The higher rate of nutrient absorption leads to a lowered output with animal voiding.

The protein content of both pig and broiler feed in Sweden was reduced as a consequence of the ban of AFA enforced in 1986 (Wierup, 1996). This has been possible since several crystalline amino acids are available at competitive price levels. Although the alteration in feed composition was primarily aimed at avoiding intestinal disturbances, there were also benefits in terms of lowered nitrogen excretion.

When discussing nitrogen load it is also important to remember that inorganic fertilisers added to agricultural soil contain a substantial amount of nitrogen. As an example, the amount of nitrogen fertilisers used in France, Germany and Sweden expressed in kg/hectare cultured land in 1995 was 120, 140 and 70 respectively (SCB, 1997). Finding ways of a better utilisation of the nitrogen in manure as a fertiliser, thereby reducing the need for inorganic fertilisers, would perhaps be a better way of reducing total nitrogen load than reducing the nitrogen content of manure.

Presence of non degraded AFA in manure or wastewater will impair the microbial activity in the recipient habitats, lowering the turnover capacity of the microbiota.

The usage of AFA leads to lower excreta nutrient discharges in pigs and poultry. Any given diet that improves nutrient absorption will also reduce nutrient losses with animal voiding.

6.4   Antimicrobial resistance genes in the environment

Faeces from animals fed AFA contains not only bacteria carrying genes coding for resistance against these substances, but also the substance itself, thereby providing a selective pressure that may further the spread of the genes. This is not primarily a problem for the environment, although some possible implications will be discussed in 6.4.4.

6.4.1 Persistence of resistant bacteria in the environment

In order to have an influence on the environmental pool of resistance genes, bacteria carrying the genes must persist for some time in the environment. Animal bacteria would be expected to disappear from the soil surface under

the influence of sunlight, but they may persist deeper in the soil for some time. Around farm buildings, enterococci and *E.coli* can be isolated from soil (Parrakova and Fratric, 1980). Mackey and Hinton (1990) investigated the survival of streptococci and enterococci on straw. After 4 weeks, the inoculated bacteria could still be isolated although their numbers were reduced. Davies and Wray (1996) found that salmonellae from infected calf carcasses could diffuse into soil and persist for up to 21 months, while bacilli and clostridia from the same source could persist in soil for more than two years. The salmonellae could also be isolated from wild-bird droppings, maggots and surface water for 4 weeks after burial of the carcasses. Hinton and Linton (1982) reported survival of multiresistant salmonellae and *E. coli* in slurry for at least 7 weeks. Thus, bacteria of animal origin (both gram-positive and gram-negative) appear to be able to contaminate soil for prolonged periods of time.

6.4.2 Gene transfer in the environment

Gene transfer occurs readily in almost any environment. It has been shown that gene transfer can occur in soil between different bacteria, by various mechanisms (Bale *et al.*, 1988; Henschke and Schmidt, 1990; Top *et al.*, 1990; Cresswell and Wellington, 1992; Lilley *et al.*, 1994). Kruse and Sørum (1994) demonstrated the transfer of multiple drug resistance plasmids between various bacterial species in diverse household microenvironments. Timoney and Linton (1982) showed that transfer of a specific plasmid between different strains of *E. coli* from calves in faeces occurred at 30°C but not at 37°C. This indicates that some transfer systems in animal bacteria are adapted only to environmental conditions.

Gene transfer to soil bacteria can occur from bacteria that will not themselves survive for long in the soil. The addition of nutrients, as when manure is spread on farmland, and the presence of plant roots, provide a favourable environment in which an increased bacterial metabolism and gene transfer would be expected (Cresswell and Wellington, 1992; Lilley *et al.*, 1994).

When studying gene transfer in the soil microenvironment, many factors may influence the result. For example, temperature, humidity and pH are important, as well as the bacterial species used in the experiments. Soil micro-organisms such as pseudomonads, bacilli and streptomycaetes are well adapted to growth in the soil environment and may transfer and maintain resistance genes in these environments better than do coliforms (Cresswell and Wellington, 1992). Methodological problems such as detection of transfer when the genes are not properly expressed and the vast amount of non-described or non-cultivable bacteria present in soil suggest that the observed transfer frequencies may only be the tip of the iceberg. One gram of

agricultural soil can contain more than 100 000 different bacterial species (Cresswell and Wellington, 1992). It seems obvious that studies on just a few of those cannot provide a basis for conclusions regarding the whole microenvironment.

6.4.3   Persistence of resistance genes in environmental bacteria

Resistance genes in soil microbes may persist for a long time, even without a selective pressure from antimicrobial substances. Gene transfer from *E. coli* to soil pseudomonads in a non-sterile, unamended, soil system has been demonstrated, with a persistence of the introduced gene in the recipient bacterium for 100 days (Cresswell and Wellington, 1992). If the expression of resistance genes is costly from a metabolic point of view, resistant bacteria are likely to disappear in the absence of a selective pressure. However, this competitive disadvantage may be overcome (see 4.4.5). Bacteria may adjust to the expression of resistance, lowering the metabolic cost. Bale and co-workers (1993) studied the survival of *E.coli* and its nalidixic acid resistant mutants on dry surfaces. Out of seven resistant mutants, five survived longer compared to the sensitive parent strains. Further, transferable genetic elements carrying resistance genes may also carry other traits of advantage. If so, the resistance genes will still be able to persist in the environment.

6.4.4   Possible implications for the environment

Co-transfer of resistance genes and virulence genes has been demonstrated, not only in animal and human pathogens, but also in plant pathogens (Amuthan and Mahadevan, 1994). Certain virulence genes in plant and animal pathogens are similar enough to suggest a horizontal spread (Alfano and Collmer, 1996). Theoretically, in the presence of a selective pressure for resistance genes, virulence of plant and wildlife pathogens might increase and non-pathogenic bacteria might acquire virulence. Although purely speculative, this merits further investigation. Similarly, possible co-transfer of resistance to pesticides such as copper or sulphur should be considered.

Transfer of genes occurs readily in the environment.

Resistance genes can persist in the environment both in their original bacterial hosts and in environmental organisms.

Possible co-transfer of resistance genes and other genes in plant pathogens should be investigated.

6.5   Summary comments

Little information is publicly available on environmental effects of AFA.
According to accessible data, antibiotic AFA present in soil are degraded by
microbes. Hence, no major toxic effects on terrestrial or aquatic fauna or on
terrestrial plants are expected. Presence of AFA in soil will transiently
disrupt the microflora with potentially negative consequences on nutrient
mineralisation etc. Further, more information is needed concerning possible
effects on modern biogas plants. Presence of AFA and genes encoding for
resistance to AFA in manure is not primarily a problem for the environment.
Such genes will form part of the resistance gene pool available for animal
and human bacteria.

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7   Coccidiostats

7.1   Introduction

Coccidiosis is a severe, world wide health and welfare problem in poultry. The disease is caused by unicellular parasites which grow and multiply in the intestinal mucosa of the birds. Commercially reared chickens are particularly vulnerable to the disease due to the intensive production systems.

Since the early 1940s various drugs have been used to treat and/or prevent coccidiosis in poultry. Coccidiostats have in fact contributed substantially to the remarkable success of modern poultry production. In the EU coccidiostats are incorporated in the feed, as feed additives, while in Sweden, coccidiostats are regarded as pharmaceutical specialities (i.e. medicated feed) and are only available on veterinary prescription. Included among the coccidiostats used in poultry are the ionophoric antibiotics and various chemotherapeutic substances. Two of the ionophores, monensin and salinomycin are also used as growth promoters, in cattle and swine, respectively.

7.1.1 The parasite and the disease

Several reviews of the coccidiosis problem in poultry have been published (Horton-Smith and Long, 1963; Macpherson, 1978; Long and Reid, 1982; Long, 1990). Since knowledge of the disease is essential for the discussion about coccidiostats in modern poultry production, some basic information about coccidia and coccidiosis will be presented in the following.

Coccidiosis is caused by intestinal protozoa of the phylum Apicomplexa. Most coccidia of domestic fowl belong to the genus *Eimeria.* Each poultry species may be infected with several different *Eimeria* species which are host specific and of varying pathogenicity. Seven different coccidial species can infect chickens. The parasites are transmitted via oocysts, shed in the faeces of infected hosts and ingested by uninfected birds. Coccidia multiply in epithelial cells in the intestinal mucosa and sometimes also in other organs of the host. The severity of the infection depends on the coccidial species, host immunity and on the dose of ingested oocysts. Young birds are highly susceptible to coccidiosis since they, to a large extent, lack passively transferred maternal immunity. Immunity is best developed by repeated exposure to small numbers of oocysts. Additionally, immunity to coccidia is

species specific which means that resistance to one coccidial species does not confer resistance to another species.

The symptoms of coccidiosis include mucoid to haemorrhagic diarrhoea, depression, emaciation and depressed growth rate. Sometimes mortality is high. The location and nature of the lesions in the host intestinal mucosa depend on the coccidial species.

Birds with clinical coccidiosis can be treated with water-soluble sulphonamides, amprolium or toltrazuril.

Coccidia are ubiquitous parasites which are easily introduced into poultry houses. Due to the persistent nature of the oocysts, and the propagation in the birds, a considerable population of oocysts will be established in the litter during the rearing period. As commercially reared young chickens, with insufficient immunity to coccidia, are kept in large numbers in high stocking density productions systems the disease becomes a severe problem if not prevented. Chickens reared in traditional, low stocking density units, such as free range backyard flocks, usually become infected by coccidia but these birds seldom develop overt clinical coccidiosis as the number of oocysts in the environment will be comparatively low and immunity develops rapidly.

Unprevented coccidiosis, even if subclinical, has a considerable impact on the economic profit in the broiler industry. The economic losses of a coccidiosis outbreak are substantial due to increased mortality, depressed growth, decreased feed efficiency, medication and increased work load.

It has been shown that coccidial infection predisposes chickens to intestinal clostridial overgrowth (Dykstra, 1978; Baba *et al.*, 1988). Interaction between coccidia and various other avian bacterial and viral pathogens has also been described (Shane *et al.*, 1985; Ruff, 1989).

Taken together, modern broiler production systems require preventive strategies for coccidiosis control, which today are synonymous with chemoprophylaxis.

7.1.2 Prevention and chemoprophylaxis

Anticoccidial drugs are classified in the Anatomical Therapeutic Chemical Classification system (ATCvet) as group QP51A, together with other antiprotozoal agents (NLN, 1995). Coccidiostats approved for use in poultry and other food-producing animals within the EU are listed in table 7.I.

In most countries, coccidiostats are incorporated in the feed to commercially raised broiler chickens and during the growth period to many replacement pullets (future layers and breeders). The by far most widespread use of coccidiostats is in broiler chickens. Ideally, the drugs should show no adverse effects on growth, feed intake, feed conversion or health, and leave no residues in meat. Furthermore, coccidiostats should minimise coccidiosis, but allow some development of coccidia in order to stimulate immunity.

The discovery in the early 1940s that the sulphonamides possessed potent anticoccidial activity made way for the development of the broiler industry in the United States. The rapid development of new and more potent drugs against coccidia as well as the development of efficient vaccines against many avian viral diseases, together with confinement rearing and genetic selection for improved growth rate, made the success of the broiler industry possible. The remarkable world-wide expansion of the poultry industry and the rapid development of coccidial resistance against coccidiostats have necessitated a continuous search for new, efficient coccidiostats (figure 7.I).
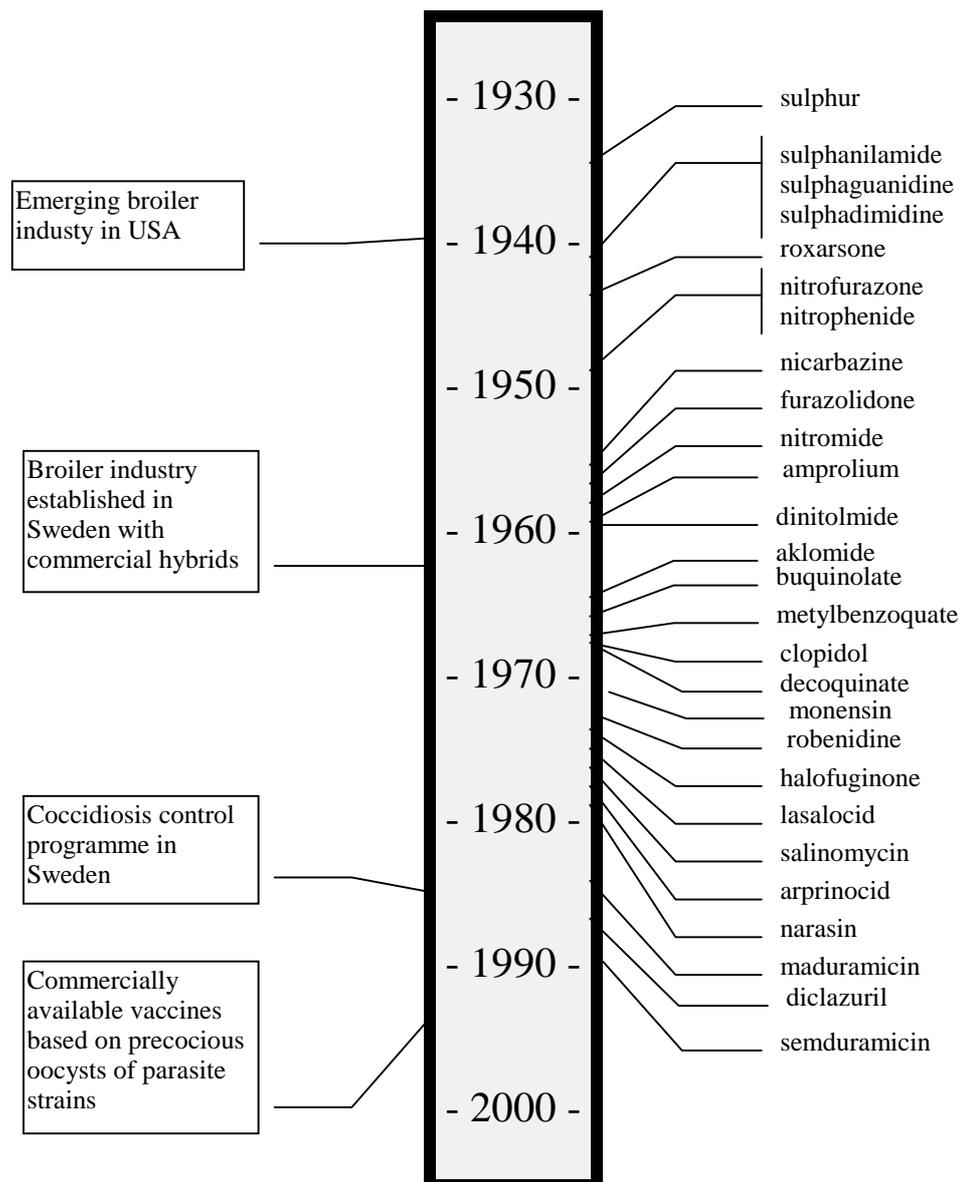
Figure 7.I. Year of introduction of representative coccidiostats.

Figur 7.I. Introduktionsår för representativa koccidiostatika.

Table 7.I. Coccidiostats approved within the European Union (Council directive 70/524)

Tabell 7.I. Koccidiostatika godkända inom den Europeiska Unionen (Rådsdirektiv 70/524)

Generic name	Chemical group	Content in feed *(mg/kg)*	Species/category	With-drawal, days	Maximum age
Amprolium	thiamine analogue	62.5-125	poultry	3	from laying onwards
Amprolium + ethopabate (ratio 25:1.6)	thiamine analogue + substituted benzoic acid	66.5-133	chickens for laying, turkeys, guinea fowl	3	from laying onwards
Arprinocid	benzylpurine	60	chickens for fattening chickens for laying	5	16 weeks
Decoquinate	quinolone	20-40	chickens for fattening	3	
Diclazuril	benzene-acetonitrile	1	chickens for fattening	5	
Dinitolmide (DOT)	dinitro-toluamide	62.5-125	poultry	3	from laying onwards
Halofuginone	quinazolinone	2-3	chickens for fattening	5	
		2-3	turkeys	5	12 weeks
Lasalocid	polyether ionophore	75-125	chickens for fattening	5	
		75-125	chickens for laying		16 weeks
		90-125	turkeys	5	12 weeks
Maduramicin	polyether ionophore	5	chickens for fattening	5	
Meti-clorpindol	pyridone	125	chickens for fattening	5	from laying onwards
		125	guinea fowl	5	
		125-200	rabbits	5	
Meticlorpin-dol+methyl-benzoquat	pyridone+ quinolone	110	chickens for fattening	5	
		110	chickens for laying		16 weeks
		110	turkeys	5	12 weeks
Monensin	polyether ionophore	100-125	chickens for fattening	3	
		100-120	chickens for laying		16 weeks
		90-100	turkeys	3	16 weeks
Narasin	polyether ionophore	60-70	chickens for fattening	5	
Narasin + nicarbazin (ratio 1:1)	polyether ionophore +carbanilide	80-100	chickens for fattening	7	
Nicarbazin	carbanilide	100-125	chickens for fattening	9	4 weeks
Robenidine	guanidine	30-36	chickens for fattening	5	
		30-36	turkeys	5	
		50-66	rabbits	5	
Salinomycin	polyether ionophore	50-70	chickens for fattening	5	
Semdura-micin	polyether ionophore	25	chickens for fattening	5	

7.2   Microbiological aspects

7.2.1   Parasitological aspects

Anticoccidial substances include the antibiotic group polyether ionophores (ionophoric antibiotics/ionophores) and various chemically synthesised substances (table 7.I.).

The ionophoric antibiotics are fermentation products from various *Streptomyces* spp. and *Actinomadura* spp. Ionophores make complexes with mono- or divalent cations, forming lipid soluble compounds which facilitate transport of cations through the cell membrane of the parasite (Jeffers, 1989). The facilitated transport of K^+, Na^+, Ca^{2+} and Mg^{2+} ions across the cell membrane results in secondary toxic intracellular calcium concentrations and disrupts osmotic balance. Ionophores are effective against both asexual and sexual stages of the coccidia.

Few of the chemotherapeutic coccidiostats have been sufficiently studied with respect to the mechanisms for anticoccidial activity. However, the quinolone coccidiostats are known to disrupt the electron transport in the mitochondrial cytochrome system of coccidia (Wang, 1975; Chapman, 1993). Amprolium acts as a thiamine antagonist (FASS VET., 1997). The mode of action for some other coccidiostats, such as halofuginone and diclazuril, is incompletely understood.

Coccidia have, so far, developed resistance to all coccidiostats used. In some cases the resistance problems have emerged so rapidly that the market life of new products has been too limited to justify the costs of development of the drug. The development of resistance to the ionophorous anticoccidials has been comparatively slow. However, increasing ionophore resistance problems have been noticed in many countries (McDougald *et al.*, 1986; Jeffers, 1989; Peeters *et al.*, 1994). Therefore, various anticoccidial programmes including switch, rotation or shuttle application of anticoccidial agents are applied to counteract resistance problems in many countries. There has been some debate as to whether resistance to one ionophoric substance conveys cross-resistance to all other ionophores. Reported results have been contradictory (Jeffers, 1989) However, the general view is that resistance leads to cross-resistance to all ionophores (Chapman, 1993).

Considering the efforts that are put into avoiding resistance problems, it is somewhat surprising that so little is known about the modes of action and resistance mechanisms for various coccidiostats. Explanations for the mechanisms of resistance are either speculative or unavailable (Chapman, 1993). Such information is essential for understanding how to counteract resistance and what substances would be most effective in a given situation.

7.2.2   Bacteriological aspects

Most polyether ionophores are not only active against protozoa, but also against aerobic and anaerobic gram-positive bacteria, e.g. *Lactobacillus* spp (Rada *et al.*, 1994), *Clostridium perfringens* (Dutta *et al.*, 1983; Benno *et al.*, 1988; Kondo, 1988; Elwinger *et al.*, 1992; Kyriakis *et al.*, 1995; Watkins *et al.*, 1997), *Eubacterium spp.*, *Peptococcus spp., Peptostreptococcus spp.*, *Streptococcus bovis* (Watanabe *et al.*, 1981) as well as *Acholeplasma* and *Mycoplasma spp.* (Stipkovits *et al.*, 1987). As is the case with coccidia, the mechanism for the inhibitory activity against bacteria is tthrough increased membrane cation permeability.

Microbiological manipulation of the ruminal fermentation process and consequently of the ruminal volatile fatty acid concentrations is thought to be the basis for the improved feed efficiency in ruminants when the feed is supplemented with low concentrations of ionophores (Nagaraja *et al.*, 1985; Chirase *et al.*, 1988). The changes of the ruminal fermentation process include enhanced production of propionic acid by ruminal ionophore resistant bacteria, and decreased production of acetic acid, butyric acid, lactic acid and methane by ionophore sensitive bacterial species (Schelling, 1984; Nagaraja and Taylor, 1987).

Several ionophores including monensin, narasin, salinomycin and lasalocid sodium have been shown to suppress growth of *C. perfringens*. Ionophoric antibiotics are therefore considered to be important in the prevention of necrotic enteritis (NE) in broiler chickens (Elwinger *et al.*, 1994). Together with the suppression of *C. perfringens,* a better performance has also been noted in ionophore-medicated chickens (Elwinger *et al.*, 1992; Quarles *et al.*, 1992; Elwinger *et al.*, 1994; Elwinger *et al.*, 1996; Waldenstedt *et al.*, 1997). It is likely that the reduced immunological challenge in terms of reduced load of *C. perfringens* in the intestines of chickens explains the increased growth rate of the medicated birds.

Like other antimicrobial substances that are mainly active against gram-positive bacteria, ionophores might be expected to facilitate salmonella colonisation (see chapter 4). Only one study has been found that investigates the effect of monensin in this respect (Manning *et al.*, 1994). The authors of this study concluded that monensin did not influence salmonella colonisation. Further studies, on other ionophores as well, are needed to evaluate this matter.

No information about bacterial resistance to ionophores has been found.

Anticoccidial drugs include ionophoric antibiotics and chemoterapeutic substances.

> Coccidial drug resistance is an emerging problem in particular for chemoterapeutic substances but also for ionophores.
>
> More information about modes of action and resistance mechanisms for various coccidiostats is needed.
>
> Most ionophores are active against gram-positive bacteria.
>
> Several ionophores suppress growth of *Clostridium perfringens* - the causative agent of necrotic enteritis in chickens.

7.3   Toxicological aspects

Coccidiostats are generally considered to be free from side effects and toxicity as long as they are used in their target species at the correct dosage. However, the ionophoric antibiotics and some of the chemotherapeutic coccidiostats have a narrow range of safety and there are many reports in the literature of accidental intoxications of target and non-target species with various anticoccidial drugs. However, most such events can be avoided if good manufacturing practices (GMP) are maintained.

   Intoxications with coccidiostats have been reported in association with poor mixing of the feed resulting in uneven cocccidiostat concentration, failure to properly dilute the coccidiostat concentrate, and in cases of incorrect identification of the product or the feed (Szancer, 1989; Novilla, 1992). The feed can become cross-contaminated at the feedmill and pet food has on occasion been shown to contain traces of coccidiostat (Wilson, 1980; Wheeler, 1996). Toxicity has also been noted when giving poultry feed containing coccidiostat to animal species for which the feed was not intended and when chicken manure has been used as a source of nitrogen in cattle feed (Muylle *et al.*, 1981; Perl *et al.*, 1991; Perelman *et al.*, 1993).

   Diagnosis of coccidiostat intoxication is sometimes difficult due to reversibility of clinical signs and/or the variability of pathological lesions associated with toxicity of coccidiostats of different chemical groups. Feed analysis is often not possible to perform because the feed has already been consumed and/or representative feed samples are not available.

7.3.1   Toxicity of ionophores

Many review articles on the toxic effects of ionophores in target and non-target species have been published during the last decades (among others Beck and Harries, 1979; Galitzer and W., 1984; Langston *et al.*, 1985; Szancer, 1989; Novilla, 1992).

The toxic effects of ionophores are directed mainly against skeletal and/or cardiac muscle. *In vitro* studies have shown that myopathy occurs as a consequence of disturbances in intracellular calcium homeostasis followed by increased intracellular Na^+ concentration (Shier and Dubourdieu, 1992; Sandercock and Mitchell, 1995). The LD_{50} values for the ionophores are generally low, often not higher than 2 to 3 times the recommended dosage in various species (Loyd-Evans, 1991). The diagnostic approach to ionophore intoxication relies on the combination of non-pathognomonic clinical signs, histopathological changes, recovery of the animals when the feed is changed, and analysis of potentially toxic levels of coccidiostats in the feed. There is no efficient, specific treatment for ionophore intoxication in any species (Langston *et al.*, 1985). Withdrawal of the suspected feed and supportive treatment is recommended (Langston *et al.*, 1985).

Accidental intoxication with ionophores has been described in many different animal species, e.g. chickens, turkeys, ostriches, cattle, sheep, deer, pigs, horses, dogs and cats (Muylle *et al.*, 1981; Glover and Wobeser, 1983; Galitzer and W., 1984; Langston *et al.*, 1985; Chalmers, 1988; Novilla, 1992; Baird, 1997). There is considerable species variation in susceptibility to ionophore toxicity (table 7.II). Horses are very susceptible to ionophores and great care is warranted at the feed mill to ensure that cross contamination of equine feed is avoided.

Table 7.II. Species and substance variation of toxicity following oral dosing of monensin and lasalocid (mg/kg body weight) (from Galitzer and W., 1984; Fowler, 1995)

Tabell 7.II. Variation i toxicitet mellan arter och substanser efter oralt intag av monensin och lasalocid (mg/kg kroppsvikt)(från Galitzer and W., 1984; Fowler, 1995)

Species	Monensin LD$_{50}$	Monensin LD$_0$	Lasalocid LD$_{50}$
Chicken	200	150	72
Mouse	125 or 61-110	*	146
Rabbit	40	*	40
Rat	35 (25-43)	*	122
Goat	24-26	10	*
Sheep	12	3-4	*
Cattle	22-80	10	*
Swine	16-17 or 50	4 or 8	*
Horse	2-3	1	22
Dog	20	20	*

* data not available

Ionophore toxicity in poultry

Intoxication with ionophores is a well known problem in poultry (among others Howell *et al.*, 1980; Hanrahan *et al.*, 1981; Halvorson *et al.*, 1982; Braunius, 1989; Dowling, 1992). The prevalence of ionophore toxicity in broiler chickens may be higher than what is generally considered, but hidden due to diagnostic difficulties.

When exposed to sub-lethal toxic doses of ionophoric antibiotics chickens show growth depression and decreased feed intake (Langston *et al.*, 1985). At higher doses clinical signs include anorexia, leg-weakness, incoordination, drowsiness, depression, diarrhoea and sometimes even deaths. Sternal recumbence with neck and hind legs outstretched may also be seen (Langston *et al.*, 1985). Gross pathology lesions are often inconclusive. Pathological changes include emaciation, congestion, myocardial ventricular dilatation, pallor and streaking of skeletal and heart muscle, and ascites (Langston *et al.*, 1985). Histopathological changes are variable. In many cases scattered foci of acute myonecrosis can be seen. In other cases severe muscle and myocardial degeneration and necrosis are observed (Langston *et al.*, 1985). The clinical signs are reversible and usually disappear once the feed containing toxic levels of the ionophore is removed.

Ionophores may also negatively interfere with reproduction in poultry. Reduced fertility (Jones *et al.*, 1990; Perelman *et al.*, 1993), reduced or completely lost egg production (Howell *et al.*, 1980; Fowler, 1995) and reduced hatchability (Howell *et al.*, 1980; Jones *et al.*, 1990; Perelman *et al.*, 1993) have been reported. This is not a problem in commercial production, since breeders and layers are not treated with ionophores during the egg-laying period. However, in cases when layer feed has been contaminated with ionophores reproductive disturbances have been reported (Perelman *et al.*, 1993).

Remarkable differences in toxicity may be seen within the group of ionophores, when used in the same animal species. For example, in turkeys, ionophore intoxication has been recorded when standard dosages of narasin and salinomycin have been used (Davis, 1983; Horrox, 1984), while other ionophores, such as monensin, maduramicin and semduramicin, are better tolerated.

Ionophore toxicity in other animal species

As mentioned, ionophore toxicity has been described in many non-target species including ostriches (Baird, 1997), sheep (Nation *et al.*, 1982; Confer *et al.*, 1983), captive white-tailed deer (Glover and Wobeser, 1983), horses (Muylle *et al.*, 1981; Kamphues *et al.*, 1990), dogs (Chalmers, 1988; Karsai and Papp, 1990; Safran *et al.*, 1993) and cats (Wheeler, 1996). Intoxication

with ionophores in non-target species can cause a variety of clinicopathological changes.

Horses may develop hypovolemic shock and toxic tubular nephrosis, as well as cardial and skeletal muscle changes when given low doses of ionophores. As a lingering effect of ionophore toxicity in horses cardiac fibrosis has also been reported (Muylle *et al.*, 1981). There are also clear indications that cats show remarkable sensitivity to ionophores. In 1996 an outbreak of feline neuropathy was linked to cat feed containing traces of salinomycin (detection limit 2 ppm Wheeler, 1996).

Ionophore intoxication has also been described in cattle (Galitzer *et al.*, 1986; Schlosberg *et al.*, 1992). A primary cardiomyopathic syndrome has been described in beef cattle fed dried broiler manure containing ionophore residues (Perl *et al.*, 1991; Schlosberg *et al.*, 1992). This syndrome is characterised by sudden death, exercise intolerance and subcutaneous oedema. There are also reports describing intoxication with ionophores in swine (Van Fleet *et al.*, 1983).

Interactions of ionophores with other medicinal substances

Some ionophores interact with antibiotics and chemotherapeutic agents (Meingassner *et al.*, 1978; Frigg *et al.*, 1983; Umemura *et al.*, 1984; Miller *et al.*, 1986; Miller, 1990; Fowler, 1995 and others). The mechanism of interaction between ionophores and other chemically defined substances has not been fully clarified. However, in an *in vitro* study the elimination of monensin in rat liver was reduced by 60% when tiamulin was added (Meingassner *et al.*, 1978). In combination with the narrow safety margin for ionophores the reduced elimination of ionophores poses an increased risk of intoxication. Monensin, narasin and salinomycin can interact with antibiotics such as chloramphenicol, erythromycin and oleandomycin (Prescott and Baggot, 1993). Other examples of interaction include lasalocid with chloramphenicol, lasalocid and monensin with furaltadone and furazolidone, lasalocid with sulphadimethoxine, and monensin with sulphaquinoxaline, sulphamethazine and sulphadimethoxine (Frigg *et al.*, 1983; Miller, 1990).

7.3.2   Toxicity of other coccidiostats

Non-specific signs of toxicity, such as depressed growth rate, reduced feed intake, weakness and depression, have been observed in birds and mammals following exposure to some of the non-ionophoric substances (Fowler, 1995). Furthermore, more specific toxic signs are reported with certain substances. One such example of toxicity is the reduction of the skin tensile strength of broiler chickens by the interference of halofuginone with the collagen synthesis (Angel *et al.*, 1985; Granot *et al.*, 1991; Christensen *et al.*,

1994). As a consequence, the birds suffer skin tears during slaughter and processing. In a recent study it was also shown that halofuginone supplementation is associated with an increase in skin scratches and sores in the birds *in vivo* (Pinion *et al.*, 1995). Unpublished Swedish observations when this product was used in the middle of the 80s are consistent with these findings. Due to problems with carcass quality that were put in connection with the use of halofuginone, the product was abandoned by the industry (Björn Engström, personal communication, 1997[1]). The animal welfare aspect of halofuginone toxicity should also be considered.

Halofuginone and arprinocid are known to be toxic for ducks and geese (Behr *et al.*, 1988). Robenidine, arprinocid and in particular halofuginone have been reported to be toxic to chicken embryos (Atef *et al.*, 1989; Granot *et al.*, 1991; Christensen *et al.*, 1994). Additionally, halofuginone is toxic to fish (Fowler, 1995).

Nicarbazin interferes with the thermoregulatory balance at high ambient temperatures which can, in a dose dependent way, cause decreased weight gain and feed efficiency as well as increased mortality (Wienernusz and Teeter, 1995). At therapeutic dosage nicarbazin also interferes with the reproduction of hens by causing decreased egg production and hatchability (Jones *et al.*, 1990).

7.3.3   Residues

To avoid residues of coccidiostats in meat, withdrawal periods before slaughter of the animals are assigned (table 7.I.). Eggs are, however, a special problem. Maximum age levels and restrictions on usage in layer feed may not always be enough to ensure that eggs are free of coccidiostats. Laying might commence shortly after, or even before, the maximum age is reached. Moreover, withdrawal right before laying starts may not be sufficient for substances that persist for a long time in the tissues of the animals.

Ionophores

It has been suggested that residues of ionophores in food could cause adverse effects on the health of humans since these substances possess potent cardiovascular properties (Kabell *et al.*, 1979; Fahim and Pressman, 1981). Monensin induces coronary vasodilatation in dogs and rabbits at low concentrations. Inotropic effects of lasalocid on human heart muscle has been demonstrated *in vitro* (Levy and Inesi, 1974) but there are no reports in

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the literature describing ionophore toxicity in humans associated with meat consumption.

Ionophores are absorbed from the gut and may be found in various organs at zero withdrawal time (Davison, 1984; Donoho, 1984; Lynch *et al.*, 1992; Atef *et al.*, 1993). However, if withdrawal times are respected, presence of toxic residues would not be expected.

For narasin, passage into egg yolk has also been reported (Catherman *et al.*, 1991).

Other substances

Some of the non-ionophoric coccidiostats are absorbed from the gut of the target animals. However, most substances appear to have low toxicity, why residues would not be expected to give rise to any toxic effects.

Decoquinate is only absorbed to a small extent (Seman *et al.*, 1989) and is reported to have very low toxicity (Fowler, 1995).

Low toxicity is also reported for diclazuril (Fowler, 1995).

Feeding of radiolabelled arprinocid at concentrations between 60 and 80 ppm has been reported to result in residues of 0.1-0.5 ppm in liver after 3-5 days withdrawal time (Jacob *et al.*, 1982).

In one study, meticlorpindol in chicken feed at 125 ppm for 34 days resulted in residues in liver and muscle of 0.5 ppm and 0.1 ppm, respectively, at 2 days withdrawal (Ekström *et al.*, 1984). Thereafter, there was a slow decrease in residue concentrations, which were measured until 10 days after withdrawal. Low toxicity of meticlorpindol after oral intake is reported (Fowler, 1995).

Amprolium, may be found in eggs up to 10 days after withdrawal from the feed (Kan *et al.*, 1989). Amprolium is reported to be fairly atoxic, and it is not permitted in the feed after the beginning of egg laying, but no withdrawal time has been assigned for eggs.

Passage of halofuginone into eggs has also been reported (Lindsay and Blagburn, 1995). No withdrawal time for eggs has been established.

For some substances, such as lasalocid, maduramicin, narasin and halofuginone, withdrawal times are shorter in Swedish national regulations than in EU regulations. As a conservative approach may be preferable in this case, it is advisable to adjust the Swedish withdrawal times to the EU regulations.

7.3.4 Allergy

Ionophores may cause irritation and allergic reactions in humans, and may thereby be an occupational hazard for feedmill workers and other people that come into contact with these substances on a daily basis (Mancuso *et al.*,

1990; Fowler, 1995). Protective clothing and dust masks are recommended when handling ionophores. Dust formation might be reduced by changes in the galenic formulation used. For some coccidiostats the maximum dusting properties allowed when handling the substances, as determined by the Stauber Heubach method, have been fixed.

Amprolium, meticlorpindol, robenidine and halofuginone may cause irritation of the skin and eyes by direct contact or through aerosols (Mancuso *et al.*, 1990; Fowler, 1995) and care should be taken to avoid contact, by using masks and protective clothing while handling these substances. Clinical reports of allergic reactions to non-ionophoric coccidiostats appear to be less frequent. However, this does not necessarily mean that such reactions are rare.

The toxicological safety margins of ionophores are comparatively narrow.

Ionophores are toxic for many non-target species.

Some ionophores interact with antibiotics and chemotherapeutics which may increase the risk of intoxication.

Some non-ionophore coccidiostats are toxic for ducks, geese and fish.

Halofuginone causes skin damage in the target species. This is unacceptable from an animal welfare point of view.

Most coccidiostats are absorbed from the gut, and may be found in various tissues at zero withdrawal. It is therefore important that withdrawal times are respected.

For some substances, withdrawal times for eggs should be considered.

Ionophores, and some non-ionophores may cause irritation and/or allergic reactions in man.

7.4   Environmental aspects

Since poultry manure is extensively used as fertilisers, coccidiostats and their degradation products are likely to reach soil and water. Therefore, it is important to identify potential effects on terrestrial and aquatic environments, including flora, fauna and microbes. However, very few studies have been published regarding the environmental fate of coccidiostats, which makes it difficult to evaluate their possible impact in this respect.

7.4.1   Environmental aspects on ionophores

Ionophores are excreted in poultry manure (Donoho, 1984; Perl *et al.*, 1991; Schlosberg *et al.*, 1992). Biodegradation studies indicate that monensin is degradable, under aerobic conditions, in soil with or without manure (Donoho, 1984). No traces of monensin were found in soil 33 days after the deposition and the monensin concentrations declined in manure piles during natural weather conditions (Donoho, 1984). However, in manure piles under relatively anaerobic conditions monensin levels declined slowly (Donoho, 1984). It is, however, reasonable to presume that the microbiological activity in the soil may be altered initially, which may influence the release of nutrients.

As ionophores are active against anaerobic bacteria, interference with anaerobic microbial processes, such as in biogas production or wastewater treatment might be expected. A reduction in the biogas conversion efficiency of UASB-(Upflow Anaerobic Sludge Blanket) reactors by 20% when monensin was added has been reported (Thaveesri *et al.*, 1994). Additionally, the acclimatisation period was twice as long and total volatile acid concentrations were twice as high compared to the control.

Direct effects on plants, fruits and vegetables are generally not expected, but an inhibitory effect of monensin on apple pollen has been reported (Speranza and Calzoni, 1992; Ciampolini *et al.*, 1993).

No published information about persistence or effects of ionophores in aquatic environment have been found.

7.4.2   Environmental aspects on other coccidiostats

It has been shown that some non-ionophore coccidiostats, e.g. decoquinate and amprolium, are excreted in active form in poultry manure (Warman *et al.*, 1977; Hobson-Frohock and Johnson, 1983).

During simulated field conditions it was found that amprolium was adsorbed in the upper layers of the soil and amprolium could be recovered from the soil up to 80 days post deposition (Warman *et al.*, 1977). No effect on soil respiration or soil microflora was shown.

Halofuginone is known to be acutely toxic to fish at low doses (acute toxicity to carp at 0.7 ppm) (Fowler, 1995). The possible impact of this on the aquatic environment is not known.

Available data indicate that ionophores are degradable under aerobic conditions.

Ionophores may interfere with anaerobic systems such as biogas production

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> Some non-ionophoric coccidiostats may persist longer in the environment.

7.5   Alternatives to coccidiostats

During the last fifteen to twenty years concern has been expressed regarding the future long-term prospects for chemical control of coccidiosis. The rapid and continuous development of coccidial drug resistance and the slow and expensive development of new anticoccidial drugs will probably limit the usefulness of chemical control in the future. Awareness of potential hazards for feed mill workers, farmers, target and non target animals and the environment will probably also increase the interest in alternative means of control. Finally, consumers have become increasingly concerned about feed additives, food safety, food quality and welfare problems in meat producing animals and egg laying hens. On the other hand, new housing systems, such as the keeping of layers in deep litter production systems, are being introduced, and larger economical demands increase the need for efficient and safe coccidiosis control. Several possibilities for non-chemical coccidiosis control are being investigated. These include improved hygienic conditions, genetic selection of more resistant animals and further development of immunological control.

7.5.1   Improved hygiene

One of the motives for keeping birds on wire floor is to separate the birds from their faeces and thereby avoiding contact with oocysts. It is, however, very difficult to eradicate avian coccidia by means of strict hygienic measures during commercial farming conditions with birds maintained on the floor. Once the coccidia have been introduced into the poultry house the residual oocyst contamination spreads to the following flock of birds (Loyd-Evans, 1991). Good hygiene and dry litter reduce the number of oocysts, but do not eliminate the risk of rapid spread of the parasites. Recently, a new oocidal disinfectant has become commercially available (Sainsbury, 1991).

7.5.2   Genetic resistance to coccidiosis

Infection with coccidia in chickens induces immunity to the parasite as demonstrated by a reduction of parasite replication in the birds (Horton-Smith and Long, 1963). The immune response depends on several factors linked to the parasites as well as to the host. Two important host factors are the age and breed of the birds. It has been known for some time that the breed of chickens influence immunity to infection by coccidia (Wakelin, 1978; Lillehoj, 1986; Lillehoj, 1988). Differences are seen both in humoral

and cell mediated immunity. Studies in inbred chickens have confirmed that this is a genetically heritable trait linked to the MHC genes and modulated by other genes (Clare *et al.*, 1985; Johnson and Edgar, 1986; Lillehoj *et al.*, 1989).

Although it has been shown in experimental systems that genetically determined resistance to chicken coccidiosis represents a possible alternative to chemical prevention, this finding still awaits commercial application. Commercial broiler and layer hybrids are primarily selected and bred for high productivity by a limited number of multi-national breeding companies. The efficacy and low cost of the anticoccidials, and the priority of production qualities in the breeding programmes of commercial hybrids have impeded the progress of development of genetic immunity to diseases in the birds.

7.5.3   Immunoprophylaxis

During recent years live vaccines against coccidiosis in chickens have become commercially available. Vaccines based on infectious and pathogenic oocysts given at carefully controlled doses followed by post-exposure chemical prevention with coccidiostats are used in breeders in some countries. The need for continuous chemical prevention is thereby avoided. This kind of vaccination programme is not used in broiler chickens.

Live, attenuated vaccines based on oocysts of so-called "precocious" strains of the chicken coccidial species are sometimes used in litter-based production systems, e.g. in breeders and some layer chickens. These vaccines provide adequate immunity to coccidiosis. Such an attenuated vaccine has been used with good results in breeders in Sweden. The major drawback of live vaccines is their relatively high cost which limits their use in broiler chickens and replacement layers.

In broilers, however, the results of vaccination trials with precocious strains in a coccidia-free environment have shown that vaccinated chickens reared without coccidiostats and antibacterial feed additives show significantly lower live weight at 13, 28 and 36 days of age compared to animals receiving narasin (70 mg/kg feed) or virginiamycin (20 mg/kg feed). Both vaccinated birds and unvaccinated controls showed higher numbers of *C. perfringens* than birds given either narasin or virginiamycin (Waldenstedt *et al.*, 1997). Suppression of subclinical necrotic enteritis (NE) by narasin and virginiamycin is the most likely explanation for the differences in performance. This indicates that unless subclinical NE can also be properly controlled, vaccination against coccidiosis may not be economically feasible in broiler chickens. Further research in the field of non-chemotherapeutic prevention of coccidiosis and NE will be needed if broilers are to be raised without coccidiostats.

No non-live vaccines are commercially available at the moment. Subunit vaccines based on immunogenic polypeptides possibly carried by live vectors may be a possibility in the future.

Long term prospects for chemical control of coccidiosis are uncertain.

Improved hygiene will reduce coccidiosis.

More research efforts are needed on the development of suitable vaccines and other preventive methods for the control of coccidiosis and necrotic enteritis.

7.6   Summary comments on coccidiostats

Coccidiostats are used specifically for the control of coccidiosis, but may also suppress subclinical necrotic enteritis.

At the moment it does not appear feasible to rear broilers without coccidiostats.

Breaches in good manufacturing procedures may result in toxic effects of coccidiostats in both target and non-target species.

In order to achieve an effective coccidiosis control and proper prevention of NE, anticoccidials must be used carefully and their effectiveness should be monitored continuously. While alternative measures for the control of coccidiosis and NE are developed, coccidiostats should be available on veterinary prescription and used under veterinary supervision.

In spite of the wide use of anticoccidial substances, little is known about resistance mechanisms. Further research into this area is needed in order to effectively combat the increasing problem of resistance.

7.7   Addendum: dimetridazole, ipronidazole and ronidazole

7.7.1 Introduction

Dimetridazole, ipronidazole and ronidazole all belong to the 5-nitroimidazole group, which also includes metronidazole. These substances are active against obligate anaerobic and microaerophilic bacteria, amoebas and flagellated protozoa (Breccia, 1980). In animals, the 5-nitroimidazoles are used for treatment of infections with anaerobic bacteria and protozoa (Prescott and Baggot, 1993).

Metronidazole was introduced for the treatment of anaerobic bacteria and *Trichomonas vaginalis* in human medicine. It is mainly used against anaerobic infections in humans (Prescott and Baggot, 1993). In some countries, metronidazole is also used in food-producing animals.

The main veterinary use for 5-nitroimidazoles is for the treatment of histomoniasis, a disease for which few alternative drugs are available.

7.7.2   Histomoniasis - the parasite and the disease

Blackhead, also known as histomoniasis, is a disease of gallinaceous birds which is caused by unicellular protozoa called *Histomonas meleagridis* (McDougald, 1991). The organism may infect various bird species, e.g. turkeys, peafowl, pheasant, quail, grouse, partridge guinea fowl and chickens. The turkey is, however, the most susceptible species. The birds develop inflammations of the caeca and liver, and mortality rates may be high. The parasite is often carried by clinically healthy birds such as chickens, by earthworms, various arthropods and by the caecal nematode *Heterakis gallinarum* (McDougald, 1991).

The control of histomoniasis is based on appropriate management. Turkeys should not be reared on ground where chickens have been farmed. Several years must elapse before turkeys can be kept on land where chickens have been reared. Confinement rearing of turkeys reduces the incidence of the disease. Histomonads are extremely delicate organisms and they do not survive outside their host, earthworms or heterakid eggs for more than a few minutes (McDougald, 1991).

Preventive chemotherapy is practised in many countries in growing turkeys but not in chickens, except on problem farms. Chemoprophylactic medication in the feed with arsenicals, nitrofuran or 5-nitroimidazoles is often used. Efficient therapeutic and preventive alternative drugs are at present lacking.

Histomoniasis has not been a problem in Swedish commercial turkey farming during the last decade. The disease is sometimes diagnosed in small turkey flocks reared outside, and occasionally in other bird species such as peafowl and pheasant. Chemoprevention is not applied in Sweden.

7.7.3   Toxicological aspects on 5-nitroimidazoles

Pharmacokinetics

Nitroimidazoles are well absorbed after oral administration to monogastric animals (Prescott and Baggot, 1993).

Dimetridazole is rapidly absorbed from the gastrointestinal tract in the target species. In turkeys approximately 88% is eliminated within three days and in swine approximately 76% of the dose is eliminated within seven days (FAO/WHO, 1989). The metabolism of dimetronidazole leads to reduction of the 5-nitro group, fragmentation of the imidazole ring, and formation of covalently bound residues (FAO/WHO, 1989). At the 34th JECFA meeting (FAO/WHO, 1990) it was found that the use of dimetridazole at permitted concentrations to poultry and swine gives rise to residues that deplete below detectable levels at 2-3 days postdosing. However, due to the possibility of bound residues, it was stated that the total residue in tissues of poultry and swine had not been characterised.

After oral administration of radiolabelled ipronidazole to rats, approximately 92% of the total radioactivity was excreted in urine, bile and faeces (FAO/WHO, 1989). Two hydroxylated metabolites still containing the 5-nitro group have been identified in the faeces of turkeys and rats after oral administration of ipronidazole (FAO/WHO, 1989). The total residues have not been characterised (FAO/WHO, 1989).

Ronidazole is absorbed from the gastrointestinal tract in both laboratory and target animals (FAO/WHO, 1990). In studies using radiolabelled ronidazole, the radioactivity was found to be widely distributed in tissues and eliminated via the urine, faeces and expired air of the animals (FAO/WHO, 1989). The exact nature of ronidazole metabolites has not been determined, but there appears to be a certain amount of bound residue (FAO/WHO, 1990).

Toxic effects

The antibacterial and antiprotozoal properties of 5-nitroimidazoles involves the reduction of the 5-nitro group which results in short-lived hydroxylamine derivatives that bind covalently to proteins and DNA, as a result of which DNA strands may also break (Edwards, 1977). Following therapeutic treatment with metronidazole, single strand-breaks have been demonstrated in the DNA of peripheral human lymphocytes (Reitz *et al.*, 1991a).

For dimetridazole, short-term toxicity in the form of clinical effects on the nervous system and testicular atrophy has been demonstrated in rats fed high doses of the substance (FAO/WHO, 1989). Maternal toxicity has been demonstrated in rabbits fed high doses of dimetridazole (FAO/WHO, 1989). Dimetridazole and its urinary metabolites gave positive results in mutagenicity tests on strains of *Salmonella* Typhimurium with nitroreductase activity, but negative results in a variety of other mutagenicity tests (FAO/WHO, 1989). In a study evaluating the mutagenic and genotoxic potential of 48 nitroimidazoles and related imidazole derivatives, dimetridazole was found to have both mutagenic and genotoxic potential (De

Meo *et al.*, 1992). In this study it was also found that the presence of a nitro substituent at the 5-position is essential for the genotoxic activity of the imidazole derivatives. Increased incidence of benign mammary tumours in rats fed dimetridazole, as compared to nonmedicated control rats, has been reported, as well as a dose-dependent increase in the incidence of benign mammary tumours (FAO/WHO, 1989). In the absence of results from carcinogenicity studies in a second animal species, JECFA could not establish an ADI (Acceptable Daily Intake) in 1989 (FAO/WHO, 1989)

The 34th JECFA meeting also stated that ipronidazole showed mutagenic properties in bacterial test systems, but because of inadequate study design of studies in mammalian test systems the genotoxic potential could not be properly evaluated (FAO/WHO, 1989). Studies on chronic toxicity and carcinogenicity indicate an effect of ipronidazole on mammary tumour formation in female rats given high doses of the substance, and a decrease in body weight along with other signs of toxicity in rats and dogs fed high doses (FAO/WHO, 1989). In 1989, JECFA could not establish an ADI, because the rat carcinogenicity study was inadequate for determining a no-effect level for ipronidazole.

Ronidazole has been found to have mutagenic potential in various bacterial assays (Hite *et al.*, 1976; FAO/WHO, 1989). The results of several *in vivo* tests were variable, with both positive and negative results (Hite *et al.*, 1976; FAO/WHO, 1989). However, a range of metabolites gave negative results in the Ames' test (FAO/WHO, 1989). Ronidazole has been found to increase the incidence of benign mammary tumours in rats and lung adenomas and carcinomas in mice (FAO/WHO, 1989). A temporary ADI was established at the 34th JECFA meeting (FAO/WHO, 1989), but this was not extended at the 42nd JECFA meeting, since requested additional data were not made available (FAO/WHO, 1995).

Metronidazole has been shown to cause DNA strand breaks in human lymphocytes in both *in vitro* and *in vivo* studies (Reitz *et al.*, 1991b; Reitz *et al.*, 1991a; Elizondo *et al.*, 1996). Similar genotoxic effects have been demonstrated in lymphocytes from metronidazole-treated sheep (Ostrosky-Wegman *et al.*, 1994). The study in sheep also showed that increased susceptibility to the genotoxicity of metronidazole may be associated with individual variations in pharmacokinetics. In earlier publications it has sometimes been stated that there was no evidence of any genotoxic effects of metronidazole in mammalian cells (Olsen and Hebjorn, 1982), but this has now been disproven in the more recent studies. This may be explained by the fact that most tests for mutagenicity demonstrate lesions due to a defective DNA repair, while DNA single strand-breaks partly demonstrate DNA damage before DNA repair (Reitz *et al.*, 1991a). Therefore, detection of DNA single strand-breaks can be a more sensitive indicator. Such studies on the other 5-nitroimidazoles have not been found, but there is no evidence that

they would be any different in this aspect, as they all share the reactive 5-nitro group.

7.7.4   Summary comments on nitroimidazoles

The strong indications of genotoxic and carcinogenic potential of 5-nitroimidazoles, combined with the lack of appropriate studies needed to fully evaluate the risk, have been regarded as enough motive for a ban on their therapeutic use in animals within the EU. Withdrawal times are supposed to ensure that no residues will be present in meat. However, when the risk cannot be fully evaluated, neither ADI nor Maximum Residue Limits (MRL) can be established. Consequently it is not been possible to determine withdrawal times necessary to mitigate the risk for the 5-nitroimidazoles. When 5-nitroimidazoles are used as feed additives, the potential hazards to human health are not restricted to the consumers. Farmers and feedmill workers will be exposed on a regular basis to these substances. Considering the strong indications of genotoxicity and carcinogenicity of the 5-nitroimidazoles, this must be regarded as totally unacceptable.

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8 Assessment of the usage of antimicrobial feed additives

8.1 Introduction

As mentioned earlier, the authorisation of feed additives is regulated under Council Directive 70/524/EEC. In article 3a of the latest amendment (Concil Directive 96/51/EC) important conditions that must be fulfilled are listed. An assessment of the use of antimicrobial feed additives must consequently include an appraisal of its fulfilment of this article. Such an assessment has been made pre-approval of the products concerned and the conditions have, according to available evidence at that time, been deemed to be met. However, as society and animal production change over time and new scientific and technical knowledge is available, it is natural that the result of re-assessment can be different.

Directive 70/524/EEC as amended in 96/51/EC, Article 3a

Community authorisation of an additive shall be given only if:

a) *when used in animal nutrition it has one of the effects referred to in Article 2 (a)[1];*

b) *taking into account the conditions of use, it <u>does not adversely affect human or animal health or the environment</u>, nor harm the consumer by altering the characteristics of livestock products;*

c) *its presence can be monitored:*
    - as an additive per se
    - in premixtures
    - in feedingstuffs or, where appropriate, in feed materials;

d) *at the level permitted, <u>treatment or prevention of animal disease is excluded</u>; this condition does not apply to additives belonging to the group of coccidiostats and other medicinal substances;*

e) *<u>for serious reasons</u> concerning human or animal health its <u>use must not be restricted to medical or veterinary purposes.</u>*

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[1] *In article 2 of this directive, favourable effects of additives are specified*

In the following, topics with relevance for the phrases underlined will be discussed. That methods appropriate for the control of the presence of the substances in feedingstuffs are available has been taken for granted.

Absence of adverse effects has been interpreted as safety. As there is no such thing as a zero risk, safety is understood as a risk below the acceptable level. The administration of AFA and coccidiostats is typically over a long period and large groups of animals are involved. The assessment of possible risks concerning AFA and coccidiostats must therefore be made with utmost care.

Issues of safety are usually dealt with by the tools provided in risk analysis. The question of whether the use of AFA substances should be restricted to medical or veterinary purposes is linked to the issue of safety, but is rather to be considered as a form of risk management.

The question of prophylactic or therapeutic effects of AFA is related to regulatory issues, not to safety.

Below, an assessment of the use of antimicrobial feed additives, based on the background provided in chapters 3-7 and annexes A to G is presented. The substances included are, of course, very different. Notwithstanding, to facilitate the discussion on important general issues, a horizontal approach has been chosen.

8.2   Some comments on risk analysis

Risk analysis is the science which addresses risk assessments and decisions regarding risks. These processes are part of everyday life, but in complex situations where demands for consistency, flexibility and transparency are constantly increasing, a formal approach to risk analysis and decision-making is warranted.

Health risk analysis relies heavily on epidemiology and statistics as well as other sciences, but is not identical to these disciplines. Risk analysis involves predictions or projections into the future based on the historical past and careful analysis of recent events. Predictive science is never perfect, and when a prediction is not upheld, the data on which the judgement was based must be re-examined, and the model reviewed. A careful evaluation of the prediction, and adjustments in the model, may help in producing more precise predictions in the future.

Nomenclature in risk analysis is in a state of confusion, partly because it is a new and rapidly developing field, and partly because it involves many scientific disciplines which may use the same words in different ways. There is not yet unanimity with regard to the general field of risk analysis, but attempts have been made to define some terms that are commonly used in risk studies (Ahl *et al.*, 1993). According to these definitions **risk analysis** is the process which includes risk assessment, risk management and risk communication. **Risk assessment** is the process of identifying a hazard and evaluating the risk of a specific hazard, in absolute or relative terms, including estimates of uncertainty. **Risk management** is the pragmatic

decision-making process concerned with selecting and implementing procedures to mitigate the risk and **risk communication** is an open, two-way exchange of information and opinions about risk, leading to better understanding and better risk management decisions.

Hazards are events or elements which represent potential harm, i.e. what might go wrong and how this might happen. **Risk** consists of two components; the likelihood and magnitude (of the consequences) of occurrence of an adverse event (i.e. a measure of the probability of harm), and the impact (biological, economical end environmental) of the adverse effects. **Safety** is the degree to which risks are judged acceptable and is, thus, a subjective measure of the acceptability of risk.

There are several specific models of the various steps included in risk analysis. Most risk assessment models begin with hazard identification, i.e. identifying the risk agents and the conditions under which they potentially produce adverse consequences. This is followed by describing and quantifying the potential of a risk source to release the risk agents (e.g. dose-response relationships), the exposure of human and animal populations to risk agents, and the economic and health consequences associated with the exposure to the risk agents. Finally an estimation of the risk, integrating the previous steps, is made, providing a quantitative measure of the likelihood, timing, nature and magnitude of adverse consequences.

It has been proposed that risk assessment and risk management should be separated as far as possible, to reduce conflict of interest between the two processes (FAO/WHO, 1997). Interactions between risk assessors and risk managers are essential for practical application and evaluation procedures, but it should be recognised that risk assessment and risk management are separate entities in risk analysis, and must be so in order to avoid confusing the issues.

Another important thing to take into account in risk management is the uncertainty in the risk assessment output. The apparent precision of a point estimate gives the misleading impression that the value presented is the one and only answer, although it has been derived from evaluating many possible outcomes and represents only an estimate of one potential outcome among many (Miller *et al.*, 1993). In view of this, it is of importance that the risk assessment is transparent. The risk estimate should, whenever possible, include a numerical expression of uncertainty. A highly uncertain risk estimate may require a more cautious risk management decision.

Risk analytic procedures are not fully objective and quantitative, both because they often have to be based on uncertain assumptions, and because elements of social and moral choice almost always intervene. The assessment of risk is a process that occurs in time and is subject to change simply because of historic and statistical processes (Albanese, 1992). For example, the entire statistical distribution concerning adverse health outcomes can

change following a change in detection technologic procedures or, simply, through the accumulation of data and theory. Unless all risk has been linked to a measurable cause with high accuracy, its assessment will have to change with time. The further into the future risks and benefits are projected, the less certain are the predictions. However, long term views are often necessary. In short, risk assessment tries to answer the following questions:

1.   *What can go wrong?*
2.   *How likely is this to happen?*
3.   *What would the consequences be if things went wrong?*

If available information is reliable and sufficient enough to at least suggest the answers to these questions, one can go on to the processes of risk management and risk communication.

The discussion on financial benefits from AFA belongs in the process of risk management, when trying to determine the reasonable cost for eliminating or diminishing the risk presented in the risk assessment. Risk communication applied to this issue would include communicating the risks to the public so as not to jeopardise consumer confidence in animal products.

8.3   Safety for animals and humans

The possible adverse effects (hazards) associated with usage of AFA for animals and/or humans can be divided into those related to the antibacterial effects of the substances (microbiological aspects) and those related to the chemical nature of the substance (toxicological aspects). In table 8.I, possible hazards, outcomes and the groups affected by the outcomes have been listed. These possible hazards are reflected in the information required for approval according to the guidelines provided (Commission Directive 94/40/EC).

Microbiological risks, specifically those related to bacterial resistance, are more difficult to assess at pre-approval level. Development of resistance in bacterial populations requires two factors to be present; the antibacterial substance and a corresponding resistance determinant. If resistance determinants are not present in the microflora of experimental groups used, most likely no development of resistance will be observed. In such a situation, the experiments do not necessarily provide a basis for accurate projections into the future. Once resistance determinants have gained entry into the microbiota of the animal species in question, the outcome might be different. Unfortunately, the application of post-approval risk assessment to the issue of the microbiological aspects of AFA is frustrated partly by the lack of valid data, partly by the complex interactions of the factors involved.

Pre-approval risk assessment of hazards related to chemical aspects of the substances is well established. It is based on results from a variety of toxicological studies in cell cultures, laboratory animals and target species.

For residues, the establishment of an acceptable daily intake reflects the acceptable risk level and if risk mitigation is needed, withdrawal times is the management mostly used.

Table 8.I. Possible hazards of the usage of AFA and their outcomes for animals or humans

Tabell 8.I. Tänkbara faror med bruk av AFT och dessas konsekvenser för djur eller människor

Hazard	Main outcome	Applicable to:		
		Humans	Target species	Non-target species
Microbiological				
Increased resistance	impairment of therapy	yes	yes	yes
Suppression of pathogens	disease not detected	(no)[1]	yes	(no)
Increased colonisation with enteric pathogens	increase of food-borne infections	yes	(no)	(no)
Chemical				
Organ "toxicity"	organ malfunction	no	yes	(no)
Residues in animal products	toxic reactions, allergic reactions	yes	no	yes
Repeated exposure	allergic reactions	yes	no	no

[1] Parenthesis denotes that there may be special circumstances where this hazard is applicable

In the following, the elements of a risk assessment based on available data on AFA with special emphasis on microbiological aspects are outlined. The existing background information has been covered in chapters 4-7 and in annexes A-F.

8.3.1   Increased resistance

Exposure of bacteria to AFA substances selects for resistance to these substances, and sometimes also for resistance against other antimicrobial substances (see Chapter 4). The proportion of the microbiota resistant to the substance will increase. Bacterial resistance is not in itself a problem but associated loss of therapeutic efficiency of the antibacterial substance or group of substances is. Hence, the impact will be dependent on whether the use of a specific additive contributes to resistance in potential pathogens to antibacterials used in therapy of infections caused by these bacteria. If this is the case in target animals, the consequences are direct. Transmission of resistance genes between microbes of the target animal and those of other animals or humans can and does occur. The probability of such transfer and the impact of subsequent indirect effects needs to be evaluated.
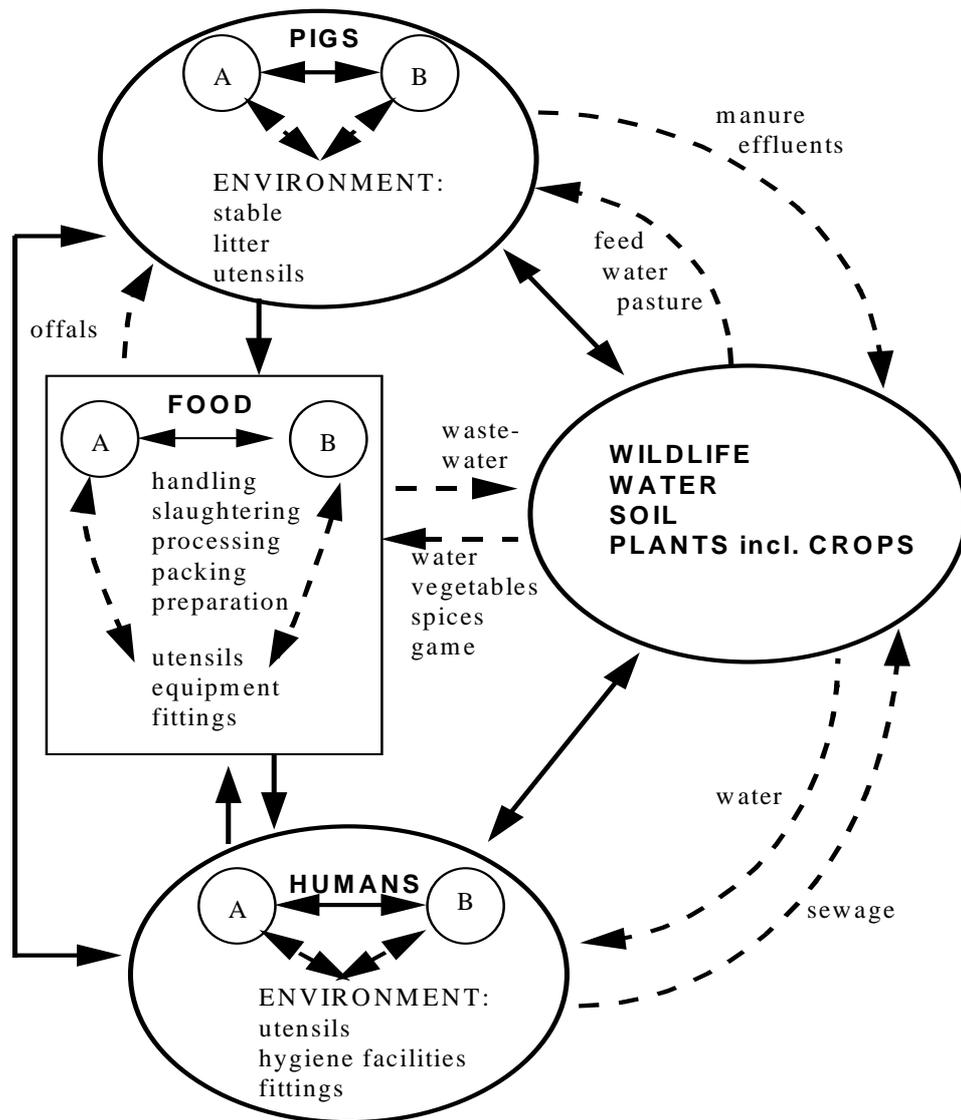
Figure 8.I. Some routes by which resistance genes can spread among animal and human microbiota

Figur 8.I. Några vägar via vilka resistensgener kan spridas bland djur- och människomikrober

There are numerous routes by which resistance genes can spread among animal and human microbiota. Figure 8.I presents a schematic view of some of these routes. Modern society is, of course, not so simple. Animals, animal feed, animal products and animal waste are transported between regions, people travel all over the world and our daily food originates from various

regions. To illustrate the latter a menu was retrieved from
http://www.ica.se/skafferiet/index.htm (as per week 29, 1997), including 5
simple one-course evening meals with one fruit for dessert. Assuming the
ingredients were chosen on a best-price basis in the two leading Swedish
supermarket chains, the basket contained food from 11-15 different
countries. The survey was done in summer, when the proportion of nationally
produced vegetables is comparatively high.

   In order to assess the degree to which the animal usage of AFA contributes
to the increased resistance in human bacteria, an estimate of the frequency of
transfer between animal and human bacteria and its subsequent effects is
needed. When risks are diffuse and pertinent data cannot be obtained, risk
analysis usually employs assumptions to estimate the magnitude of the risk.

Probability of transmission of resistance genes

To further illustrate one of the dissemination routes in figure 8.I, the path
from animal to consumer via the food-chain was examined. The scenario
discusses the acquisition of gene *xxx*A, coding for resistance against
antibiotic X, through consumption of animal product Y. The following
assumptions were made on basis of data available for glycopeptides,
glycopeptide resistance, enterococci and broiler meat :

- the consumption of antibiotic X is low, 100 defined daily doses (DDD)
  per 10 000 inhabitants and year (based on total human glycopeptide
  consumption in Sweden 1995)
- the consumption of product Y is high, occurring 50 days per year and
  individual (corresponding to a consumption of 12.5 kg chicken per
  capita and year)
- the prevalence of gene *xxx*A in/on product Y is 50% (estimated from
  Kruse, 1995)
- one meal of product Y weighs 250 g
- product Y contains 1000 colony forming units (CFU) of bacteria/g
  carrying gene *xxx*A (estimated from Kruse, 1995)
- the transfer rate of gene *xxx*A from animal to human bacteria is
  10^{-6}/donor.

   Further, as the optimal conditions of transfer are unknown, the model was
restricted by the assumption that:

- transmission will only occur if the individual has a contact with product
  Y while being treated with antibiotic X.

The probability (P) of an individual having contact with product Y contaminated with bacteria carrying the gene *xxx*A while being treated with antibiotic X, at least once in 365 days, can be expressed as:

$$P = 1- (1-p)^{365}$$

it is further assumed that:

$$p = k * d$$

where; k is the probability of having contact with product Y on any
randomly selected day
d is the probability of being treated with antibiotic X on any
randomly selected day

Table 8.IIa. Probability of having contact with product Y containing gene *xxx*A while being treated with antibiotic X - assumptions and calculations

Tabell 8.IIa. Sannolikhet för kontakt med produkt Y innehållande genen xxxA samtidigt som behandling med antibiotikum X sker - antaganden och beräkningar

		Calculated as	Result
Antibiotic X consumption, humans			
Defined daily doses (DDD) per 10000 individuals and year			100
Consumption of animal product Y			
Consumption of product, days/year			50
Prevalence of gene *xxx*A in/on product			0.5
Consumption of product with *xxx*A, days/year		0.5*50	25
Occurrence and transfer of *xxx*A			
CFU with *xxx*A/g of product			10^3
No. *xxx*A in 250g			$2.5*10^5$
Transfer rate of *xxx*A/donor bacterium			10^{-6}
Total transfers per 250g		$250*10^3$	0.25
Probabilities			
Probability of treatment any selected day	d	100/(10000*365)	0.00003
Probability of having contact with product with *xxx*A any selected day	k	25/365	0.07
Probability of having contact and being treated	p	p=k*d	0.000002
Probability of having at least one contact with product containing *xxx*A while being treated during one year (365 days)	P	$P=1-(1-p)^{365}$	0.0007
Probability of successful transfer of *xxx*A when contact and treated		P*0.25	0.0002

   With the assumed values P = 0.0007, i.e. 7 individuals in 10 000 will have at least one contact with product Y while being treated with antibiotic X. As the number of successful transfers from the bacteria in one portion of product Y will be 0.25, a successful transfer of *xxx*A will be expected to occur in ¼ of the contacts, i.e. in 2 out of 10 000 individuals.

   A sensitivity analysis was performed to test the impact of each assumed variable on the outcome of the calculations (see table 8.IIb).

Table 8.IIb. Sensitivity analysis of estimate of probability of transfer of gene xxxA expressed as number of individuals colonised bytransconjugant per 10 000 individuals and year

Tabell 8.IIb. Sensitivitetsanalys av skattning av sannolikheten för överföring av genen xxx*A uttryckt som antal individer som koloniseras av transkonjuganten per 10 000 individer och år*

	Assumptions:			Results for assumptions:		
	Initial	Low	High	Initial	Low	High
Consumption of antibiotic X	*100*	10	10 000	*2*	0.2	166
Consumption of product Y, days/year	*50*	20	90	*2*	0.7	3
Prevalence of *xxx*A in/on Y	*0.5*	0.1	0.7	*2*	0.3	2
No. of bacteria with *xxx*A, CFU per g of Y	*1000*	1	100 000	*2*	0.002	171
Transfer rate	10^{-6}	10^{-8}	10^{-4}	*2*	0.02	171

   Neither the number of days/year that the product is consumed, nor the prevalence of products with bacteria carrying the resistance gene had any major influence on the results. Of much greater importance were the rate of transfer of the resistance gene between different bacteria, the consumption of the particular antibiotic and the number of resistant bacteria in the product. The two latter factors are also those most likely to vary with time and geographic region.

   The antibiotic consumption in this example is assumed to be low. If the consumption is increased to 10 000 DDD per 10 000 inhabitants and year the final number of successful transfers will be 2 in 100 inhabitants per year, maintaining the original bacterial content in the product. This higher figure for consumption is only slightly higher than the human macrolide consumption in the Nordic countries (Apoteksbolaget, 1996; DANMAP, 1997; MAF, 1997).

   The transfer rate will be dependent on the transfer mechanisms of the resistance gene in question as well as on the donor-recipient combination. A transfer rate of 10^{-6} can not be considered as unusually high (see Chapter 4).

All the bacteria in one portion of product Y were assumed to be in a condition capable of transferring *xxx*A. When the product is actually consumed, this might not be the case. *Enterococcus faecium* has been reported to survive for 10 minutes at 65°C (Panagea and Chadwick, 1996) but a thorough heat treatment of the product will reduce or eliminate its content of viable bacteria. When the number of bacteria in the example was lowered to 1 colony forming unit per gram of the product, the final amount of successful contacts was 2 out of 10 000 000 individuals per year. On the other hand, zoonotic food-borne illnesses with infectious doses in the range of 10^6-10^9 organisms do occur in spite of heat treatment. This shows that higher numbers of viable bacteria of animal origin do occur in food. Recontamination and lax enforcement of basic hygienic rules provide opportunities for microorganisms to multiply. Numerous occasions for transfer are encountered before heat treatment (kitchen utensils etc.) and the gene might survive heat treatment. The prevalence of re-contamination in the case of resistance genes is not known. Further, the dose required for successful colonisation of an individual with for instance resistant enterococci is largely unknown.

In the above calculations, it was assumed that transmission will only occur when the individual is consuming the relevant antibacterial. The occurrence of individuals colonised with, for instance, vancomycin resistant enterococci (VRE) without prior exposure to vancomycin indicates that this is not always the case (Van der Auwera *et al.*, 1996). However, experimental evidence from a mouse model shows that exposure to vancomycin is important in establishment of a long term colonisation of VRE (Whitman *et al.*, 1996). Therefore, the restriction seems prudent in order not to overestimate the risks.

To summarise the probability of at least one transfer of a resistance gene to human bacteria via one type of product in the food-chain was estimated to occur in 2 out of 10 000 individuals each year, ranging from 2/100 to

2/10 000 000. It is important to note that this figure does not account for secondary transmissions between humans.

Impact of transmission of resistance

In the calculations above, it was assumed that the consumption of the antibacterial was low. Such antibacterials are likely to be of use only in special situations, and most likely in hospitals (e.g. vancomycin, quinpristin-dalfopristin). Therefore, it is probable that the individuals in the example above are hospitalised when the effective contact occurs.

The likelihood of secondary spread of resistant bacteria in antibiotic dense surroundings such as hospitals is high (Salyers, 1995). In the case of VRE, numerous reports on spread to both patients and staff within hospital wards

have been published (for a review see Woodford *et al.*, 1995). For example, Wade (1995) reported acquisition of VRE by 110 patients in a hospital ward during a 22 month period. Five ribotypes accounted for 78% of the isolates. Duration of admission was the only independent risk factor for acquisition of VRE in the study. This concurs with the findings of Garber (1989) who found that not only patients treated with ampicillin, but also other patients in the ward experienced a higher risk of infections with ampicillin resistant bacteria.

Transmission of and subsequent colonisation with resistant bacteria is not in itself a problem but disease caused by resistant bacteria is. Multiple factors predispose a person to infection with VRE, but colonisation precedes most infections (Edmond *et al.*, 1995). In another study on VRE epidemiology (Montecalvo *et al.*, 1995), the colonisation rate was 16.6 patients per 1000 patient-hospital days. Colonisation persisted for at least 7 weeks and sometimes for as long as 1 year. The colonisation rate was 10.6 times greater than the VRE infection rate. Using the figures from the scenario above, this would mean that 2/1000-2/100 000 000 individuals would experience clinical problems due to the transmission from product Y. This does not account for cases resulting from secondary spread.

The control of nosocomial infections is primarily based on isolation of cases and hygienic measures. Repeated new introductions into a hospital would in most cases complicate such control measures.

Consequences for health and health economics

The adverse economic and health effects of drug resistant bacterial infections can only be roughly quantified. The evaluation has to be based on the disease consequences as the human carriage of resistant bacterial strains does not in itself constitute a direct cost. Naturally, the two issues are linked as an increased prevalence of carriers is linked to an increased probability of diseases due to infections with resistant bacteria. According to Holmberg and co-workers (1987) the mortality, likelihood of hospitalisation and length of hospital stay was generally at least twice as great for patients infected with drug resistant strains as for those infected with drug susceptible strains of the same bacterial species.

In table 8.III, some of the items that can lead to costs in relation to disease caused by resistant bacteria in humans and animals have been listed (adapted from Salyers, 1995).

It has been argued that with adequate diagnostics, identifying correctly the drug susceptibility of the infectious organism, the patient would get the correct treatment and resistance would not be a problem. However, apart from extending the time span between clinical diagnosis and treatment, laboratory diagnostics will also involve increased costs. Further, if a resistant

strain is introduced into a hospital ward it is likely to spread, thereby increasing the number of patients in need of alternative drugs.

Table 8.III. Negative effects of increased resistance in human and animal pathogens

Tabell 8.III. Negativa effekter av ökad resistens hos human- och djurpatogener

Humans	Animals
Short time effects	**Short time effects**
Patient suffering	Animal suffering
Cost of new antibiotics	Cost of new antibiotics
Cost of increased laboratory testing	Cost of increased laboratory testing
Longer course of disease	Longer course of disease
Higher costs for medical consultations	Higher costs for veterinary consultations
Long term effects	**Long term effects**
Irreversible damage to internal organs	Irreversible damage to internal organs
Days missed from work	Production losses
Costs for alternative measures for control and prevention	Costs for alternative measures for control and prevention
Reduced compliance with other recommendations or preventive measures due to loss of confidence in physicians	Reduced compliance with other recommendations or preventive measures due to loss of confidence in veterinarians
Measures to protect health care workers	
Shorter life span of new therapeuticals	Shorter life span of new therapeuticals
Cost of increased monitoring	Cost of increased monitoring
	Loss of consumers confidence
Disruption of patients family life	

The difference in cost between older drugs, such as erythromycin, and newer alternatives may be 6-fold (Nightingale and Quintiliani, 1997). However, some newer drugs are less likely to produce adverse reactions and might have other advantages which will reduce the final treatment cost (Norinder *et al.*, 1997).

The cost of increased resistance as a consequence of antimicrobial use at society level was calculated by Phelps (1989). The extra societal cost arising from any use of antibiotic was assumed to be the product of the expected loss in therapeutic value of the antibiotic times the proportional effect of the drug use on microbial resistance. For an estimated 150 million annual antibiotic prescriptions (United States), this cost appeared to be between $0.35 billion and $35 billion. In the lower end of the estimate, no mortalities were expected while the higher figure represents 1 death due to resistance per 1000 infected patients with a value of a premature loss of life assigned to $1 million. The calculations depend heavily on unknown parameters but even if the assumptions are only partly true, the estimate shows that the

unrecognised cost is substantial. Critical points in the analysis are the proportion of infections resisting treatment, the numerical relation between use and development of resistance over time and the proportion of bacterial infections by resistant bacteria leading to death. Further analysis on these issues is needed in order to obtain valid estimates to be used both in risk management and in risk communication. It has been suggested that VRE infections may lead to premature death in one per 1 000 000 inhabitants in the EU (Baquero, 1996).

   The relative impact of resistance genes dervied from animal production on the extra society costs discussed above will depend on the proportion of usage in different animal populations and in humans. In a situation where a drug is widely used in human medicine, the impact added by animal usage will probably be low. For resistance to substances, such as quinpristin-dalfopristin (streptogramins), that have recently been introduced more widely in human medicine, the situation is different. Following the usage of related substances (virginamycin) as AFA, a pool of resistance genes is available in animals. The effective lifespan of the new drug may therefore be substantially shortened. This is even more pronounced for substances that are yet under development for human therapy. Everninomycins is currently a promising candidate for human therapy, belonging to a class that has never been used in humans therapy. Everninomycins belong to the orthosomycins class and are structurally closely related to the feed additive avilamycin. This emphasises substances used as AFA may be useful templates for future drugs. However, cross-resistance is likely to occur.

No studies concerning the cost of antimicrobial resistance in animals have been found. As an example, the cost of a newer drug such as tiamulin is about 2-fold compared to an older drug such as tylosin. The comparison refers to one tonne of medicated feed at recommended therapeutic concentrations for swine. This may not be the major cost involved, as production losses including mortality and the consequences of increased withdrawal times generally outweigh the cost of therapeuticals. Obviously, morbidity is associated with high costs in pig production. Wallgren (1994) reported significantly ($p<0.05$) better production data, in terms of higher daily weight gain, lower mortality, larger litters and lower feed consumption per kg weight gain from specific pathogen free (SPF) herds than from conventional pig herds. This was ascribed to differences in incidence of infections. Similar results have been reported by other authors (Young *et al.*, 1959; Caldwell *et al.*, 1961; Jorgensen, 1987).

   Losses due to swine dysentery in Australia have been estimated to about 2.5 % of the annual gross national pig production (Hampson, 1991). This figure includes costs for medication, i.e. it assumes that there are available therapeutic drugs. In the future this may not be the case. Resistance to tiamulin, as well as to tylosin, in *S. hyodysenteriae*, or banning of the use of

pleuromutilin drugs in veterinary therapy, could quickly change the prospect. It has already been suggested that the use of quinolones in animal husbandry should be reassessed (Jacob-Reitsma *et al.*, 1994). It is not to be expected that innovations in the field of antimicrobial therapy will primarily be made available as veterinary drugs, or that the use of such new substances would necessarily be economically feasible in food producing animals.

8.3.2   Suppression of pathogens

Some reports indicate that the use of AFA may suppress the excretion of *Serpulina hyodysenteriae*, thereby lowering the sensitivity of diagnostic tests (Ronne and Jensen, 1992; Fellström *et al.*, 1996). If methods for control of swine dysentery by improved bacteriological diagnostics were to become available, removal of at least certain AFA before testing would be advisable.

   Theoretically, AFA with effects on gram-negative bacteria, such as the quinoxalines and possibly flavomycin, would be expected to suppress the shedding of for example salmonella as long as resistance has not developed. This could be looked on as an advantage as the burden of zoonotic pathogens originating from animals in the final product would be lower. On the other hand, when control programs are instituted, the increased number of false negatives could, depending on diagnostic methods used, frustrate identification of infected animals or herds. This would influence the cost of such programmes.

8.3.3   Favouring of enteric pathogens

All food-borne infections pose a considerable threat to human health and the economy of individuals, families and nations (quoted from WHO, 1996). The major causes of food borne infections in EU are *Salmonella* and *Campylobacter* infections. Eggs, broiler meat, meat and meat products and raw sprouts are the main sources for salmonellosis within the EU. Verotoxin producing *E.coli* have lately emerged as serious new enteric pathogens. Other zoonotic food borne pathogens of interest are *Listeria monocytogenes* and *Yersinia enterocolitica.*

   The use of some AFA is likely to enhance colonisation and shedding of zoonotic enteric pathogens. Relevant literature on this subject has only been found for salmonella (for a critical appraisal of published studies see 4.8).

   Most studies have been focused on prevalence of shedding of salmonellae over time, as an indicator of the likelihood of the individual animal shedding salmonella at the time of slaughter (for a review see Gustafson, 1983). The results of these studies are conflicting. However, a dose-dependent response

for salmonella shedding of chickens fed avoparcin has been established by Barrow (1989). For other AFA, no dose-response studies have been found.

The sample sizes in most of these studies examined are rather small. Studies designed to detect small differences in this aspect inevitably involve large group sizes. In table 8.IV, sample sizes needed in order to detect small differences with a 95% confidence level (type I error of 0.05) and an allowance for a type II error of 0.2 have been calculated.

Table 8.IV. Sample sizes needed to detect differences in prevalence between groups of animals at various levels of prevalence in the control group

Tabell 8.IV. Antal prov som krävs för att upptäcka skillnader i prevalens mellan grupper av djur vid olika prevalens i kontrollgruppen

Difference to detect	Sample size required when the prevalence in the control group is:		
	25%	50%	75%
1%	30 000	39 000	29 000
5%	1 300	1 600	1 100
10%	300	400	300

Obviously, studies of this size may be impractical. Not only would the group sizes be very large, but all animals in each group must be sampled. For smaller studies information on the smallest difference that could have been detected (detection level) should be included. Alternatively, the allowances for type I and type II errors could be reversed (see 4.8).

An equally important but less studied issue, related to consumer exposure, is whether the use of AFA affects the likelihood of a flock becoming infected at all when the animals are exposed to low doses of organisms. The epidemiologic unit of interest in this case would be the flock, not the individual animals. A reduction of the number of salmonellae needed in order to establish colonisation following avoparcin exposure has been shown (Smith and Tucker, 1980). Such a reduction would increase the risk of flocks getting infected in natural settings.

In countries where investments are being made in control programs for salmonellosis, any effect in terms of increased likelihood of colonisation of animals will negatively affect the benefit/cost ratio of the programs.

The prevalence of shedding by individual animals may not be the best way to examine the impact of AFA on consumer exposure to salmonella. Intensive rearing of broilers, where thousands of birds are kept together is conducive to the spread of salmonellae and other zoonotic pathogens. Transfer of salmonella between birds occurs during transport and between carcasses and parts during processing. Inoculation of tracer bacteria into chicken carcasses has shown that a high degree of spread of these bacteria to other carcasses occurs during slaughter (Stewart, 1965, cit. by Bryan and Doyle, 1995). Under such conditions, a few infected birds can easily spread

pathogens to many animals or animal products. Thus, even a low prevalence within a flock at the time of slaughter will result in most of the carcasses becoming contaminated unless rigorous hygienic control measures are applied. In pigs, contamination during slaughtering is easier to avoid.

No studies on the effects of AFA on prevalence in the final product has been found. To examine the effects of an increased prevalence of salmonella in animal products, the resulting increase in cases of salmonellosis in humans has been calculated (table 8.V). The calculation was made as:

additional cases = increased prevalence in product * P(i) * N

where; P(i)= the probability of getting infected if contact with contaminated product

N= population size, 100 000

It was assumed that all members of the population were exposed to the product on a yearly basis. On basis of data from Canada, Todd and Harwig (1996) calculated the ratio of humans diseased from/exposed to salmonella contaminated poultry products to 1/80 or approximately 1%. P(i) was therefore assumed to be 1%.

The cost for one reported case of salmonellosis was calculated by Engvall and co-workers (1994) for Sweden to approximately 14 000 SEK. This figure does not include costs due to premature deaths. The estimated average for both reported and non-reported cases was approximately 8 000 SEK. If the use of AFA increases the prevalence of enteric pathogens in the final product by 5% and the likelihood of getting diseased if exposed is 1%, this will result in 50 additional cases per 100 000 inhabitants at a society cost of 400 000 SEK.

Table 8.V. Effect of increased prevalence of salmonella on risk of contracting salmonellosis through contact with animal products

Tabell 8.V. Effekten av ökad förekomst av salmonella för risken att insjukna i salmonellos efter kontakt med djurprodukter

Increased prevalence in product	Additional cases per 100 000 inhabitants at P(i)[1]		
	0.1%	1%	5%
1%	1	10	50
5%	5	50	250
10%	10	100	500

[1] Probability of getting infected if contact with infected product
[1] *Sannolikhet att bli infekterad om man har kontakt med produkten*

The example above illustrates that even a seemingly marginal increase of the prevalence of enteric pathogens in animal products can have a significant impact on human health.

A further quantification of the risk cannot be performed as hitherto published studies on the possible effects of AFA on enteric pathogens have not addressed the issue of consumer exposure (see also 4.8).

8.3.4   Toxicological aspects

Target species

Most AFA are not absorbed from the gut and/or are given at comparatively low dosages. Toxic reactions in the target species would therefore normally not be expected. Exceptions are the quinoxalines (see chapter 5), halofuginone, nicarbacin and the ionophores (see chapter 7).

The quinoxalines cause adrenal damage in pigs leading to hypoaldosteronism at doses and exposure times well within the range of those permitted for growth promotion. This has been clearly documented in experimental settings but no field reports have been found. Symptoms of intoxication may be overlooked as they typically consist of dry faeces and increased thirst.

Halofuginone interferes with collagen synthesis resulting in loss of skin tensile strength. This leads to skin scratches and sores in live birds and skin tears during slaughter and processing, leading to reduced carcass quality. Unpublished Swedish observations when this product was used in the middle of the 80s are consistent with these findings. Due to problems with carcass quality that were put in connection with the use of halofuginone, the product was abandoned by the industry (Engström, B.[2] personal communication 1997)

Nicarbacin interferes with the thermoregulatory balance at high ambient temperatures. The effect is dose dependent. In regions where climatological conditions are such that critical temperatures are seldom reached, this problem is not likely to occur.

The ionophores have a narrow safety margin, often in the range of 2-3 times the recommended dose. Toxic effects are directed mainly against skeletal muscle and/or cardiac muscle. Numerous reports on intoxication of poultry due to accidental overdose with ionophores have been published. The prevalence of this condition may be underestimated due to diagnostic difficulties.

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[2] Björn Engström, Department of Poultry, National Veterinary Institute (SVA) Sweden

Exposure of non-target species, e.g. by accidental intake, may have serious consequences but is not likely to present a substantial risk under good manufacturing practices.

Residues

As mentioned above, most AFA are not absorbed from the gut and/or are given at comparatively low dosages. Residues at levels potentially harmful for the consumer would therefore normally not be expected. Exceptions to this are the quinoxalines and the nitroimidazoles.

Quinoxalines and their metabolites are mutagenic *in vitro* and are potentially genotoxic (FAO/WHO, 1995). Genotoxic substances cause chromosomal aberrations in eukaryotic cells. Due to the additive effects of such aberrations, even a very low dose may pose a risk. Acceptable daily intakes (ADI) have not been set.

Nitroimidazoles have shown mutagenic and genotoxic potential in several tests and their carcinogenic potential cannot be ruled out. Neither JECFA, not CVMP could establish an ADI.

Low, or very low concentrations of AFA residues in food might elicit allergic reactions in sensitised individuals. Such low concentrations might be present in or on animal products due to absorption from the gut or to faecal contamination. The population at risk for such allergic reaction is likely to be very small and the cause of the reaction would be difficult to determine. No reports of food related allergy implicating AFA approved in EU have been found.

Occupational hazards

Several AFA (macrolides, quinoxalines, bacitracin) are potent antigens and occupational exposure on a daily basis can lead to sensibilisation. Occupational contact dermatitis and/or asthma seem to be rather frequent as judged by reports in the literature. Allergic reactions have also been reported for amprolium, meticlorpindol, robenidine and halofuginone. Airborne antigen or direct contact is thought to be the main cause for these reactions. As the handling of feed and litter inevitably involves formation of airborne particles, the exposure of the population at risk must be regarded as high. Usage of special galenic formulations of premixes may reduce the exposure.

As for the remaining AFA and coccidiostats, no reports on allergic reactions have been found. However, the causal diagnosis of such reactions can be extremely difficult. At least in Sweden, there is no report system for side-effects in humans caused by substances used in animals. Thus, the absence of reports does not exclude the possibility of such hazards.

Concerning the potential genotoxicity of nitroimidazoles and quinoxalines, not only the safety for the consumer should be considered. When these substances are used on the farm, humans are exposed to low doses on a daily basis. The exposure by inhalation or other routes during the handling of feed and manure is not known. The possibility of additive effects has to be borne in mind, as farmers are often exposed not only to these but to many, potentially offensive chemicals.

8.4   Safety for the environment

AFA are typically administered over a long period. Large groups of animals are involved and many additives are poorly absorbed and therefore excreted to a considerable extent. The effects will depend on the length of time that the substance in question persists in the environment. Persistence may lead to increase in concentrations over time, due to bioaccumulation.

In order to assess the exposure in various environmental compartments, the fate of the AFA in the ecosystem has to be known. A model for antibiotic release from feedlots was presented by Addison (1984) in which volatilisation, degradation, diffusion, adsorption to sediment, losses to ground water and streams etc. is calculated. This model clearly illustrates the complexity of the matter.

Table 8.VI shows some hazards for the environment related to AFA usage that can be envisaged.

Table 8.VI. Potential hazards and consequences of AFA usage for the environment

Tabell 8.VI. Potentiella faror och konsekvenser för miljön orsakade av AFT användning

Hazards	Consequences	Likelihood
Toxic effect on terrestrial fauna	loss of diversity reduced soil fertility reduced decomposition	low
Toxic effect on aquatic fauna	loss of diversity reduction of fish numbers	low
Toxic effect on plants	loss of diversity reduction of crops	low
Toxic effect on microflora	loss of diversity reduced soil fertility	high but transient
Co-transfer of genes	increased virulence of plant pathogens	unknown

Most AFA are likely to be degraded by soil microbes, as they are antibiotics, produced by microorganisms. According to available literature, most antibiotic substances appear to have a half life in soil of about 2-3 weeks at 20°C, while lower temperatures generally cause a slower

degradation. As light and temperature are important variables regarding degradation times, separate risk assessments may be necessary for different geographic areas. Synthetic substances such as quinoxalines are more likely to persist in the environment.

Concentrations in manure would be expected to be too low for any toxic effects on either terrestrial or aquatic fauna, provided that no bioaccumulation occurs. If the slurry concentration of the substance is 50 ppm, the concentration in soil would be roughly 1 ppm with an application of manure of 25 tonnes/hectare and a ploughing depth of 10 cm (table 8.VII). This is in most situations unlikely to have an effect on eukaryotic cells. However, as the minimum inhibitory concentration for naturally susceptible soil microbes is in the same magnitude, a temporary disruption of the microflora would be expected. This effect has been demonstrated as a transient reduction in nitrification when a low concentration of tylosin was added to soil (Bewick, 1978).

Table 8.VII. Predicted environmental concentration (ppm) in soil at various concentrations of AFA in slurry[1]

Tabell 8.VII. Uppskattade AFT koncentrationer i jord (ppm) efter gödsling med gödsel med olika AFT-koncentrationer[1]

AFA in slurry (ppm)	Predicted environmental concentration (ppm) at plough depth of:	
	5 cm	10 cm
1	0.03	0.02
10	0.3	0.2
25	0.8	0.4
50	1.7	0.8
75	2.5	1.3
100	3.3	1.7

[1]Application of 25 tonnes slurry per ha, soil density $1.5g/cm^3$
[1] *Applikation av 25 ton flytgödsel per ha, jorddensitet 1,5g/cm^3*

As discussed in chapter 4 and 6, the environment will also serve as a reservoir for resistance genes and bacteria carrying these genes. Resistance in itself is not a problem for the environment, as the consequence of resistance is impairment of therapy. If the resistance genes are located on DNA segments harbouring virulence genes, transfer between microorganisms present in soil or water could theoretically lead to an increase in virulence of plant or wildlife pathogens. This could ultimately increase the prevalence or severity of diseases. No information is available on the occurrence of such events.

Accurate risk-assessment of environmental effects of AFA is hampered by the circumstance that the most extensive data bearing on the effects of AFA are those gathered by commercial organisations when seeking regulatory approval, and that these data are dealt with confidentially between the

organisations and the regulatory agencies. It would be in the public interest, and would help to strengthen confidence in regulatory decisions, if these data were made publicly available under arrangements that protect the legitimate commercial interests of the companies concerned.

8.5   Prophylactic effects of AFA

Many authors have discussed increased animal health problems due to infectious diseases when antibacterial feed additives are withheld (e.g. McOrist, 1997; Viaene, 1997). This poses questions as to what degree antibacterials at their permitted dosages for growth promotion also have therapeutic and/or prophylactic effects.

Permitted AFA dosages are usually around 20 ppm (range 1-100 ppm) which for most substances is substantially lower than therapeutic doses, but there is an overlap between dosages permitted for growth promotion and recommended doses for prophylaxis and therapy. Even at a "low" dosage they will have antimicrobial effects in the intestine, since minimum inhibitory concentrations for normally sensitive bacteria are around 1-5 ppm (see 3.2 and 4.2)

The Swedish experience from banning antibacterial growth promoters in 1986 was the emergence of clinical problems and disturbances of the health status of piglets and broilers which initially created an increased demand for medicated feed with antibiotics at therapeutic dosages. By changing feed composition, improvements in hygiene, changing rearing strategies etc. this is now largely corrected for (Wierup, 1996).

Important animal diseases for which preventive effects of AFA at the dosages given for performance enhancement have been described include necrotic enteritis in poultry, swine dysentery, bacterial enteritis and porcine proliferative enteritis (see chapter 3.2). Several antibacterial feed additives also have indications as therapeutic drugs for these animal diseases.

According to the available literature there are no indications that the basic mode of action of AFA is different from that of antibacterials when used for prevention or therapy. The growth promoting effect can be ascribed to a reduction of pathogen load and the animal's response to this. This prophylactic effect is applicable whether the manifestastion of the pathogen is clinical or subclinical.

Documentation on prophylactic and/or therapeutic effects of AFA is not included in the application for approval and such effects are therefore not evaluated in the approval process.

8.6   The risks and the benefits

8.6.1   The benefits

The major beneficial effect of AFA is their prophylactic and therapeutic effects in relation to some bacterial diseases. However, according to article 7 in directive 70/524/EEC such properties are only allowed for coccidiostats and other medicinal substances. It is questionable whether this lack of coherence between regulations and the actual situation caters for consumer confidence built on transparency.

   Usage of AFA improves feed utilisation and growth rate. This will generate an economic profit and, depending on its distribution, producers, consumers or others may benefit. Other sequels to AFA usage are reductions in manure and nitrogen output. This may be seen as beneficial from an environmental point of view. However, all these positive effects can also be achieved by other means.

8.6.2   Indirect long-term losses and animal welfare aspects

AFA have been used for over 4 decades and this has undoubtedly contributed to the development of the current systems for animal production.

   Use of AFA caters for high stocking rates which from an economic point of view is favourable but from a disease point of view raises serious questions about disease control. Examples of situations where animal density create problems are the control of outbreaks of epizootic diseases (e.g. swine fever), zoonotic diseases (e.g. salmonella) and production diseases (e.g. swine dysentery). Only certain diseases in the latter group are prevented by AFA usage. These high density production systems have also been questioned from the point of view of animal ethics and animal welfare. Neither animal welfare nor animal health are created through administration of antibacterials. A sound approach from an animal welfare point of view is to rear animals employing management strategies and animal care of high standard. A modern Animal Welfare Act is also an Animal Health Act.

   AFA are considered to be part of a production model in which productivity is over-emphasised. Clearly, a development towards more health-orientated production systems is needed.

   Agriculture must, as all other sectors, aim towards a sustainable development. This means the development of production systems that meet the need of the present without compromising the ability of future generations to meet their own needs. In essence, sustainable development is a process of change in which the exploitation of resources, the direction of

investments, the orientation of technological development, and institutional change are all in harmony and enhance both current and future potential to meet human needs and aspirations.

In view of the above, it is questionable whether continued reliance on usage of AFA favours a sustainable development of animal production systems. Non-usage of AFA in a region or a country would contribute to an innovative and dynamic climate for finding measures to raise healthy animals in the absence of AFA without compromising the efficiency of the production.

The future of the Common Agricultural Politics was addressed by Mr. Jacques Santer, President of the European Commission, in a speech to the European Parliament on February 18, 1997. He made the following statement:

"The starting point for the reform will be the idea that there must be a greater focus in European agriculture on quality, protection of the environment, animal welfare, a return to more natural production methods and a simplification of Community law."

8.6.3   The risks

The main hazard associated with use of AFA is increased resistance of commensals and pathogens. The consequence of this might be impairment of therapy in both animals and man. Transmission of resistance genes between animal and man can and does occur. The risk for loss of effectiveness of therapeutical drugs has been estimated to be far from negligible. The estimate is uncertain due to the complexity of the problem and to lack of pertinent data. Consequently, the magnitude of the risk can presently not be fully established. In order to reduce some of the uncertainties, the following information needs to be on hand:

Exposure
- exposure of animals to AFA
- exposure of animals to therapeutic antimicrobials
- exposure of man to therapeutic antimicrobials
- prevalence of relevant resistance genes in animals
- prevalence of relevant resistance genes in animal products
- prevalence of relevant resistance genes in the environment
- prevalence of relevant resistance genes in man
- dose response relationship between antimicrobial use and prevalence of resistant genes

Transmission/epidemiology
- identification of relevant resistance genes
- identification of transfer mechanisms of relevant resistance genes
- transfer rates of relevant resistance genes between animal and human bacteria in realistic settings
- transfer rates of relevant resistance genes between animal and environmental bacteria in realistic settings
- transfer rates of relevant resistance genes between human and environmental bacteria in realistic settings
- persistence of relevant resistant genes in human or animal hosts in the absence and presence of a selective pressure

Consequences
- incidence of co-transfer of resistance and other relevant genes
- probability of acquiring an infection caused by a bacterium carrying the relevant resistance gene
- probability of the infection above requiring therapy with the relevant substance

Needless to say the above information is required for each resistance gene and for each antimicrobial substance. The minimum time required to undertake this research would be 5-10 years. Whenever the conditions change or new information or techniques become available parts of the above given studies will have to be repeated and the risk assessment updated.

The economic benefits of AFA in livestock production in EU has been estimated to 2 billion ECU annually. In view of this it is surprising that the data necessary for a risk assessment of the most controversial hazard are still lacking as information on the level of risk is essential for the consumer. A search through scientific databases shows that the number of publications available on performance enhancing effects of AFA is more than 10-fold the sum of publications on microbiological effects. At the present stage it is impossible to fully quantify the risk.

Risk-assessment of environmental effects of AFA is hampered by the fact that the information necessary is not publicly accessible. Based on available information, the usage of most AFA does not appear to be associated with major risk for the environment.

Other hazards that can be identified for some AFA and medicinal feed additives (MFA) are toxic effects. Halofuginone and the quinoxalines have toxic effects on the respective target species. In addition, many AFA and MFA are potent allergens and thereby present an occupational hazard and possibly a consumer hazard. The risk for the latter is assumed to be very small.

Quinoxalines and nitroimidazoles are potentially genotoxic. The use of such substances in animal production is highly questionable. The primary

risk group is persons handling the substances including feedingstuffs. The nitroimidazoles have been banned as veterinary drugs for food-producing animals through listing in annex IV of Council Directive 2377/90/EEC. This lack of coherence between regulations is also associated with a risk of losing public credibility.

8.6.4   Conclusions

It is the overall opinion of this commission that the benefits of antibacterial feed additives do not outweigh the risks. With regard to coccidiostats and other medicinal substances, they are specifically used to prevent disease and therefore they should be treated as pharmaceutical specialities, i.e. as medicated feed. Our opinion can be summarised as follows:

- Antibacterial feed additives have favourable economic effects on livestock production, but from a long term perspective these are questionable, especially regarding animal welfare and animal health. AFA can, at levels permitted in feedingstuffs, be used for treatment or prevention of animal diseases, which is in violation of Council Directive 70/524EEC.
- Further, the quinoxalines and the nitroimidazoles are potentially genotoxic thereby posing an occupational hazard.
- Halofuginone and the quinoxalines are toxic for target species. This is deleterious for the well being of the animals.
- The risk for increased resistance associated with the general use of antibacterials as feed additives is far from negligible and the potential consequences are serious for both animal and human health. Antibacterials that are not yet used as therapeuticals in human or veterinary medicine are valuable templates for future drugs. As emergence of resistance is considered to be a threat to animal and human health, all AFA should be restricted to medical and veterinary purposes only.

8.6.5   Considerations for risk management decisions

Although it is somewhat beyond the scope of this assignment, some preliminary ideas relating to possible actions to mitigate the risks involved have been noted for consideration.

As the risks involved are of uncertain magnitude, the decisions on risk management are particularly difficult. The risk can obviously not be excluded with certainty, nor can it be determined as acceptable. Scientists may declare that the information is inadequate for decision making, but for the policymakers, failure to take action is not a neutral position but represents a positive decision to do nothing. In a climate of uncertainty it is preferable to show caution.

The determination of what level of risk is acceptable is established by the public, not by scientists. Communication on the subject of risk, in form of a two-way exchange of information between scientists, decision-makers and consumers, is essential in order to establish the level of acceptance. In general, the risk acceptance tends to increase considerably if there is a possibility of control (i.e. choice) involved compared to a situation were one can not actively avoid the risk.

Another important aspect on risk management is to decide on who is to bear the cost - the risk maker or the risk taker. In the event of an over-cautious decision, the risk maker may suffer a cost through reduced benefits. On the other hand, inadequate risk management may lead to a cost for the risk taker.

In terms of risk management, a range of measures involving various degrees of intervention are available. Since AFA, like coccidiostats, are medical products they should be approved and used as pharmaceutical specialities and not as feed additives. Dual authorisations are not acceptable. A single system would reduce the administrative burdens. Further, the transparency of the system would increase. It is understood that animal production would need time to adjust to the situation, allowing for knowledge on appropriate changes in management and feeding practices to expand and to be implemented. Countries which today have restrictions in the use of AFA should be allowed to maintain these until the regulatory system can be fully harmonised.

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Annex A: Avilamycin

A.1   Introduction

Avilamycin is a mixture of oligosaccharides of the orthosomycin group, that are produced by *Streptomyces viridochromogenes*. Other members of this group include curamycin and everninomycins (Wolf, 1973). Avilamycin is mainly active against gram-positive bacteria. The compound is used for growth promotion in swine and poultry, at dosages ranging from 5 to 40 ppm for swine and from 2.5 to 10 ppm for poultry. It has never been used for therapeutic purposes in either human or veterinary medicine but a related compund, everninomycins, has been suggested for evaluation in human therapy (Chopra *et al.*, 1997; Nicas *et al.*, 1997). In rats and swine, oral avilamycin is primarily excreted in faeces (Magnussen *et al.*, 1991).

A.2   Mode of action and resistance mechanisms

Avilamycin acts on the bacterial ribosome, by inhibiting the binding of formylmethionyl-tRNA to the 30 S ribosomal subunit (Wolf, 1973). This blocks the formation of the 70 S initiation complex in bacterial protein synthesis. This inhibition occurs in *in vitro* ribosomal systems from both gram-positive and gram-negative bacteria (Wolf, 1973), so the difference in avilamycin susceptibility of gram-positive and gram-negative bacteria is probably due to differences in factors outside the protein synthesizing system, like cell wall composition. Experimental data indicate that avilamycin interferes with the attachment of tRNA to the ribosome by binding to the 30 S subunit (Wolf, 1973).

   No data has been published on mechanisms of resistance, but it seems logical to assume that structural changes in the ribosomal 30 S subunit or ribosomal protection could confer resistance to avilamycin in naturally susceptible bacteria. In view of the ease and speed at which resistance to some other anti-ribosomal antimicrobials (e.g. aminoglycosides and tetracyclines) has emerged, it is very important to investigate possible resistance mechanisms to avilamycin and how these may interfere with the effect of other anti-ribosomal substances.

   Full cross-resistance to everninomycins would be expected in avilamycin-resistant bacteria, as the two compounds are structurally very similar (see figure A.I). Cross-resistance to unrelated antimicrobials in avilamycin-resistant bacteria has not been reported, but there is no information on

The figures in this report is only available in the printed version

Figure A.I. Tentative sketch of avilamycin and everninomycins

possible cross-resistance to antimicrobials with a similar site of action (i.e. the binding site for formylmethionyl tRNA), such as viomycin and capreomycin, among others. Cross-resistance between structurally unrelated compounds may be seen when these compounds have the same site of action. Even when the sites of action are not identical, cross-resistance could, theoretically, appear if structural changes in the ribosome, or protecting proteins, affect both antimicrobial binding sites. In view of this, knowledge of resistance mechanisms is necessary in order to asses possible risks regarding resistance.

A.3   Development of resistance

Unfortunately, only two publications concerning bacterial susceptibility to avilamycin have been found. This is hardly enough to illustrate the prevalence of avilamycin resistance, but the data from these studies are compiled in table A.I.

Table A.I. Reported prevalence of avilamycin resistance

Bacterial species	Source of isolates	No. Of isolates	Year(s)	Resistance in %	Reference	Country
Clostridium perfringens	various	95	1991	0	Devriese *et al*, 1993	Belgium
Staphylococcus hyicus	swine	71	1995-96	0	DANMAP, 1997	Denmark
Staphylococcus aureus	cattle	211	1995-96	0	DANMAP, 1997	Denmark
Coagulase negative staphylococci	cattle	371	1995-96	0	DANMAP, 1997	Denmark
Enterococcus faecalis	swine	225	1995-96	1	DANMAP, 1997	Denmark
Enterococcus faecium	swine	58	1995-96	2	DANMAP, 1997	Denmark
Enterococcus faecium	poultry	54	1995-96	69	DANMAP, 1997	Denmark
Enterococcus faecium	cattle	13	1995-96	0	DANMAP, 1997	Denmark

In Denmark, 84 % of the annual consumption of avilamycin in feed is used in poultry, which is reflected in the differences in resistance to this compound in *E. faecium* from cattle swine and poultry, respectively. The consumption of avilamycin increased four-fold between 1994 and 1995, coinciding with its introduction into poultry feed. Unfortunately, there is no data available on avilamycin resistance in *Enterococcus faecium* isolates collected before this increase in the consumption. As no data has been published on the development of resistance, it is not possible to evaluate the risk of this. An adequate amount of this type of data is essential in risk evaluation and should be made available as soon as possible.

A.4   Acquisition of resistance

No published information has been found about genes conveying resistance to avilamycin, transfer of avilamycin resistance, or bacterial hosts for resistance genes. If no such investigations have been undertaken, they should be planned immediately.

A.5   Impact of resistance on animal and human health

As avilamycin is not yet used for therapy in humans or animals, resistance would not be expected to cause clinical problems unless cross-resistance to other substances was present. However, in the future orthosomycin compounds may well be of interest for clinical use. For example, everninomycins have been suggested as a new important candidate for use in human therapy (Cormican and Jones, 1996; Urban *et al.*, 1996; Chopra *et al.*, 1997; Nicas *et al.*, 1997). The impact of avilamycin resistance on the life span of future drugs is difficult to assess, but could be substantial.

A.6   Other effects on the microflora

A.6.1   Salmonella

Hinton (1988) investigated the effect of in-feed avilamycin on salmonella colonisation in chickens. Groups of 10 chickens were given avilamycin in the feed at concentrations of 2.5 or 10 ppm, alone or together with monensin. Medicated birds were compared to non-medicated controls. Four replicate experiments were performed, in two study designs, where the birds were either initially infected on the day of purchase or one week later. All birds

were infected with *Salmonella* Kedougou in the feed for two weeks, at concentrations from 1.6 to 176 bacterial cells per g feed. All birds in the same replicate experiment received the same dose of organisms. Samples for bacterial culture were taken on day 7 and 14 after the introduction of infected feed. The author concluded that no evidence was obtained to suggest that avilamycin, at concentrations of 2.5 or 10 ppm in the feed, favoured colonisation of the intestinal tract in chickens with *S.* Kedougou when they were challenged with this organism in the feed.

   This is the only published study on the effect of avilamycin on intestinal salmonellae. One single study, no matter how well performed, is hardly enough to form the basis for any definite conclusions, especially when this study did not result in any clear evidence as to whether avilamycin presents a risk in this aspect or not.

A.6.2   Other enteric pathogens

No publications on the effects of avilamycin on other enteric phogens have been found.

A.7   Effects on specific animal diseases

Avilamycin at growth promoting levels has been shown to reduce the amount of *Clostridium perfringens* in the intestinal tract of chickens (Elwinger *et al.*, 1993; Elwinger *et al.*, 1995) and may thus be used prophylactically against necrotic enteritis in poultry. Kyriakis (1989) investigated the effect of avilamycin at 40 or 80 ppm in the control of stress-induced post-weaning diarrhoea in piglets. The author stated that avilamycin at 80 ppm had a significant (p<0.05) effect in reducing diarrhoea and mortality in newly weaned piglets. Even at the level of 40 ppm, which may be used for growth promoting purposes in piglets, avilamycin notably reduced diarrhoea, although these figures were not statistically significant. Mortality was, however, significantly (p<0.05) reduced in the piglets that received 40 ppm avilamycin in the feed. At 40 ppm, avilamycin did not have a growth promoting effect in this experiment. Thus, in this study design, with piglets that were already sick, avilamycin served as a therapeutic or prophylactic agent and not as a growth promoter.

A.8   Toxicological aspects

No information about possible toxic effects either on the target species or on humans has been found. Such effects may be totally absent or, as it is a

comparatively new compound, reports on allergy and other side effects may not yet have appeared.

One publication (Magnussen *et al.*, 1991) has been found concerning residues. The results in this study indicate that avilamycin fed to swine at a concentration of 60 ppm gives rise to small but measurable residues in tissues. At zero withdrawal time the residue levels were 0.14 ppm in muscle, 0.66 ppm in liver, 0.34 ppm in kidney and 0.55 ppm in fat. There is no available information to suggest that this would present a risk for the consumer.

A.9   Environmental effects

Source separated municipal solid waste and agricultural waste can be utilised for biogas production. Substances with an antimicrobial effect against anaerobic bacteria could disturb this process. In studies on manure from pigs and poultry fed avilamycin, Sutton (1989) reported efficient operation of experimental and large mesophilic digesters. The presence of avilamycin appeared to alter the metabolism of the microflora, increasing the efficiency to degrade volatile solids.

No other publications on possible environmental effects of avilamycin have been found. As the compound is produced by a soil microbe, it would be expected to be microbially degraded in soil.

A.10   Summary comments

Use of avilamycin in poultry appears to have caused an increase in avilamycin resistance in *E. faecium* from this animal species. This resistance might have appeared after only a few years of avilamycin use. Avilamycin is closely related to everninomycins, and cross-resistance could shorten the therapeutic life span of everninomycins if they were to be used in human therapy. The overall information about the possible effects of avilamycin in various aspects is much too scarce to form the basis of a risk assessment.

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Annex B: Bacitracin

B.1   Introduction

Bacitracin is a complex mixture of cyclic polypeptides produced by *Bacillus subtilis* and *Bacillus licheniformis*. The compound has bactericidal effect on gram-positive bacteria but little activity against gram-negative organisms (Prescott and Baggot, 1993). It is most commonly used in complex with zinc which seems to stabilise the antibiotic complex (Quinlan and Gutteridge, 1989).

Bacitracin is poorly absorbed from the gastrointestinal tract (Donoso *et al.*, 1970; Froyshov *et al.*, 1986), as well as from skin and mucosal surfaces. Absorbed bacitracin is excreted by glomerular filtration (Prescott and Baggot, 1993).

The substance is used in human therapy, mostly for topical treatment of superficial infections of the skin and mucosal surfaces. However, its effectiveness against vancomycin-resistant enterococci has led to an increase in its use for oral treatment (O'Donovan *et al.*, 1994; Chia *et al.*, 1995). In veterinary medicine, bacitracin has been suggested for the treatment and/or prevention of proliferative adenomatosis in swine (Kyriakis *et al.*, 1996), swine dysentery (Jenkins and Froe, 1985), and for clostridial infections in man (Caputo *et al.*, 1994) and various animal species (Carman and Wilkins, 1991; Prescott and Baggot, 1993). Two diseases may be of particular interest in this respect, namely proliferative adenomatosis in swine and necrotic enteritis in poultry (Prescott and Baggot, 1993).

Recommended dosages for prophylaxis and therapy in poultry are in the range between 50 and 200 ppm, and for swine around 250 ppm (Prescott and Baggot, 1993). The corresponding dosages permitted for growth promotion are between 5 and 100 ppm for poultry, and between 5 and 80 ppm for swine. Growth promoting dosages for calves, lambs and kids are in the range of 5-80 ppm and for fur animals 5-20 ppm.

Bacitracin is widely used for laboratory purposes, in selective media for bacterial culture and in cell culture media. It is also used experimentally as a protease inhibitor (Fukuda *et al.*, 1995).

The figures in this report is only available in the printed version

Figure B.I. Tentative sketch of bacitracin

B.2   Mode of action and resistance mechanisms

Bacitracin inhibits the formation of bacterial cell wall peptidoglycan by complexing directly with the lipid isoprenyl pyrophosphate (IPP) carrier, inhibiting the dephosphorylation reaction that is required for its regeneration. This leads to accumulation of phospholipids inside the cell and inhibition of cell wall formation. Proposed resistance mechanisms include active efflux, increased production of IPP kinase, suppression of autolytic systems, reduced membrane permeability and suppressed exopolysaccharide secretion (see below).

   No information about cross-resistance to other substances has been found. Cross-resistance might be expected in some cases, like when autolytic systems are suppressed. This would be expected to convey cross-resistance to other cell wall inhibitors. Reduced membrane permeability might convey cross-resistance to substances with similar diffusion properties or mechanisms for uptake.

   It has been claimed that bacitracin "cures" resistance against other antimicrobials and that its use as a feed additive would therefore be purely beneficial from a resistance point of view (Walton, 1978; Gedek, 1981; Walton, 1984; Walton and Wheeler, 1987). However, due to weaknesses in study design in relation to the specific question to be answered, as well as

inconsistent results, the only conclusion that can be drawn from these studies is that bacitracin does not seem to induce resistance to any of the other, unrelated, antimicrobials tested. However, as resistance to bacitracin was not determined or characterised, the results provided little information about the potential for cross-resistance. Some of the studies mentioned show synergistic effects of bacitracin together with other antimicrobials, but do not investigate any effects on actual resistance genes. Undoubtedly, antimicrobials interfering with cell wall synthesis, such as bacitracin, may act synergistically with other antimicrobials that exert their effects inside the bacterial cell. One must still keep in mind that this most likely has to do with the combined mechanisms of the two antibacterials and does not mean that any resistance genes have been eliminated.

B.3   Development of resistance

B.3.1   Prospective studies

Only two studies that deal specifically with development of bacitracin resistance over time have been found (Linton *et al.*, 1985; Kaukas *et al.*, 1988). These include one experimental study and one field study.

   In the study by Kaukas *et al.* (Kaukas *et al.*, 1988), groups of 10 chickens were fed different antibiotics, with one group serving as a non-medicated control group. The bacitracin group was given a dosage of 40 ppm bacitracin in the feed. Enterococci were isolated from cloacal swabs, identified and analysed for antimicrobial susceptibility. When comparing the incidence of bacitracin resistance in enterococci from medicated birds to that in enterococci from non-medicated birds, bacitracin exposure seems to have had little effect on the incidence of resistance in *Enterococcus faecalis* and *E. gallinarium*. *E. faecium*, however, showed an incidence of resistance to bacitracin as high as 47% in the medicated group, while in the non-medicated group the incidence was 29%. This difference was statistically significant ($p<0.005$), and indicates that bacitracin in the feed selects for resistant strains of *E. faecium.* Further, the incidence of resistance to therapeutic antibiotics, expressed as the Antibiotic Resistance Index (ARI) was significantly ($p=0.003$) higher in all groups receiving antibacterials, as compared to the control group. This increase was most likely associated with an increase in the proportion of *E.faecium* in the enterococcal population of the treated birds.

   In the publication by Linton *et al.* (Linton *et al.*, 1985), the first part is a field survey comparing five commercial premises and a total of nine batches of broiler chickens. One farm, where one batch of birds was reared, used feed with bacitracin at the recommended level for growth promotion. No non-medicated controls were included and no information on whether

coccidiostats were used was provided. Cloacal swabs were taken at the beginning and at the end of the rearing period and isolated enterococci were tested for antimicrobial susceptibility. The percentage of strains resistant to bacitracin, among the strains isolated from birds fed bacitracin, increased from 87% to 100% during the rearing period. However, the enterococci were not identified to the species level and no information is provided on how many birds were sampled in each group in this experiment. This, taken together with the fact that there was no non-medicated control group, makes the results hard to evaluate.

The second part of the same publication describes an experimental study, where enterococci were also tested for their susceptibility to bacitracin. Bacitracin was not a factor of the experiment but one of the four groups of chickens was a non-medicated control. In this group, the proportion of resistant strains varied between 20% and 90% during the rearing period, which indicates that a day to day variation is not unusual and must be taken into account when evaluating this type of data.

B.3.2   Point-prevalence studies

Data from different studies on prevalence of resistance to bacitracin have been compiled in table B.I. Unfortunately, most studies do not report MIC values and there seems to be some confusion about what should be the break-point value for different bacteria. Further, the number of strains included are often inadequate for an estimate of the prevalence of resistance. Bearing this in mind, it is noteworthy that the proportion of enterococcal isolates with presumably acquired resistance shows a range from 3-77% , varying between different animal species and different countries. To fully assess the possible importance of this, information on resistance mechanisms and transferability of resistance in enterococci is needed, as well as consumption figures for different animals and different countries.

Table B.I. Resistance to bacitracin in various bacterial species, reported in different studies

Bacterial species	Source of isolates	No. Of iso-lates	Year	Resis-tance in % *	Reference	Country
Clostridium perfringens	cattle	32	1991	*9*	Devriese et al, 1993	Belgium
C. perfringens	poultry	31	1992	*6*	Devriese et al, 1993	Belgium
C. perfringens	poultry	80	1984-86	*>50*	Benno et al, 1988	Japan
C. perfringens	swine	32	1992	*0*	Devriese et al, 1993	Belgium
Clostridium spp.	cattle	*	1979-82	*9*	Dutta and Devriese, 1984	Belgium
Clostridium spp.	poultry	*	1979-82	*6*	Dutta and Devriese, 1984	Belgium
Clostridium spp.	swine	*	1979-82	*1*	Dutta and Devriese, 1984	Belgium
Enterococcus faecalis subsp *liquefaciens*	poultry	23	1980	*21*	Dutta and Devriese, 1982	Belgium
E. faecalis	poultry	8	1980	*62*	Dutta and Devriese, 1982	Belgium
E. faecalis	poultry	60	1977	*17*	Barnes et al, 1978	UK
E. faecalis	swine	225	1995-96	*3*	DANMAP, 1997	Denmark
Enterococcus faecium	poultry	15	1980	*67*	Dutta and Devriese, 1982	Belgium
E.faecium	cattle	13	1995-96	*8*	DANMAP, 1997	Denmark
E.faecium	poultry	54	1995-96	*41*	DANMAP, 1997	Denmark
E.faecium	poultry	13	1977	*77*	Barnes et al, 1978	UK
E.faecium	swine	58	1995-96	*31*	DANMAP, 1997	Denmark
Enterococcus spp.	humans	9	1992-1993	*11*	Everett et al, 1995	USA
Streptococcus spp.	humans	50	1992-1993	*2*	Everett et al, 1995	USA
Staphylococcus aureus	cattle	211	1995-96	*0*	DANMAP, 1997	Denmark
S. aureus	various	324	1970-80	*<1%*	Devriese, 1980	Belgium
S. aureus, methicillin resistant	humans	106	1989	*2*	Maple et al, 1989	various
S. hyicus	swine	71	1995-96	*0*	DANMAP, 1997	Denmark
Coagulase positive staphylococci	humans	119	1992-93	*17*	Everett et al, 1995	USA
Coagulase negative staphylococci	cattle	371	1995-96	*0*	DANMAP, 1997	Denmark
Coagulase negative staphylococci	humans	261	1992-93	*6*	Everett et al, 1995	USA

* total number of strains for cattle, poultry and swine = 192

B.4   Acquisition of resistance

Published information on bacitracin resistance is sparse, but some hitherto identified mechanisms of resistance to bacitracin are shown in Table B.II.

Table B.II. Mechanisms of bacitracin resistance

Bacterial species	Gene	Mechanism	Reference
Bacillus licheniformis	*bcr*	active efflux	Podlesek et al, 1995
plasmid pXV62, original bacterial source not given	*bac* A	production of IPP kinase	Cain et al, 1993
Enterococcus spp.	not identified	suppressed autolytic system	Krogstad and Pargwette, 1980*
various gram-positive and gram-negative bacteria	not identified	reduced membrane permeability	Mukherjee et al, 1989
various gram negative bacteria	not identified	suppressed exopolysaccharide secretion	Pollock et al, 1994

*this is the mechanism proposed although not proven in the article

   Podlesek and co-workers (Podlesek *et al.*, 1995) characterised a resistance gene, *bcr*, in *Bacillus licheniformis* that codes for proteins forming an ATP-binding transport system in the cell membrane. The proposed action of this transport system is active efflux of the bacitracin molecule. Another resistance gene, located on plasmid pXV62, named *bac*A (Cain *et al.*, 1993), proposedly encodes a phosphokinase involved in IPP metabolism. A similar enzyme has been characterised in *Staphylococcus aureus* (Sandermann Jr and Strominger, 1971), although its possible effects on the bacitracin susceptibility of this bacterium was not investigated. Resistance to bacitracin due to altered cell membrane permeability has been reported (Mukherjee *et al.*, 1989). Resistance due to suppressed autolytic enzyme systems has also been suggested (Krogstad and Pargwette, 1980). Suppression of autolytic enzymes makes the bacterium resistant to substances that inhibit peptidoglycan synthesis (Tomasz *et al.*, 1970; Krogstad and Pargwette, 1980).
   Other mechanisms for reduced bacitracin susceptibility that have been suggested include increased production of the carrier IPP, which may competitively overcome the inhibitory effect of bacitracin on peptidoglycan synthesis, and cessation of exopolysaccharide synthesis (Pollock *et al.*, 1994). The excretion of polysaccharides require the same carrier IPP that is needed for the synthesis of peptidoglycan and a halt in this excretion will leave more IPP available for cell wall synthesis. This will also occur in the

absence of essential components required for exopolysaccharide synthesis, e.g. in an environment depleted of certain sugars (Pollock *et al.*, 1994).

Transfer of bacitracin resistance seems to have attracted little attention from researchers. Transduction between strains of *Streptococcus pyogenes* has been shown to occur (Stuart and Ferretti, 1978). However, this study did not investigate what gene(s) and mechanisms were involved in the observed resistance. No other studies, concerning transfer or non-transfer of bacitracin resistance have been found. Bacitracin, like other substances that inhibit late stages in peptidoglycan synthesis, has been shown to induce the expression of the vancomycin resistance gene, *van*A in enterococci (Allen and Hobbs, 1995; Lai and Kirsch, 1996). The practical aspects of this are not clear but it is not likely to be of any clinical importance.

B.5   Effects on specific animal diseases

Some reports indicate that bacitracin, even at concentrations used for growth promotion, may prevent necrotic enteritis in poultry (Wicker *et al.*, 1977; Prescott *et al.*, 1978; Stutz *et al.*, 1983). Stutz and co-workers (1983) found that supplementing a soybean protein and sucrose-based diet with levels of 5.5, 16.5, or 55 ppm of bacitracin significantly reduced the number of *Clostridium perfringens* organisms in the ileal contents of chicks (p<0.05). Prescott and co-workers (1978) reported that inclusion of bacitracin at 200 or 400 mg/gallon in the drinking water was effective in treating experimentally induced necrotic enteritis in chickens and incorporation of 100 mg/gallon in the drinking water prevented its occurrence. Wicker and co-workers (1977) found a significant (p<0.01) decrease in mortality due to necrotic enteritis in chickens given 11, 33 or 55 ppm bacitracin in the feed, as compared to non-medicated birds. These studies suggest that bacitracin at the concentrations used for growth promotion also has prophylactic and therapeutic effects on necrotic enteritis in poultry.

Some authors have investigated the possible effects of orally administered bacitracin on the immune response to certain forms of challenge (Harmon *et al.*, 1973; Wasinska, 1980). Harmon and co-workers (1973) compared non-medicated pigs to pigs fed bacitracin at a concentration of 55 ppm during various lengths of periods. After repeated intraperitoneal injections of sheep red blood cells or phenolised *Salmonella* Pullorum, serum antibody titers were tested by agglutination and hemagglutination. The authors stated that the immunological response to sheep erythrocytes was little affected by bacitracin in the feed, whereas the antibody response to *Salmonella* Pullorum was significantly enhanced by medicated feed. Various explanations for this were discussed in the paper.

Wasinska (1980) investigated the antibody response and resistance to infection after vaccinating pigs against erysipelas, colibacillosis and swine

fever. In addition, non-vaccinated controls were included in the study. Pigs were fed bacitracin at a concentration of 70 ppm for six months, and compared to pigs fed no antimicrobial. It was concluded that the supplementation of feed with bacitracin did not exert any negative effects on the antibody response in vaccinated pigs, nor did it decrease the effectiveness of immunisation, as determined by experimental infection.

B.6   Impact of resistance on animal and human health

Increased resistance to bacitracin in clostridia and enterococci could lead to therapeutic failures when bacitracin is used for the treatment of infections with these organisms in animals and humans. Without further information about the extent of the therapeutic use of bacitracin, the impact on human and animal health of such incidents is impossible to assess.

B.7   Other effects on the microflora

B.7.1   Effects on salmonella colonisation

Only a few published studies investigating the association between salmonella colonisation of the gut and bacitracin in the feed have been found (Nurmi and Rantala, 1974; Smith and Tucker, 1975; Smith and Tucker, 1980; Latour and Barnum, 1981; Humbert *et al.*, 1991; Manning *et al.*, 1994). All use poultry as the experimental animal species and in-feed bacitracin at the concentrations used for growth promotion. Unfortunately, like other studies in this area, most of these suffer from weaknesses in study design and the results are often inconclusive and inconsistent. The results in the study by Manning and co-workers (1994) indicated that in chickens fed 490 ppm bacitracin, a significantly ($p<0.05$) larger proportion were infected with salmonella at the end of the experiment, as compared to non-medicated birds. All animals were kept on used litter, and challenged with 10^6 organisms of *Salmonella* Enteritidis. The concentration of bacitracin used was considerably higher than what is used for growth promotion, and the selective culture technique used appears to be based on visual examination only. This makes the results difficult to evaluate.

  In the study by Humbert and co-workers (1991) bacitracin was given at a concentration of 50 ppm to groups of chickens, with chickens receiving no antimicrobial in the feed serving as controls. Another factor in the experiment was treatment with bacterial flora from 12-week old Specific Pathogen Free-chicks (competitive exclusion, CE). All birds were challenged

with 10^4-10^5 organisms of *Salmonella* Typhimurium. Half of the animals were killed on day 3 for bacteriological examination of the caeca. The rest were killed and examined on day 6. The results show a small increase in the amount of salmonella shed by medicated birds, but a slight decrease when the medicated birds were also given CE. However, in the groups not receiving CE treatment, the proportion of salmonella positive birds was 100% in both medicated and non-medicated groups. Thus, no conclusions on the influence on the prevalence of colonisation can be drawn and the study period of 6 days is too short for conclusions about differences in excretion time. The bacterial counts are presented as log means for each group, so it is not possible to determine whether the differences are caused by changes in the amount of excretion in just a few individuals or if it is an overall effect. Another minor weakness is the selective method used for salmonella isolation where the recording of salmonella was based on visual inspection and without further confirmation.

Nurmi and Rantala (1974) compared the re-isolation of *Salmonella* Infantis, after experimental infection, from the caeca of non-medicated birds and of birds fed 10 ppm or 20 ppm bacitracin with and without concurrent treatment with CE. The results indicate a decrease in the amount and prevalence of *Salmonella* shedding in birds receiving bacitracin, as compared to non-medicated birds. The feeding of bacitracin did not inhibit the positive effect of the CE treatment. The groups were very small and the percentage of salmonella shedding in the non-medicated control group varied between experiments making interpretation of the results difficult.

Smith and Tucker reported two studies including bacitracin (Smith and Tucker, 1975; Smith and Tucker, 1980). In the first (Smith and Tucker, 1975) birds were fed bacitracin at concentrations of either 10 or 100 ppm and inoculated with *S.* Typhimurium. Faecal samples were compared with samples from similarly infected non-medicated birds. It was found that the feeding of bacitracin only slightly increased the prevalence and length of salmonella shedding.

In another study by the same authors (Smith and Tucker, 1980) chickens were challenged with five different salmonella serovars (*S.* Heidelberg*, S.* Infantis*, S.* Oranienburg*, S.* Senftenberg and *S.*Typhimurium). The percentage of re-isolation of the challenge organism from faeces and caecal contents of birds fed bacitracin (10 ppm) was compared to that of non-medicated birds until 50 days of age. The study also included a comparison of salmonella infection in medicated and non-medicated birds of four different breeds fed four different feed mixes. The results indicate that bacitracin at worst slightly favours salmonella colonisation. No remarkable differences were noted between the various bacterial strains used for challenge, or between different feed mixes and different poultry breeds. The isolation procedure used in this study includes selective culturing and

identification by visual examination only. As in the study by Humbert and co-workers, it cannot be excluded that some coliforms can be mistaken for salmonella. Since one cannot be sure that this error would be the same in all groups, there is a slight risk that this may have affected the outcome of the study.

Latour and Barnum (1981) used ducks as experimental animals. Two concentrations of in-feed bacitracin were tested, 10 and 100 ppm, and medicated birds were compared to non-medicated birds. The challenge organism was *Salmonella* Typhimurium, 10^7-10^{10} organisms per bird, and cloacal swabs were taken throughout the experiment. The results did not show a definite trend. In two experiments there was little difference between the bacitracin-medicated groups and the controls and in two other the samples from medicated birds yielded significantly ($p<0.05$) more salmonellae than the untreated animals. However, the groups were small and the variability between the control groups in the various experiments regarding re-isolation of the infecting organism make the results difficult to evaluate.

Bailey and co-workers (1988) investigated resistance to salmonella infection in broilers fed various AFA. However, all groups of animals received combinations of AFA, together with competitive exclusion microflora, and therefore the effect of a single antibiotic (in this case, bacitracin) could not be determined.

No studies investigating the effect of bacitracin on the infectious dose necessary to achieve establishment of salmonella colonisation have been found, nor any studies investigating dose-response relationships or the effect on prevalence of salmonella in animal products.

B.7.2 Other enteric pathogens

No studies on the possible effects on colonisation by other enteric pathogens have been found.

B.8 Toxicological aspects

Bacitracin is highly nephrotoxic when administered parenterally (1993), but as it is poorly absorbed from the gut, no adverse effects would be expected after oral administration.

B.8.1 Adverse effects in ruminants

Bacitracin is used as a feed additive in calves, lambs and kids. Adult cattle, however, react adversely to bacitracin in the feed. Sudden milk drops, in

herds fed concentrate feed contaminated with bacitracin at the feed mill, have been reported (Woodger, 1979). Higher mortality and decreased effectiveness of antimicrobial therapy was noticed in calves fed 50 ppm bacitracin in the milkreplacement in a study by Jonson and Jacobsson (1973). It is discussed whether resistant bacteria or immunosuppression in the medicated animals were the cause, but no investigations to determine the cause were included in the article. In sheep, biotransformation of plant toxins, that occurs in the rumen of some alkaloid-resistant sheep, can be impaired by oral administration of bacitracin (Wachenheim *et al.*, 1992). No conclusions can be based on these few reports, but there seems to be cause for further investigations into the possible risks for adverse effects when using bacitracin in ruminants.

B.8.2 Allergy

Allergic reactions to bacitracin are frequently reported (Katz and Fisher, 1987; Grandinetti and Fowler, 1990; Knowles and Shear, 1995). Both anaphylaxis, eczema, urticaria and delayed reactions may be seen (Katz and Fisher, 1987). Most reports concern patients treated with bacitracin ointment. It has been stated that bacitracin is the topical agent most commonly implicated in anaphylactic reactions (Katz and Fisher, 1987). Considering this, it is somewhat surprising that no reports have been found on allergic reactions in people who come in contact with bacitracin professionally, such as farmers, hospital personnel and people working in the pharmaceutical industry.

B.9   Environmental effects

Like for other AFA, if bacitracin reduces the amount of feed consumed per kg weight gain in the target animal, it would also be expected to reduce the amount of nitrogen output per kg weight gain.

Very little of ingested bacitracin is absorbed and most is excreted unmetabolised in the faeces (Donoso *et al.*, 1970; Froyshov *et al.*, 1986). Gavalchin and Katz (1994) studied the degradation of bacitracin in sandy loam from a non-agricultural area, mixed with chicken faeces, at different temperatures. Sterile soil-faeces mixtures were used as controls. At 4°C, inactivation of bacitracin occurred rapidly, only 23% remained after 30 days. At 20°C, however, 33% of the initial concentration was still present after 30 days of incubation. The half life for bacitracin at 4 and 20°C was calculated to 12.5 and 22 days, respectively. The inverse temperature relation of the degradation was tentatively explained by the isolation of a psycrotrophic pseudomonad capable of degrading bacitracin from the soil-faeces matrix.

Similar results have been obtained earlier by Jagnow (1978) reporting half life in soil of 22,5 days at 20 °C and 12 days at 30°C .

Vogtmann and co-workers (1978) reported that bacitracin had no adverse effects on composting but detected some depression of plant growth when fresh manure containing bacitracin was used as a fertiliser.

B.10   Summary comments

Bacitracin has a bactericidal effect mainly on gram-positive bacteria, by inhibiting the formation of bacterial cell wall peptidoglycan. It is used, albeit not to any large extent, in both human and animal therapy. Lately it has been increasingly used for the treatment of vancomycin-resistant enterococci in humans.

In-feed bacitracin affects the antimicrobial resistance of the intestinal microflora, mainly in *E. faecium* but possibly also in other species.

Data available on colonisation by enteric pathogens in animals fed bacitracin is too inconsistent and too scarce to form the basis of any firm conclusions about the effects of bacitracin.

Bacitracin administered at growth promoting concentrations has prophylactic and therapeutic effects on necrotic enteritis in poultry.

Allergic reactions to bacitracin are documented in humans undergoing bacitracin treatment. People who are exposed to the substance on a daily basis may be at risk of being sensitised.

Bacitracin is degraded in soil. The environmental degradation appears to be inversely related to soil temperature.

In conclusion, available information is to scarce for an assessment of the possible risks of bacitracin usage to human and animal health. Bacitracin usage does not appear to represent any substantial danger to the environment.

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Annex C: Flavomycin

C.1   Introduction

Flavomycin, also known as flavophospholipol, bambermycins or moenomycin, is a phosphorus-containing glycolipid, mostly obtained as complexes of very similar components. It is produced by a group of *Streptomyces spp*, including *S. bambergensis*, *S. ghanaensis*, *S. geysirensis* and *S. ederensis* . At the moment it is used for growth promoting purposes only and is not included in any therapeutic drug either in human or veterinary medicine. Flavomycin inhibits cell wall synthesis, mainly in gram-positive bacteria.

Concentrations used for growth promotion are between 1 and 20 ppm for swine, 0.5-20 ppm for poultry, 2-4 ppm for fur animals, 2-16 ppm for cattle and 2-4 ppm for rabbits.

Flavomycin is not absorbed to any great extent after oral administration. When parenterally administrated it is excreted in the urine at a very slow rate and has, therefore, a prolonged activity in blood. The antimicrobial activity is reduced by serum and is optimal at pH 5.0 - 6.5. (Huber, 1979)

C.2   Mode of action and resistance mechanisms

Flavomycin exerts its effect by inhibiting bacterial cell wall synthesis. The transglycosylation reaction necessary for peptidoglycan synthesis, that is catalysed by the penicillin-binding protein PBP 1b, is impaired in the presence of flavomycin (van Heijenoort *et al.*, 1987, Huber, 1979 #339). In this reaction the lipid-bound N-acetyl glucosaminyl-N-acetylmuramyl-pentapeptide is transferred to the peptidoglycan.

No reports on mechanisms for resistance to flavomycin have been found. This is somewhat surprising, since investigations on resistance mechanisms and resistance genes might determine whether cross-resistance to flavomycin and other antibiotics is at all possible in any bacterial species. Hudd (1983) claimed that no cross-resistance had been found in staphylococci, but without presenting any data or information on how these results had been obtained.

Flavomycin is reportedly mainly active against gram-positive bacteria. However, several publications indicate that the compound is sometimes active against gram-negative organisms, such as *Salmonella* spp. and *Escherichia coli* (Dealy and Moeller, 1976; Dealy and Moeller, 1977b; Dealy and Moeller, 1977a; Witte, 1996). Unfortunatley, pure flavomycin for

laboratory use is not commercially available. Thus, no experiments could be performed to clarify this issue.

The figures in this report is only available in the printed version

Figure C.I. Tentative sketch of flavomycin

C.3   Development of resistance

C.3.1   Prospective studies

Unfortunately, and somewhat surprising, most studies where antimicrobial resistance patterns are determined in faecal bacteria, before and after the supplementation of feed with flavomycin, do not include any investigations on susceptibility to flavomycin itself in these bacteria.

A study by Dealy and Moeller (1977a) shows a significant ($p < 0.001$) increase in the percentage of flavomycin-resistant *E. coli* isolated from calves fed flavomycin at growth promoting levels, as compared to non-medicated calves. Since flavomycin is active mainly against gram-positive bacteria it would be interesting to see similar data on, for instance, enterococci.

Table C.I. Prevalence of resistance to flavomycin in various bacterial species

Bacterial species	Source of isolates	No. Of iso-lates	Year	Resistance in %	Reference	Country
Clostridium botulinum	various	*	1979-82	*0*	Dutta and Devriese, 1984	Belgium
Enterococcus faecalis subsp *liquefaciens*	poultry	23	1980	*0*	Dutta and Devriese, 1982	Belgium
E. faecalis	poultry	8	1980	*0*	Dutta and Devriese, 1982	Belgium
E. faecalis	poultry products	54	1995-1996	*0*	DANMAP, 1997	Denmark
E. faecalis	swine	38	1986-1995	*0*	Devriese and Haeseb-rouck, 1996	Belgium
E. faecalis	swine	225	1995-96	*0*	DANMAP, 1997	Denmark
E. faecalis	pork	38	1995-96	*6*	DANMAP, 1997	Denmark
E. faecalis	beef	21	1995-96	*19*	DANMAP, 1997	Denmark
Enterococcus spp.	swine	21	1986-1995	*71*	Devriese and Haeseb-rouck, 1996	Belgium
Coagulase negative staphylococci	cattle	371	1995-96	*0*	DANMAP, 1997	Denmark
Staphylococcus hyicus	swine	71	1995-96	*0*	DANMAP, 1997	Denmark
Staphylococcus aureus	cattle	211	1995-96	*0*	DANMAP, 1997	Denmark
S. aureus	various	792	1970-1980	*0*	Devriese, 1980	Belgium
Streptococcus spp.	swine	19	1986-1995	*21*	Devriese and Haeseb-rouck, 1996	Belgium

* total number of strains: *C.perfringens* = 142, *C. sporogenes* = 6, *C. botulinum* = 3

C.3.2   Point-prevalence studies

Data on prevalence of resistance to flavomycin from various studies are compiled in table C.I.

Some bacterial species are reported to be naturally resistant to flavomycin, including *Clostridium perfringens*, *Clostridium sporogenes* and *Enterococcus faecium* (Dutta and Devriese, 1984). However, susceptible strains of *E. faecium* have been reported (DANMAP, 1997). These discrepancies could be due to methodological differences regarding breakpoints etc.

C.4   Acquisition of resistance

No published investigations on resistance genes, transfer of resistance or resistance determinants in different bacterial hosts have been found.

C.4.1   Influence on resistance against other antimicrobials

George and Fagerberg (1984) investigated the effect of flavomycin on plasmid-mediated antimicrobial resistance in *E.coli,* and found that flavomycin decreased the transfer frequency of some R plasmids, while it increased transfer frequency of others. It also selectively inhibited growth of bacteria harbouring some R plasmids, but not others. The reason for this suppression of bacteria carrying some R plasmids is discussed in the article and the possibility of sex pili and pilin precursor proteins in the bacterial cell wall causing increased susceptibility to flavomycin in these bacteria is suggested. This theory is supported by the fact that some *E. coli* harbouring plasmids depressed for pili synthesis were not suppressed by flavomycin. If this is really the case, it would mean that flavomycin may have a suppressing effect on the spread of certain resistance plasmids. However, it would not affect transfer of resistance by transduction or transformation, nor would it decrease the spread of transposons carrying resistance genes.

A similar investigation, with similar conclusions was conducted by Sepulchre (1979). This study also includes *in vivo* experiments on effects of flavomycin in the feed on resistance in enteric microflora in pigs. However, the decrease in resistance observed in bacteria isolated from medicated pigs was also seen in the non-medicated control group, so the actual effect of flavomycin could not be determined.

Another study, by Pohl (1975), also showed decreased transfer frequency of some, but not all, R plasmids between *E.coli* strains in the presence of flavomycin.

Brophy (1988) conducted an *in vivo* study on the effect of in-feed flavomycin on prevalence of R plasmid-carrying *E.coli* in faecal samples from calves. In this experiment the introduction of flavomycin into the diet brought about a marked increase in the incidence of R plasmid-carrying *E. coli* and the number of these organisms isolated from the treated group was at any time during the trial notably higher than from the non-medicated control group. However, the results indicate that the incidence was higher in the treated group already at the beginning of the experiment. If this is correct, comparing the two groups without adjusting for the initial differences may be misleading.

Corpet (1984) used a mouse model to study changes in chlortetracycline resistance of faecal *E. coli* after supplementing the drinking water with flavomycin. The percentage of chlortetracycline-resistant isolates was lower in the flavomycin-fed group than in the control group. It is doubtful, though, whether this mouse model is practically applicable for food producing animals.

The proposed reason for flavomycin's limited action against gram-negative bacteria is that the antibiotic cannot penetrate the outer membane (Huber, 1979). If the presence of R-plasmids in some gram-negative organisms causes alteration of the cell wall surface, this may facilitate the uptake of flavomycin into the cell and thereby render these cells susceptible to flavomycin.

C.5   Effects on specific animal diseases

The fact that all *C. perfringens* seem to be flavomycin-resistant may cause some concern regarding necrotic enteritis in poultry. Stutz and Lawton (1984) fed flavomycin at 55 ppm to chickens whithout noticing any increase of *C. perfringens* in ileal contents. Brenes et al (1989) conducted a similar experiment with similar results. However, 55 ppm is more than twice as much as the maximum dose used for growth promotion in chickens and would therefore not quite correspond to the real-life situation. No published studies evaluating the risk of increasing necrotic enteritis by feeding flavomycin at lower levels have been found.

C.6   Impact of resistance on animal and human health

No flavomycin-related substances are used in either human or aimal therapy. Thus, at present no adverse effects would be expected from an increased resistance to flavomycin among human and animal bacteria.

Several other phosphorus-containing glycolipids with similar chemical properties and antibacterial spectra have been described (Huber and Nesemann, 1968; Meyers *et al.*, 1968; Huber, 1979). Some of these are prasinomycin, diumycin (macarbomycin), 11837 R.P, quebemycin, 19402 R.P., ensanchomycin, prenomycin and pholipomycin. As these substances are all highly active against gram-positive organisms, have extremely low toxicity and a prolonged acticity in blood after parenteral administration, it is remarkable that they have not yet been used for therapeutic purposes. In the present situation, with increasing antimicrobial resistance in both pathogenic and opportunistic bacteria, these substances would appear to be a welcome addition to the therapeutic arsenal.

C.7   Other effects on the microflora

C.7.1   Salmonella

A few studies on the effects of in-feed flavomycin on *Salmonella* colonisation have been published (Smith and Tucker, 1975; Dealy and Moeller, 1976; Dealy and Moeller, 1977b; George *et al.*, 1982; Humbert *et al.*, 1991).

Dealy and Moeller (1976; 1977b) investigated the shedding of *Salmonella* Typhimurium in experimentally infected calves and pigs. Animals given flavomycin at growth promoting levels were compared to non-medicated animals. The results indicate that the use of flavomycin reduced the duration and prevalence of *Salmonella* shedding in both pigs and calves. However, the experimental groups are rather small and, oddly enough, the challenge strain of *S.* Typhimurium used in the trials was susceptible to flavomycin and did not develop resistance during the experimental period. Since there are no reports on the regular susceptibility of *Salmonella* spp. to flavomycin, it cannot be determined whether this is unusual or not. If the infecting strain is sensitive to flavomycin, feeding this drug would be expected to reduce shedding. This might be regarded as therapy and not growth promotion, though.

In the study by Humbert and co-workers (1991), flavomycin was given to one of the experimental groups. In this study, chicks were challenged with *S.* Typhimurium and re-isolation of the organism was compared between medicated and non-medicated birds. The medicated groups included some feed additives, either in combination with or without competitive exclusion (CE) microflora. The results were very variable and the authors concluded that, due to interaction between the CE treatment and the feed additives, it was not possible to identify any antibiotic effect. Some objections may be

given as to the design of this study, regarding culturing methods, presentation of the results and length of study period (see annex B).

George and co-workers (1982) studied the effects of in-feed flavomycin on incidence, shedding, and antimicrobial resistance pattern of *S*. Typhimurium in experimentally infected chickens. The authors concluded that flavomycin had no effect on body weight, duration of salmonella shedding, number of salmonellae shed, tissue recoverability and total number of resistance patterns. Resistance to flavomycin was not tested.

Smith and Tucker (1975) also compared flavomycin-fed chickens to non-medicated chickens after experimental infection with *S*. Typhimurium and found a slight increase in salmonella shedding in medicated birds. This study includes the same selective technique for re-isolating the salmonellae as the study by Humbert and co-workers, where selective culture and visual examination only is used to identify salmonellae, which may lead to coliforms being mistaken for salmonellae.

C.7.2   Other enteric pathogens

No studies regarding the effects of in-feed flavomycin on other enteric pathogens have been found.

C.8   Toxicological aspects

The toxicity of flavomycin is very low, even after intravenous administration (Huber, 1979), and is only absorbed from the gut in small quantities (Sambeth *et al.*, 1974).Therefore, no toxic effects would be expected in either animals or humans due to the usage of flavomycin in animal feed.

C.8.1 Allergy

Frese and Blobel (1973) studied the antigenicity of flavomycin in rabbits and found that neither oral administration nor subcutaneous injection of the substance produced any antibody responses or anaphylactic reactions. This is the only published report on flavomycin in association with allergy. It is hard to say whether data obtained from rabbit experiments are applicable to people exposed to the substance.

C.9   Environmental effects

Like other AFA, if flavomycin reduces the amount of feed consumed per kg weight gain in the target animal, it would also be expected to reduce the amount of nitrogen output per kg weight gain.

Flavomycin shows a high degree of stability through the procedure of pelleting feed (Waals, 1973), which would suggest heat stability. The substance is degraded in soil and manure, but at a slow rate (Jagnow, 1978). According to Jagnow (1978), it takes 17 weeks for complete aerobic degradation of 10 ppm flavomycin in fresh manure, and 2 weeks in a soil mixture. Galvachin and Katz (1994) found that at 20°C or more, flavomycin, in a soil-faeces mixture, was degraded within 25 days. At 4 °C little or no degradation occured during the study period, which was 1 month. However, flavomycin does not seem to be a problem as far as environmental residues are concerned except for, possibly, areas with a constant temperature below 4°C.

C.10   Summary comments

Flavomycin appears to be a very attractive substance for therapy, as it is fairly atoxic and has good pharmacokinetic properties. However, if its use for growth promoting purposes causes increased resistance among animal bacteria, both flavomycin and related substances may be rendered useless for therapy in animal and human medicine. In general, very little information about flavomycin is available. As the substance has been in use for more than 20 years it is remarkable that so few investigations have been published on resistance in various bacterial species, various animal species and in different geographical regions. For substances not used in therapy, such investigations are essential, since resistance will not be noticed in clinical practice.

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Annex D: Ardacin and avoparcin

D.1   Introduction

Ardacin and avoparcin are glycopeptide antibiotics. The glycopeptides are large molecules produced by a variety of bacterial genera including *Streptomyces*, *Actinoplanes, Nocardia* and *Kibdelosporangium*. Avoparcin and ardacin are both compounds of two or more substances with similar molecular structure.

Chemically, the glycopeptides all have a common core and differ in the four side chains (figure D.I). The basic peptide structure possesses a nucleus of seven amino acid residues and five amino acids. Sugars and amino sugars, linked to the core structure, are mainly located on the outside of the molecule. They do not markedly affect the antimicrobial activity, but give the substances different pharmacokinetic properties (Reynolds, 1990).

Glycopeptides are active against gram-positive bacteria such as staphylococci, streptococci, enterococci, corynebacteria, clostridia and *Listeria* spp. Presently known glycopeptides are not active against gram-negative bacteria because the antibiotic molecules are unable to pass through the outer membrane and hence cannot reach their target (Reynolds, 1990).

Many glycopeptides, including avoparcin, are poorly or not at all absorbed from the gastrointestinal tract (Zulalian *et al.*, 1979; Hudd *et al.*, 1983).

Avoparcin is, in accordance with Directive 97/6/EC, presently not approved in the EU. Similarly, ardacin has been approved for growth promotion in annex II of Directive 70/524/EEC according to Directive 94/77/EEC, but there are indications that the authorisation will not be prolonged.

Vancomycin and teicoplanin (formerly teichomycin) are well known substances that are used therapeutically in human medicine for the treatment of severe infections caused by gram-positive bacteria.

D.2   Mode of action and resistance mechanisms

The bacterial cell wall is composed of a three dimensional web of cross-linked peptidoglycans. Precursors for this web are transported to the outer surface of the cell membrane on a lipid carrier. The assembly of precursors by cross linking takes place on the outer surface of the cell membrane. At this step, glycopeptides inhibit cell wall formation by binding to peptide stems of the precursor ending with D-alanine-D-alanine (D-Ala-D-Ala). The

The figures in this report is only available in the printed version

Figure D.I. Tentative sketch of ardacin, avoparcin, vancomycin and teicoplanin

The figures in this report is only available in the printed version

Figure D.I continued

binding is the result of five hydrogen interactions between D-Ala-D-Ala and the backbone structure of the glycopeptide. This results in the accumulation of precursors inside the cell and an arrested peptidoglycan assembly, eventually leading to the death of the bacterial cell. As the mode of action of glycopeptides derives from the core structure of the molecule, it can be assumed to be basically the same for all substances within the group (Arthur *et al.*, 1996).

Bacteria producing peptidoglycan precursors not ending in D-Ala-D-Ala will be partially or completely resistant to glycopeptides. The intrinsic resistance to high levels of vancomycin and teicoplanin seen in most *Lactobacillus* spp is due to the production of precursors ending in D-Ala-D-lactate (D-Lac) to which glycopeptides will not bind.

Enterococci belonging to the *Enterococcus gallinarium* group are resistant to low levels of vancomycin but sensitive to teicoplanin (VanC phenotype). These enterococci produce peptidoglycan precursors where D-Ala is substituted by D-serine (D-Ser). Vancomycin, but not teicoplanin, have a lower affinity for precursors ending in D-Ser (Arthur *et al.*, 1996).

In enterococci with acquired resistance, two main phenotypes have been described. Both these resistance phenotypes (VanA and VanB, see D.4) are due to the production of peptidoglycan precursors ending in D-Ala-D-Lac in stead of the normally produced D-Ala-D-Ala. As a result of this substitution, the complex between glycopeptides and the precursors will not be formed. This will allow the bacteria to grow in presence of the antibiotic. Recently, a phenotype designated VanD has been described in a strain of *E. faecium* (Perichon *et al.*, 1997). The described strain was constitutively resistant to vancomycin and to low levels of teicoplanin. Apparently, this phenotype is also the result of the production of peptidoglycan precursors ending in D-lactate.

D.3   Development of resistance

Experimental studies

In a study by Walton (1978), the effects of avoparcin on resistance in faecal streptococci (enterococci) and staphylococci was investigated. Avoparcin was fed to chickens at dosages of 10 and 100 ppm and birds on a non-supplemented feed were used as control group. In all groups, there was a wide variability in the total viable count of bacteria as well as in the proportion of resistant strains. Avoparcin-resistant enterococci and staphylococci were isolated from both avoparcin-fed birds and from the control group. Similarly, vancomycin-resistant strains were detected in all groups. The author stated that there was no evidence of cross-resistance

between avoparcin and other antibiotics tested (vancomycin, among others). The results are not presented in a form that makes it possible to substantiate this claim. No species identification of resistant isolates was performed.

Kaukas and co-workers (1988), monitored the effect of several antibiotics given at growth promoting dosages. Avoparcin was fed at 20 ppm to small groups of chickens and the results were compared to those from birds given non-supplemented feed. The incidence of resistance to therapeutic antibiotics, expressed as the Antibiotic Resistance Index (ARI), was higher (p=0.003) in all groups receiving antibacterials, as compared to the control group. This increase could be associated with an increase in the proportion of *E. faecium* in the enterococcal population of the treated birds. The proportion of strains resistant to avoparcin (defined as having a minimum inhibitory concentration > 4µg/ml) in *E. faecium* and *E. faecalis* was higher in the control group than in the avoparcin group. The authors commented that avoparcin resistance was an overall common finding also in the other experimental groups (22-52% in birds given different antibacterial feed additives and 42% in control birds).

Cohort studies

An association between the use of avoparcin and prevalence of vancomycin resistant enterococci (VRE) in animals has been reported (Aarestrup, 1995; Klare *et al.*, 1995a; Kruse, 1995; van den Bogaard *et al.*, 1996). In studies from USA where avoparcin has never been used, and from Sweden where avoparcin has not been used for 10 years, no VRE were found in samples from animals when selective techniques were used (Coque *et al.*, 1996; Greko, 1996). Thus, in the absence of avoparcin, the prevalence of VRE in animals is, at most, very low.

Bager and co-workers (1997) investigated poultry and pig farms in Denmark in a retrospective cohort study. The relative risk for occurrence of high level vancomycin resistance in *E. faecium* was 3.3 (0.9-12.3) for pig herds exposed to avoparcin. The corresponding figure for poultry flocks was 2.9 (1.4-5.9).

Taken together, there is strong evidence of a causal relationship between avoparcin use and occurrence of high level vancomycin resistance.

Point prevalence studies

Shortly after the introduction of avoparcin, no glycopeptide resistance was found among 15 strains of *E. faecium* isolated on vancomycin-free media (Dutta and Devriese, 1982), nor was resistance found in other enterococcal species. In table D.I, results concerning prevalence of glycopeptide resistance from Denmark and Sweden are presented. The results in these studies were

obtained without the use of antibiotic containing media in the course of monitoring studies.

   Studies using media favouring resistant isolates indicate that enterococci with high level resistance to glycopeptides are widespread among animals, including pets and horses (Bates *et al.*, 1994; Klare *et al.*, 1995b; Devriese *et al.*, 1996).

   The lack of earlier data on VRE in animals precludes conclusions on whether the resistance trait was present in animal populations at the time of introduction of avoparcin in animal husbandry.

Table D.I Prevalence of glycopeptide resistance

Bacterial species	Animal source	No. of isolates or samples	Year	Resistance in %	Reference	Country
E. faecium	cattle	13	1995-96	0	DANMAP, 1997	Denmark
E. faecium	poultry	54	1995-96	59	DANMAP, 1997	Denmark
E. faecium	swine	58	1995-96	20	DANMAP, 1997	Denmark
E. faecalis	cattle	35	1995-96	0	DANMAP, 1997	Denmark
E. faecalis	poultry	225	1995-96	29	DANMAP, 1997	Denmark
Enterococcus spp.	swine	46	1995	0	Greko, 1996	Sweden
Enterococcus spp.	poultry	60	1995	0	Greko, 1996	Sweden
Enterococcus spp.	swine	218	1996	0	Greko, 1997	Sweden
Enterococcus spp.	poultry	207	1996	0	Greko, 1997	Sweden

D.4   Resistance genes and gene transfer

Resistance to high levels of vancomycin, teicoplanin, avoparcin and, presumably, ardacin (the VanA phenotype) in enterococci is mediated by a cluster of genes designated the *van*A-gene cluster (Arthur and Courvalin, 1993; Arthur *et al.*, 1996). A dissociated resistance phenotype with various levels of vancomycin resistance and sensitivity to teicoplanin characterises the VanB-type of resistance encoded for by the *van*B-gene cluster (Arthur *et*

al., 1996). The recently described VanD phenotype is encoded by a gene cluster designated *van*D (Perichon *et al.*, 1997).

As shown in table D.II, there is a high degree of similarity between the main mechanisms mediating the VanA and VanB phenotypes. The explanation for the difference in teicoplanin susceptibility between these phenotypes is likely to be derived from differences between the regulatory products vanS-vanR and $vanS_B$-$vanR_B$ (Arthur *et al.*, 1996).

Table D.II. Genes encoded for by Tn*1546* (the *van*A-gene cluster) and Tn*1547* (the *van*B-gene cluster), their products and main functions (based on information from Arthur *et al.*, 1996)

Genes in cluster		Product type of respective gene	Function of respective gene	Amino acid identity between homologous
VanA	**VanB**			**products (%)**
*van*H	$van\text{H}_B$	dehydrogenase	Formation of D-Lac from pyruvate	67
*van*A	*van*B	ligase	Binding between D-Ala and D-Lac	76
*van*X	$van\text{X}_B$	dipeptidase	Hydrolysis of D-Ala-D-Ala	71
*van*Y	$van\text{Y}_B$	carboxypeptidase	Hydrolysis of terminal D-Ala	30
*van*Z	-	unknown	Confers teicoplanin resistance by unknown mechanism	-
*van*R	$van\text{R}_B$		Initiation of transcription of vanH, vanX and vanA/B	34
*van*S	$van\text{S}_B$		Regulation of *van*R	23
-	*van*W		Unknown function	-
ORF1	-		Open reading frame, transposase	
ORF2	-		Open reading frame, resolvase	

Production of VanH, VanA and VanX (VanHAX), encoded for by the genes in the operon *van*HAX, is normally inducible. This means that they are

only produced in the presence of a suitable inducer such as a glycopeptide. Synthesis of VanHAX is thought to be activated (induced) by the phosphorylated form of VanR (the product of the gene *van*R). Recent evidence indicate that VanS, encoded for by the *van*S gene, controls the effect of VanR negatively by dephosphorylation in the absence of glycopeptides. VanS is likely to have a domain acting as a membrane associated sensor. Elimination of the VanS gene results in high-level constitutive activation of the *van*HAX operon (Arthur *et al.*, 1997). VanA-type resistance is induced by vancomycin, teicoplanin and apparently also by avoparcin (Klare *et al.*, 1995b). The ability of ardacin to induce VanA-type resistance has not been investigated. However, considering that the molecular structure of ardacin is partly similar to both vancomycin and teicoplanin, there is no indication that it would not be an inducer.

Conflicting data about the inducing effects of other cell wall active antibiotics such as moenomycin (flavomycin), bacitracin, daptomycin, penicillin, cephalotin have been presented (Allen and Hobbs, 1995). Recent evidence indicate that apart from glycopeptides, only flavophospholipol (moenomycin/flavomycin) has the capacity to act as an inducer (Baptista *et al.*, 1996).

The regulatory system for production of $VanH_B$, VanB and $VanX_B$ in strains with VanB-type resistance is similar to the above described. However, the regulatory system $VanR_B$- $VanS_B$ is only activated by vancomycin, explaining the phenotypic susceptibility of VanB strains to teicoplanin (Evers and Courvalin, 1996). Teicoplanin resistant derivatives of VanB type strains have been reported (Hayden *et al.*, 1993; Green *et al.*, 1995). These isolates are likely to be the result of spontaneous mutations altering the specificity of the $VanS_B$ sensor domain.

Both the *van*A and *van*B gene clusters are generally located on plasmids and/or transposons (Arthur and Courvalin, 1993). High level resistance to glycopeptides mediated by the *van*A-gene cluster has been detected in *E. faecium*, other enterococcal species (Arthur and Courvalin, 1993), *Oerskovia turbata* and *Archanobacterium haemolyticum* (Power *et al.*, 1995). The gene cluster is mostly associated with the conjugative transposon Tn*1546* and/or self-transferable plasmids (Arthur *et al.*, 1996). Transfer of the *van*A-gene cluster has been shown *in vitro* from *E. faecium* to *Listeria monocytogenes*, *Staphylococcus aureus*, and to various streptococci (Leclercq *et al.*, 1989). Transfer frequencies were 10^{-4} for transfer between different strains of *E. faecium* and 10^{-6} - 10^{-9} for transfer from *E. faecium* to other species.

Resistance to glycopeptides mediated by the *van*B-gene cluster has, with respect to avoparcin and ardacin, attracted less attention. The *van*B-gene cluster is transferable either directly from the chromosome by a transposon (Tn1547) or through plasmids (Quintiliani and Courvalin, 1994; Woodford *et al.*, 1995b; Quintiliani and Courvalin, 1996) at a low frequency. The *van*B-

gene cluster has been found in *E. faecalis*, *E. faecium* and, recently, in *S. bovis* (Arthur and Courvalin, 1993; Poyart *et al.*, 1997). As mentioned, the *van*B-gene cluster is induced by vancomycin but not by teicoplanin, meaning that when strains carrying the gene cluster are exposed to teicoplanin, the gene will not be activated and the strain phenotype will remain susceptible (Arthur and Courvalin, 1993). According to available information, the *van*B-gene cluster does not seem to be inducible by avoparcin. No information is available on the capacity of ardacin to induce *van*B.

Selection of mutants expressing the *van*B-gene constitutively have been reported both from *in vitro* studies as well as in clinical isolates (Hayden *et al.*, 1993; Green *et al.*, 1995). Recently, transfer experiments with strains expressing *van*B constitutively were reported (Hayden *et al.*, 1997). The resulting transconjugants were either of constitutive or of inducible type. The use of a non-inducing antibacterial such as avoparcin, and possibly ardacin, could favour strains harbouring *van*B-gene clusters with the mutation required for the gene to be constitutively expressed should the gene be present in animal populations or their environments. Further information is needed on this topic.

VRE harbouring the *van*A gene cluster, have been isolated from humans, both in hospitals and community, from swine, rabbits, dogs, cats, horses chickens, turkeys, pheasants, ducks, foods of animal origin and sewage (Bates *et al.*, 1994; Torres *et al.*, 1994; Klare *et al.*, 1995b; Chadwick *et al.*, 1996; Devriese *et al.*, 1996; DANMAP, 1997). A polyclonal nature of the VRE strains has been demonstrated (Klare *et al.*, 1995b). As shown in table D.II, the *van*A gene cluster consists of 7 gene components. It is extremely unlikely that such a complicated gene should have developed separately in so many different host populations. Its occurrence therefore suggests an interspecies spread.

Human VRE have successfully been used to colonise mice experimentally (Whitman *et al.*, 1996). This indicates that at least certain enterococcal strains can colonise, or transiently inhabit, a variety of hosts. A report on occupational exposure provides further evidence (van den Bogaard *et al.*, 1997). The prevalence of VRE in turkeys, turkey farmers, turkey slaughterers and urban residents was found to be 50%, 39%, 20% and 14% respectively. Further investigations showed that VRE isolated from one of the farmers and his turkeys could not be differentiated by phenotypic or genotypic (pulsed-field gel electrophoresis) methods. Investigations of the *van*A-gene cluster by polymerase chain reaction (PCR) and hybridisation showed the two strains to be identical in the tested areas, having an insertion in a not previously described position, between the *van*X and *van*Y gene, and a deletion in the right end of the cluster.

The question of "identity" of genes has been a matter of debate in relation to the possible effects of the use of avoparcin in animal husbandry. The *van*A

gene cluster contains 9 genes (7 *van* and two transposition genes). Between those genes are intergenic, non-coding regions. The coding regions would be expected to be highly conserved once their sequences are optimal for function. As the intergenic regions are not essential for the function of the gene cluster, they are more likely to vary. Three recent studies have addressed the matter by amplification by polymerase chain reaction (PCR) and sequencing of the genes and/or their intergenic regions (Jensen, 1996; Haaheim *et al.*, 1997; Kirk *et al.*, 1997).

In the Norwegian study (Haaheim *et al.*, 1997), PCR for the *van*A and *van*B genes combined with restriction fragment analysis of a long PCR covering the entire gene cluster and sequencing of the intergenic *van*S-*van*H region were used to analyse the vanA gene cluster from VRE of Norwegian poultry and humans of various nationality (Swedish, Norwegian and American). In 9/12 human isolates and 7/10 poultry the results were identical, indicating horizontal transfer of the gene cluster.

Kirk and co-workers (1997), investigated 37 VRE isolates from one UK hospital and 36 VRE isolates from poultry meat bought in national supermarkets. By PCR, three intergenic regions of the *van*A-cluster were investigated (*van*S-*van*H, *van*X-*van*Y and *van*Y-*van*Z). The presence of *van*X, *van*Y and *van*Z was also determined. In the chicken isolates, all three investigated genes were amplified as well as the three intergenic regions. Evidence of a not previously described insertion sequence in various locations of the intergenic region between *van*X and *van*Y was found in some of the chicken isolates. The gene sequences of the strains from humans obviously differed from those of other described gene clusters from human strains (Arthur *et al.*, 1993; Handwerger *et al.*, 1995), as the *van*Y region as well as the intergenic regions *van*X-*van*Y and *van*Y-*van*Z failed to amplify with the primers used. Only the regions *van*S-*van*H, and the genes *van*X and *van*Z were amplified, indicating the presence of the *van*X and *van*Z genes but not the *van*Y gene. These results may be caused by an insertion or a deletion in the primer binding site for the *van*Y gene. In a recent publication by Mackinnon and co-workers (1997), a novel insertion sequence element designated IS*1476,* located within the *van*Y gene, was reported in one out of 124 clinical isolates, indicating the occasional finding of such variations. The fact that all the gene clusters from human strains in the study by Kirk and co-workers (1997) apparently had a deletion and/or insertion in the *van*Y region seems to indicate a horizontal spread of the gene within the hospital. Therefore, it is questionable whether the investigated strains can be deemed representative for human strains in general. The authors conclusion was that the results indicate that the infections with VRE in humans may not be caused by VRE from chickens. Considering the indications of a horizontal spread of the gene cluster among the human strains in this particular material and the variation between the genes of the chicken strains, indicating a

multiple origin, this study does not seem suitable for any general conclusions about the relation between human and animal strains.

Another report, cited above, of comparisons between the *van*A-clusters of different strains indicates the transmission between animal and man (van den Bogaard *et al.*, 1997). VRE isolated from a farmer and his turkeys could not be differentiated by phenotypic or genotypic methods. The *van*A gene clusters also appeared to be identical, having an insertion between the *van*X and *van*Y gene, and a deletion in the right end of the cluster.

The focus of a Danish study, reported by Jensen (1996), was slightly different. In order to investigate the degree of variation within the *van*A gene cluster, isolates from different animals and humans from a wider geographic range were investigated. Similar to Kirk and co-workers, Jensen found evidence of an insertion sequence in the *van*X-*van*Y region in 7 of 12 British human isolates. Based on sequencing of coding and non-coding regions, the remaining isolates could be divided into 3 groups, each containing isolates both from man and animals from different countries. The designation of one of the groups was based on the presence of a point mutation in *van*X and an insertion sequence (IS*1216V*) in a specific position in the transposon (Tn*1546*). The group contained isolates from humans (Denmark and USA) and pigs (Denmark and UK). Mutations within the coding regions appear to be rare. Insertion sequences are highly mobile, and frequently vary in their location. The occurrence of an insertion sequence in the same location in strains from different origins could either be interpreted as evidence of an epidemiological relationship, or as the site being a "hot spot" for insertion of the specific sequence. However, the likelihood of both a point mutation and an insertion in a specific location occurring independently in different strains is extremely low. Therefore, the genes present in those isolates must be very closely related and their presence the result of horizontal transfer.

Co-transfer of genes

As mentioned, transfer of the *van*A-gene cluster has been shown *in vitro* from *E. faecium* to *L. monocytogenes*, *S. aureus*, and various Streptococci (Leclercq *et al.*, 1989). Transfer frequencies were 10^{-4} for *E. faecium* to *E. faecium* and 10^{-6}- 10^{-9} for transfer to other species. When resistance to MLS antibiotics was also present, the two traits were transferred *en bloc* (Leclercq *et al.*, 1989). Conjugal co-transfer of resistance to high levels of glycopeptides, erythromycin and chloramphenicol, from *E. faecalis* to *S. aureus* on the skin of hairless obese mice was demonstrated in an experiment by Noble and co-workers (1992). The mice used cannot be regarded as "normal" mice. Nonetheless, they are a better model for the *in vivo* situation than a petri dish.

   The localisation of, for instance, MLS resistance determinants on mobile gene elements together with glycopeptide resistance means that selective pressures other that glycopeptides (e.g. macrolides) can result in increased or maintained resistance levels, and vice versa.

D.5   Effects on specific animal diseases

Glycopeptides are not used for therapy of animal diseases, although avoparcin can be used to prevent necrotic enteritis in chickens. Using a model with experimental infections, Prescott (1979) showed that inclusion of avoparcin at 20 ppm in the feed prevented necrotic enteritis, but 10 ppm was only marginally effective. Elwinger and co-workers (1996) showed that 15 ppm of in-feed avoparcin significantly lowered the caecal counts of *Clostridium perfringens* as compared to control birds given feed without additives.

D.6   Impact of resistance on animal and human health

Glycopeptides are used in human medicine for the treatment of infections (especially nosocomial) with multiresistant enterococci and staphylococci. Glycopeptides are also used to treat certain intestinal infections. Enterococci have a remarkable capacity for acquiring resistance to antimicrobial substances (Murray, 1990; Leclerq, 1997). In many instances, vancomycin is the sole drug with activity against these infectious agents.

   Infections with bacteria resistant to vancomycin and/or teicoplanin have been reported with increasing frequency in human medicine. VRE are emerging as a significant cause of hospital acquired infections (HICPAC, 1995; Woodford *et al.*, 1995a). Infections with VRE are associated with increased mortality (Linden *et al.*, 1996). A further threat is the possible spread of vancomycin resistance genes from their enterococcal hosts to multiresistant staphylococci. Although transfer of the *van*A gene cluster from enterococci to *Staphylococcus aureus* on the skin of mice in an *in vivo* model has been reported (Noble *et al.*, 1992), no such resistance has yet been reported in clinical isolates of staphylococci.

   Multiple factors predispose a person to infection with VRE, but colonisation precedes most infections (Edmond *et al.*, 1995). In Europe, a community reservoir has been demonstrated. The reported rates of VRE carriage in non-hospitalised humans range from 2 to 10 % (Jordens *et al.*, 1994; Gordts *et al.*, 1995; van den Bogaard *et al.*, 1996). Whether this colonisation is transient or persistent is not known.

According to the findings of Whitman (1996), administration of glycopeptides seems to be an important factor in establishing a persistent infection. Van der Auwera (1996) studied the effects of administration of oral glycopeptides to healthy volunteers. Before exposure, no VRE were recovered from the faecal samples whereas after exposure, 64% of the volunteers gave VRE positive samples. It is not clear whether these findings were due to a pre-exposure colonisation below the detection level of the methods used or to acquisition of resistant strains following the administration of the drug. Administration of other antimicrobial drugs may also alter the intestinal microflora, thereby predisposing for colonisation.

The occurrence of VRE in food of animal origin has been demonstrated (Bates *et al.*, 1994; Aarestrup, 1995; Klare *et al.*, 1995a; Wegener *et al.*, 1997). Once VRE-containing foods have been introduced into households or hospitals, numerous occasions for transfer of the resistance genes are available. Human enterococci may acquire resistance genes from animal enterococci in the environment (i.e. on towels, cutting boards, fittings of hygiene facilities etc.). Lax enforcement of hygienic rules provide opportunities for recontamination of heat treated foods. *E. faecium*, the principal bacterial host of the *van*A-gene cluster, has a high capacity for surviving heat treatment. Panagea and Chadwick (1996) showed that several isolates of *E. faecium* had the capacity to survive exposure to +65°C for 10 minutes. Thus, food-borne transmission is possible even without recontamination if food is not heated to higher temperatures for longer times. Transmission of VRE from animals to man may also occur through direct contact, as indicated by the findings of van den Bogaard and co-workers (1997, see E.4).

Numerous reports are available on the spread of VRE between patients and hospital staff (for a review see Woodford *et al.*, 1995a). Clearly, introduction into the ward from a community reservoir or through contaminated food may lead to an increased number of colonised patients, and consequently to an increased number of infections.

D.7   Other effects on the microflora

D.7.1   Salmonella

Of all AFA, avoparcin is probably the best studied regarding the possible influence on salmonella colonisation. Almost half of the studies discussed in chapter 4 include avoparcin. On the other hand, no studies have been found on the effect of ardacin on salmonella colonisation. All studies use chickens as experimental animals.

Already in 1978, Smith and Tucker (1978) demonstrated an increase in the prevalence and duration of salmonella shedding among chickens fed 10 ppm avoparcin, as compared to that of nonmedicated birds, both after direct and indirect experimental infection with *Salmonella* Typhimurium. The animals were either directly infected with 03 ml of a nalidixic acid-resistant strain of *S.* Typhimurium at a concentration of 10^9 CFU/ml, or through contact with experimentally infected chickens. Faecal samples were cultured on selective media containing nalidixic acid. However, this method also allows the growth of mutant (i.e. nalidixic acid-resistant) lactose-negative enterobacteria, and one has to bear in mind that the proportion of colonies possibly mistaken for salmonellae may not be the same in all experimental groups.

In another, similarly designed, study by the same authors (Smith and Tucker, 1980), the earlier results were confirmed. In this study, 5 different serovars were used (*S.* Typhimurium, *S.* Heidelberg, *S.* Oranienburg, *S.* Infantis and *S.* Senftenberg). Three different poultry breeds and 3 different commercial diets were also tested. All experiments gave similar results; feeding avoparcin at a concentration of 10 ppm led to a higher prevalence and a longer duration of salmonella shedding. Different inoculation dosages were also tested. It was demonstrated that, in the medicated animals, the inoculation dose required to achieve colonisation was 10-fold smaller than for the controls. This is the only study that has been found to deal with the effects on infectious dose, but it clearly demonstrates that avoparcin may increase the risk of a flock becoming infected when exposed to low doses of salmonella organisms.

In his thesis from 1981, Leuchtenberger (1981) compared the salmonella excretion of experimentally infected broilers treated with avoparcin, virginiamycin or tylosin with nonmedicated controls. In a series of experiments, 2 different concentrations of antibiotic (15 and 25 ppm avoparcin), 2 types of housing (wired cages and floor housing), 2 methods of experimental infection (direct oral inoculation or mixed in the feed) and 2 different infectious doses (10^3 or 10^4 organisms of *S.* Typhimurium) were tried in various combinations. Most experiments were performed in duplicate, with groups of 20-30 animals. Sampling was performed on several occasions throughout the trials, by cloacal swabs from live birds and from the heart, liver, duodenum and caecum of killed birds at the end of the experiment. Not all birds were sampled on all occasions, though. The author concluded that feeding avoparcin, virginiamycin or tylosin, at both levels tested, can prolong the persistence of *S.* Typhimurium infection in the intestine as well as internal organs, and significantly increases the amount of salmonella found in samples from these sites. The author also stated that the duration and frequency of salmonella excretion depends on the dose and way

of infection, as well as the frequency of dosing with infectious organisms, and on the housing system.

It is rather surprising that so many of the trials yielded significant differences between treated and non-treated groups, since the sample sizes are invariably too small for anything but large differences to be detected. However it is only in the main experiments, where cloacal swabs were taken continuously, that large enough numerical differences were recorded to be interpreted as a strong tendency towards increased salmonella excretion in treated birds, compared to non-treated controls. The trials where all birds were killed and cultures made from internal organs must be regarded as inconclusive, due to too small sample sizes and too variable numerical differences in the results.

In 1981 Gustafson and co-workers published a study where chickens fed avoparcin, virginiamycin or no antimicrobial were compared after receiving *S.* Typhimurium via the drinking water (Gustafson *et al.*, 1981). The authors tried to achieve a level of infection that corresponds to the level of natural infection and the administration of the salmonellae was distributed over several days. The results indicate that the feeding of avoparcin led to a larger proportion of animals shedding salmonella as compared to controls during the first 3 weeks after infection. This proportion of positive birds then decreased to become lower than that in the control group at about 4 weeks post infection. However, in the samples taken from the caeca after slaughtering the birds at the end of the trial, the proportion of salmonella-positive birds was higher in the avoparcin-fed group than in the control group. The sample size in this study is comparatively large, 100 animals in each group (all animals were sampled on each sampling occasion). The bacteriological methods include selective culture on media containing nalidixic acid, as the experimental strain was resistant to nalidixic acid. No further identification of the presumed salmonellae isolated in this way is reported in the article. One cannot be certain that the proportion of coliforms mistakenly identified as salmonellae would be the same in all experimental groups. The major objection against the design of this study, however, is that all birds also received 100 ppm monensin. Since monensin has antibacterial effects, this must be regarded as a possible confounder. Thus, the study only investigated the differences between birds fed monensin and birds fed monensin plus avoparcin or virginiamycin.

Another study by Gustafson and co-workers (Gustafson, 1983) reported no significant difference between avoparcin-fed birds and control birds, regarding salmonella shedding after experimental infection. On the other hand the prevalence among birds raised on clean litter was significantly higher that that among birds raised on used litter.

Linton and co-workers (1985) and Hinton and co-workers (1986) reported two salmonella studies that include avoparcin. The birds were naturally

infected via contaminated food, and all animals, except for one control group in one experiment, also received monensin. This makes the results difficult to evaluate, but in the first study the authors stated that there was no statistical difference between the medicated group and the control group (Linton *et al.*, 1985) and in the other that the results were inconclusive (Hinton *et al.*, 1986).

In 1989, Barrow (1989) demonstrated a dose-response relationship between in-feed avoparcin and prevalence of salmonella shedding among experimentally infected chickens. Infections with *S.* Typhimurium, *S.* Choleraesuis, *S.* Dublin and *S.* Arizonae all gave similar results. This is the only published study on dose-response relations between AFA and salmonella shedding. It provides an explanation to why some studies yield contradictory results; the concentrations of avoparcin used in most studies (around 10 ppm) appear to correspond roughly to the breakpoint of response-no response.

Humbert and co-workers (1991) investigated the effects of various AFA, including avoparcin, on salmonella shedding among CE- (competitive exclusion) treated chickens. The authors stated that birds fed 10 ppm avoparcin had significantly more salmonella in their caeca than control birds receiving no antibiotic. However the concurrent CE-treatment, among other things, make these results hard to evaluate.

In conclusion, avoparcin increases the prevalence of salmonella shedding among experimentally infected chickens, as demonstrated by a dose-response relationship. Avoparcin also lowers the infectious dose necessary for achieving salmonella colonisation of chickens. No studies have been found regarding ardacin, but as the two substances are very similar in activity and antibacterial spectrum, there is no reason to believe that ardacin would differ from avoparcin in this respect.

D.7.2   Other enteric pathogens

No publications have been found on the influence of either avoparcin or ardacin on the intestinal colonisation of target animals with other zoonotic pathogens.

D.8   Toxicological aspects

Glycopeptides are poorly absorbed after oral ingestion (Prescott and Baggot, 1993).

In an experiment where radiolabelled avoparcin was fed to chickens for 7 consecutive days at the dose of 1 mg/kg body weight, virtually the entire dose was retrieved in urine-faecal samples and in gastrointestinal contents

(Zulalian *et al.*, 1979). Residues in all tissues were less than 0.05 ppm. Based on these findings, the authors concluded that essentially no avoparcin was absorbed from the gastrointestinal tract of the animals. No residues are therefore to be expected. Maximum residue levels (MRL) have not been established.

Experimental feeding of broiler chickens with 15 ppm ardacin for 30 days resulted in liver residues of up to 50 µg/kg after a 7 day withdrawal period (Gottschall *et al.*, 1995). Ardacin is not biotransformed to any large extent (Gottschall *et al.*, 1995). No MRL has been established.

No information on toxicity to target species, non-target species or humans has been found. Vancomycin is ototoxic in humans, but it is not known whether this feature is shared by ardacin or avoparcin.

Occupationally derived contact dermatitis after contact with avoparcin has been reported (Barriga *et al.*, 1992).

D.9   Environmental effects

As for other AFA, if feeding avoparcin or ardacin leads to a reduction in the amount of feed consumed per kg weight gain, this would be expected to lead to a reduction in nitrogen output.

Like other naturally produced antibiotics, avoparcin and ardacin would be expected to be microbially degraded in soil, but no reports relating to the environmental fate of either ardacin or avoparcin have been found. Regarding avoparcin, that has been used for over 20 years in some countries, it is remarkable that such information is not publicly available.

D.10 Summary comments

Increased glycopeptide resistance is a human health problem. Avoparcin has been shown to select for glycopeptide resistance among animal bacteria, and there is no reason to believe that ardacin would be any different in this respect. Numerous reports indicate that transfer of glycopeptide resistance between animal and human microflora can and does occur.

Avoparcin has also been shown to affect salmonella colonisation. The lowering of the infectious dose necessary for colonisation that is seen in avoparcin-treated animals indicate that avoparcin may increase the prevalence of salmonella-infected poultry flocks, especially in areas where the exposure to salmonella is low.

The available information on toxicological and environmental aspects is too scarce to form the basis of any assessment.

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Annex E: Spiramycin, tylosin and virginiamycin

E.1 Introduction

Spiramycin and tylosin belong to the macrolide group of antibiotics, while virginiamycin belongs to the streptogramins. Macrolides and streptogramins are not chemically related but they are often grouped together because they show similar antibacterial spectra and are functionally related in their mode of action. A third class, the lincosamides is also often included for the same reason, leading to the acronym MLS (macrolides, lincosamides and streptogramins). They all inhibit protein synthesis and bind to the 50S subunit of the bacterial ribosome. They are mainly active against gram-positive aerobic bacteria, various gram-positive and gram-negative anaerobic bacteria. Lincosamides are not used as feed additives and will therefore only be discussed where appropriate.

E.1.1 Macrolides (tylosin and spiramycin)

Macrolides are derived from products from *Streptomyces* spp. and are characterised by a macrocyclic lactone ring attached to one or more sugar moieties and can, according to the ring structure, be divided into 14-, 15- and 16 lactone ring macrolides (fig. E.I). Erythromycin, a 14 membered lactone, and several newer derivatives are used in human medicine and are important in the treatment of common infections caused by bacteria such as staphylococci, streptococci, mycoplasmas and campylobacters. Macrolides have been suggested as the primary drugs of choice for a number of clinically significant infections in children (Adam, 1992). Among the 16-membered lactones, spiramycin and josamycin have also been evaluated for the treatment of certain protozoal diseases such as toxoplasmosis (St Georgiev, 1994).

In veterinary medicine, the 16-membered lactones tylosin and spiramycin have been widely used, both for growth promotion and for therapy. In the EU, spiramycin is approved for growth promotion in poultry, calves, lambs, kids and swine. Dosages range between 5 and 20 ppm for poultry, 5-80 ppm for calves, lambs and kids, and 5-80 ppm for swine. Tylosin is approved for growth promotion in swine, at concentrations between 5 and 40 ppm.

Important clinical applications for those and related substances are swine dysentery (*Serpulina hyodysenteriae*) and mycoplasmosis. They are also

The figures in this report is only available in the printed version

Figure E.I. Tentative sketch of erythromycin, spiramycin and tylosin

The figures in this report is only available in the printed version

Figure E.I. Continued; virginiamycin

second-choice antibiotics for a range of common infections, including mastitis caused by penicillin-resistant staphylococci. Another macrolide for therapeutic purposes, tilmicosin, has lately been introduced into the veterinary field, mainly for use against infections with *Actinobacillus pleuropneumoniae* in swine, *Pasteurella* spp. in cattle and mycoplasmosis in various animal species (Prescott and Baggot, 1993). Therapeutic dosages of in-feed tylosin for swine are usually around 100 ppm (Prescott and Baggot, 1993).

   The different macrolides differ to some extent with respect to their pharmacokinetic properties. Common characteristics of the group are high intracellular concentrations and a wide distribution in tissues (Prescott and

Baggot, 1993), properties which together with their activity against important pathogens make them a valuable group for therapeutic purposes in both veterinary and human medicine.

E.1.2   Streptogramins (virginiamycin)

Streptogramins are natural cyclic peptides including substances such as pristinamycins and virginiamycins (fig E.I). They are produced by a variety of *Streptomyces* spp. Streptogramins consist of two components; A (also called M) and B (also called S), each having bacteriostatic activity and acting synergistically.

Streptogramins have been sparingly used in human medicine, although there are geographic variations. Pristinamycin and virginiamycin are the most well known substances, but a new derivative, quinpristin-dalfopristin, has recently been launched (Pechere, 1992; Cormican and Jones, 1996; Nicas *et al.*, 1997). The role of these substances as therapeutic tools is expected to increase due to the increasing resistance to more commonly used substances. Quinpristin-dalfopristin has been suggested as a new option for the treatment of infections with glycopeptide- (vancomycin) and aminoglycoside-resistant enterococci (Cormican and Jones, 1996; Nicas *et al.*, 1997).

In animals, virginiamycin is mainly used as a feed additive for growth promotion. Examples of prophylactic or therapeutic applications are swine dysentery and clostridial infections such as necrotic enteritis in poultry.

Virginiamycin is permitted for growth promotion in poultry, calves and swine at concentrations between 5 and 50 ppm for poultry, 5-80 ppm for calves and 5-50 ppm for swine. Therapeutic dosages for swine are around 100-150 ppm and for prophylactic treatment of necrotic enteritis in poultry around 40-80 ppm (Prescott and Baggot, 1993; FASS VET., 1997).

There are few data available on the pharmacokinetics of streptogramins. Orally administered virginiamycin is not absorbed from the gut (Prescott and Baggot, 1993).

E.2   Mode of action and resistance mechanisms

The MLS antibiotics inhibit protein synthesis by binding to the 50 S subunit of the bacterial ribosome. By binding to, or near, the peptidyl transferase centre on the ribosome, macrolides prevent the elongation of the peptide being synthesised. Intrinsic resistance to macrolides, lincosamides and streptogramin B (MLS_B) in gram negative bacteria is due to low permeability of the outer membrane (Leclercq and Courvalin, 1991b).

The most common mechanism of acquired resistance to MLS antibacterials is alteration of the ribosomal target. Inactivation of the drug and active efflux from the bacterial cell have also been described.

Concerning streptogramin A, the mechanisms of intrinsic resistance are unclear. Described mechanisms of acquired resistance include enzymatic inactivation and active efflux.

Depending on the exact mechanism, a variety of resistance phenotypes are found, many of which are cross-resistant to most or all members of the group (table E.I). Most studies on MLS resistance have been conducted on gram-positive cocci and clostridia. Regarding macrolide and streptogramin resistance in *Campylobacter* spp. and *Serpulina* spp., little is known about the mechanisms and the genes conferring resistance.

Further information on resistance mechanisms and corresponding genes are found in E.4.

Table E.I. Examples of phenotypic and genotypic expression of some resistance genes (R=resistant, S=sensitive)

Resistance gene	Phenotype[1]						Genotype					
	Er	Ty	Sp	SgB	SgA	SgAB	Er	Ty	Sp	SgB	SgA	SgAB
erm												
-constitutive	R	R	R	R	S	S	R	R	R	R	S	S
-inducible in:												
staphylococci	R	S	S	S	S	S	R	R	R	R	S	S
streptococci[2]	R			R	S	S	R	R	R	R	S	S
E. faecalis	R				R		R	R	R	R	R	R
ere	R	S	S	S	S	S	R	S	S	S	S	S
sbh	S	S	S	R	S	S	S	S	S	R	S	S
msr	R	S	S	R	S	S	R	S	S	S	S	S
satA	S	S	S	S	R	S	S	S	S	S	R	S

[1] Er=erythromycin, Ty=tylosin, Sp=spiramycin, SgB= streptogramin B component, SgA= streptogramin A component, SgAB= streptogramin components A+B

[2] *S. sanguis* and *S. pyogenes*

E.3   Development of resistance

Most studies regarding development of resistance to macrolides and streptogramins have been investigating the association between clinical therapy and development of resistance. Thus, the dosages used are usually higher than what is permitted for growth promotion. Nonetheless, the results indicate that resistance develops in bacteria exposed to these substances. Moreover, as discussed in chapter 4, the concentrations used for growth

promotion are well above the MIC values for common intestinal bacteria. Where relevant information is lacking about the situation when growth promoting dosages are used, results from studies employing therapeutic dosages have been cited.

E.3.1   Prospective studies

Experimental studies

Several studies by the research group of Linton (Linton *et al.*, 1985; Hinton *et al.*, 1986; Kaukas *et al.*, 1987; Kaukas *et al.*, 1988) illustrate the influence of macrolides and streptogramins (tylosin, virginiamycin) at growth promotion dosages on the prevalence of resistance in enterococci. In two of these studies (Hinton *et al.*, 1986; Kaukas *et al.*, 1987), the control groups were given a diet containing growth promoters (including virginiamycin) and/or a coccidiostat with antibacterial effect. A gradual increase in macrolide (tylosin) resistance in the control groups was noted in both these studies. This is in contrast to the studies where the control groups were provided feed without antibacterial agents (Linton *et al.*, 1985; Kaukas *et al.*, 1988). Considering the potential of virginiamycin to select for MLS_B-type resistance, it cannot be excluded that the gradual increase in macrolide resistance for the control groups in the former studies was related to the antibiotics in the diet.

In one of the studies (Linton *et al.*, 1985) the enterococci were not identified to the species level, which makes the results hard to evaluate. Moreover, part of the study was conducted on commercial farms and it is not known whether any coccidiostats or other antimicrobial substances were used during the study period. In the other studies, the isolation frequencies of different species of enterococci on different sampling occasions were compared. Again, a marked difference can be observed between the two studies where the bacterial flora of the control groups was exposed to antibacterial agents and the one where it was not. The normal changes in composition of the enterococcal flora during the first weeks of life of chickens not exposed to antimicrobial agents was further investigated in another study (Kaukas *et al.*, 1986). The results show the same trend in number of isolates of different enterococcal species as was observed in the unexposed control group in the study from 1988 (Kaukas *et al.*, 1988). Thus, the confounding effect of growth promoters and anticoccidials in the other control groups is further substantiated. All animals fed antimicrobial agents seem to have had a larger proportion of *E. faecium* in their intestinal flora than animals not receiving any antibacterials.

In the following, only the study from 1988, where the control group did not receive any antimicrobials will be discussed in detail. In this study (Kaukas *et al.*, 1988), small groups of chickens were given feed containing either avoparcin, nitrovin, virginiamycin or zinc bacitracin during the first three weeks of life. The feed of the control group, as mentioned earlier, contained no additives. Each study group consisted of ten chickens and the experiment was performed in duplicate. The incidence of resistance to therapeutic antibiotics, expressed as Antibiotic Resistance Index (ARI) was significantly higher in all groups receiving antibacterials, as compared to the control group (p=0.003). This increase was associated with an increase in the proportion of *E. faecium* in the enterococcal population of the treated birds. The incidence of resistance to streptogramins in bacterial isolates from the group receiving virginiamycin and from the control group differed significantly (p<0.001) for both *E. faecalis* (46 and 25% respectively) and *E.faecium* (88 and 26% respectively). Thus, in addition to the selection of the species *E. faecium* by virginiamycin, a specific selection for resistance traits was also observed.

In a study from 1983 (Christie *et al.*, 1983) an increase of MLS resistance over time in gram positive cocci isolated from pigs fed 100 ppm tylosin was observed. Despite weaknesses in study design, sample size and methodology, and the fact that therapeutic dosages were used, this study nevertheless implies that tylosin in pig feed affects development of resistance in both staphylococci and enterococci from these animals. This study has been much criticised. Among other things, it appears that due to a feed mixing error the control group also received some tylosin early in the study (Anonymous, 1985). However, the influence of this error on the results would have been expected to be seen as a decrease in the difference between the two groups. Thus, it ought not to have caused any overestimation of the difference.

Field studies

The influence of virginiamycin on the prevalence of resistant enterococci within flocks of turkeys has been reported by Thal and co-workers (1996). Different turkeys from the same flock were found to harbour the same type of streptogramin-resistant *E. faecium* and the prevalence of resistance to both streptogramins and ampicillin increased with the age of the flock.

In a German study (Hummel and Witte, 1981) macrolide resistant staphylococci could be isolated from pigs in herds exposed to tylosin via the feed. Moreover, such resistant strains were also isolated from people who worked directly with the animals but not from their family members. All staphylococci from pigs in non-exposed herds were sensitive to macrolides. This indicates that tylosin may exert a selective pressure, not only on the microflora of animals consuming the feed with the added tylosin but also

directly on the bacteria of people handling the feed and the animals. The failure to demonstrate a spread of the resistant strains or the genes mediating resistance to non-exposed humans could indicate that a selective pressure has to be in force in order to promote further transmission. The dose of tylosin given and the precise administration principles were not detailed in the publication but from what is noted a therapeutic concentration seems most likely. In addition, all animals included in the study were fed tetracyclines, apparently on routine basis.

E.3.2   Retrospective studies

The development of tylosin resistance in *Serpulina hyodysenteriae* was reported by Molnar (1996) in an investigation from Hungary. The proportion of strains classified as resistant to tylosin in this investigation increased from 11% during 1978-82 to 40% during 1983-87. The author assumed that the high level of resistance to tylosin was to a large extent due to the fact that since the 1980s most pig rations in Hungary have contained tylosin as an additive. However, this assumption is not substantiated in the article and no data on consumption is presented.

   The prevalence of resistance to macrolides in *Clostridium perfringens* isolated from cattle, swine and poultry was investigated in two studies from Belgium (Dutta and Devriese, 1984; Devriese *et al.*, 1993). In the material included in the first study, collected between 1979 and 1982, the prevalence of resistance ranged from 1% to 21% depending on animal source. In the second study from 1991-1992, the corresponding figures were 3% to 12%. The number of animals in these studies is to small for any clear trends to be deducible, but there is no evidence of increase in the resistance rates over the years.

E.3.3   Point-prevalence studies

Enterococci

With the exception of *E. faecium*, enterococci are naturally resistant to the A component of streptogramins (Leclercq and Courvalin, 1991b). Acquired resistance in enterococci is, according to present knowledge, of the MLS_B type, encoded for by a number of *erm*-genes. In table E.II, data from several studies on resistance to macrolides in enterococci from poultry and swine have been compiled. The prevalence of resistance appears to be high in the bacterial populations studied, although somewhat lower in the material presented from Sweden.

Table E.II. Resistance in enterococci to MLS$_B$ antibacterials as reported in different studies

Bacterial species	Animal source	No. of isolates	Year	Resistance in % [1]	Reference	Country
Enterococcus faecalis subsp. *Liquefaciens*	poultry	23	1980	*83*	Dutta and Devriese, 1982	Belgium
E.faecalis	poultry	8	1980	*50*	Dutta and Devriese, 1982	Belgium
E.faecalis	swine	225	1995-96	*91*	DANMAP, 1997	Denmark
Enterococcus faecium	poultry	15	1980	*67*	Dutta and Devriese, 1982	Belgium
E.faecium	poultry	54	1995-96	*65*	DANMAP, 1997	Denmark
E. faecium	cattle	13	1995-96	*38*	DANMAP, 1997	Denmark
E. faecium	swine	58	1995-96	*91*	DANMAP, 1997	Denmark
Enterococcus spp.	cattle	34	1979-82	*26*	Rollins et al., 1985	USA
Enterococcus spp.	swine	72	1979-82	*79*	Rollins et al., 1985	USA
Enterococcus spp.	pork	50	1995	*15*	Quednau et al., 1996	Denmark
Enterococcus spp.	pork	50	1995	*2*	Quednau et al., 1996	Sweden
Enterococcus spp.	swine		1996	*33*	Greko, 1997	Sweden
Enterococcus spp.	poultry	55	1979-82	*50-67*	Dutta and Devriese, 1984	Belgium
Enterococcus spp.	poultry	93	1979-82	*67*	Rollins et al., 1985	USA
Enterococcus spp.	poultry meat	50	1995	*27*	Quednau et al., 1996	Denmark
Enterococcus spp.	poultry meat	50	1995	*10*	Quednau et al., 1996	Sweden
Enterococcus spp.	poultry	207	1996	*17*	Greko, 1996	Sweden

[1]When several macrolides have been investigated, the figures for erythromycin have been used.

Staphylococi

Data on the prevalence of macrolide resistance in animal staphylococci from various studies are presented in table E.III. A majority of these strains have been isolated from pathological lesions or from skin. As can be seen in the table, there seems to be a marked difference in the prevalence of macrolide-resistance between staphylococci from different animal species. The majority of the staphylococcal strains reported as resistant in table E.III expressed the resistance constitutively.

Table E.III. Resistance in *Staphylococcus aureus* to erythromycin as reported in different studies

Bacterial species	Animal source	n of isolates	Year	Resistance in %	Reference	Country
S.aureus	cattle	517	1971-80	*2-4*	Devriese, 1980	Belgium
S.aureus	cattle	211	1995-96	*1*	DANMAP, 1997	Denmark
S.aureus	cattle	183	1995	*1*	Nilsson, 1996	Sweden
S.aureus	poultry	399	1970-80	*6-20*	Devriese, 1980	Belgium
S.aureus	swine	124	1973-80	*27-53*	Devriese, 1980	Belgium

Campylobacter

C. jejuni is associated mainly with poultry and *C. coli* with swine. The species most commonly isolated from humans is *C. jejuni*. The incidence of macrolide resistance in *C.coli* is much higher than in *C. jejuni*. (Wang *et al.*, 1984; Burridge *et al.*, 1986; Lacey, 1988; Jacobs-Reitma *et al.*, 1994; DANMAP, 1997). It has been implied that the widespread resistance to macrolides in *C. coli* may be linked to the use of macrolides (Davies *et al.*, 1996; Moore *et al.*, 1996). One of the few studies made on MICs of tylosin for field isolates of *C. coli* (Ryden R, cit. by Burridge *et al.*, 1986) showed that the prevalence of tylosin resistant *C. coli* among nontreated pigs, or pigs given antibacterials other than tylosin, was 55%, and in pigs receiving therapeutic doses of in-feed tylosin it was 70%. In contrast, *C. jejuni* originating from poultry are rarely resistant to macrolides. The use of macrolides is presumably higher in swine as compared to poultry as tylosin is approved for growth promoting purposes only in the former species. In view of the zoonotic character of campylobacteriosis, it is remarkable that no further investigations on the possible relation between use of macrolides as growth promoters and selection of resistant strains of campylobacters have been made.

Table E.IV. Prevalence of erythromycin resistance in campylobacter in
various studies

Bacterial species	Source	n of isolates	Year	Resistance in %	Reference	Country
C. coli	swine	99	1995-96	*55*	DANMAP, 1997	Denmark
C. coli	poultry	91	1992-93	*12*	Jacobs-Reitma et al., 1994	Netherlands
C. coli	sewage	32	1995	*25*	Koenraad et al., 1995	Netherlands
C. coli	human	58	1987-91	*19*	Reina et al., 1992	Spain
C. coli	human and swine	80[1]	1984	*70*	Wang et al., 1984	USA
C. jejuni	poultry	55	1995-96	*4*	DANMAP, 1997	Denmark
C. jejuni	poultry	177	1992-93	*2*	Jacobs-Reitma et al., 1994	Netherlands
C. jejuni	sewage	121	1995	*9*	Koenraad et al., 1995	Netherlands
C. jejuni	human	614	1987-91	*2*	Reina et al., 1992	Spain
C.jejuni	human and swine	98[2]	1984	*3*	Wang et al., 1984	USA
Campylo-bacter spp.	Poultry	59	1989-90	*5*	Cabrita et al., 1992	Portugal
Campylo-bacter spp.	Swine	65	1989-90	*26*	Cabrita et al., 1992	Portugal

[1] 11 isolates from humans, 69 isolates from swine

[2] 93 isolates from humans, 5 isolates from swine

Serpulina

During the 1970s, tylosin was regarded as the drug of choice in the treatment
of swine dysentery. This is no longer the case, as resistance to tylosin now
seems to be widespread in strains of *Serpulina hyodysenteriae* (Smith *et al.*,
1991 see also E.3.2; Prescott and Baggot, 1993; Fellström *et al.*, 1996;
Molnar, 1996). A high proportion of virginiamycin resistance in this group of
bacteria has also been reported from other countries in recent years (Ronne
and Szancer, 1990).

E.4   Resistance genes and acquisition of resistance

Various genes conferring the different types of resistance mentioned above
(E.2) have been identified (table E.IV). These genes, often of a highly mobile
type, can be transferred between bacteria, within the same species and
between different species. They are often carried on "jumping genes"
(transposons) which means that they can copy themselves from, for instance,

the chromosome to a plasmid or from one plasmid to another plasmid. Further, they may be carried on self-transmissible plasmids.

Table E.IV. Examples of resistance to MLS antibacterials by different mechanisms and genes. (Data compiled from Arthur *et al.*, 1987; Brisson Noel *et al.*, 1988; Leclercq and Courvalin, 1991a; Leclercq and Courvalin, 1991b; Eady *et al.*, 1993; Mullany *et al.*, 1995; Tauch *et al.*, 1995; Weisblum, 1995a; Allignet *et al.*, 1996)

Phenotype	Mechanism	Gene	Described localisation	Example of species
MLS_B	target modification	*erm*A	Tn*554*	*S. aureus,* coagulase negative staphylococci
		*erm*B, *erm*AM	Tn*551*, pAM77, Tn*917*, Tn*1545*, plus various other plasmids and transposons	*S. aureus, S. intermedius, S. hyicus, S. pneumoniae, Streptococcus spp., E. faecalis, Lactobacillus* spp.
		*Erm*C	pE194, plM13, pE5, pNE131	*S. aureus*, coagulase negative staphylococci, *S. hyicus, Bacillus subtilis*
		*erm*CX	Tn*5432*	*Corynebacterium xerosis*
		*erm*D	chromosome	*B. licheniformis*
		*erm*BC	pIP1527	*E. coli*
		*erm*F	pBF4	*Bacteroides spp.*
		*Erm*E	chromosome	*Streptomyces erythreus*
		*erm*P, *erm*Q		*Cl. perfringens*
		*erm*Z	Tn*5398*	*Cl. difficile*
		*erm*J		*Bacillus anthracis*
		*erm*M		*S. epidermidis*
14-M	drug inactivation	*ere*A	pIP1100	*E. coli*
		*ere*B	pIP1527	*E. coli*
L		*lin*A	pIP855	*S. haemolyticus*
		*lin*A'	pIP856	*S. aureus*
S_B		*sbh*	pIP524	*S. aureus*
		vgb	pIP680	*S. aureus*
S_A		*satA*	pAT424	*E. faecium*
		vat	pIP680, IS257	*S. aureus*
		vatB	pIP1156	*S. aureus*
14-M	active efflux	*erp*A		*S. epidermidis*
$14\text{-}MS_B$		*msr*A		*S. epidermidis*
S_A		*vga*	plasmids	*S. aureus, S. epidermidis*

E.4.1   Target modification

Cross-resistance to all MLS_B antibiotics due to target modification is widespread although the prevalence differs regionally (Duval, 1985; Arthur *et al.*, 1987). The bacteria equipped with this property produce rRNA methylases which are enzymes capable of altering the ribosomal binding site of MLS_B-antibiotics. The alteration inhibits binding of the antibiotic and hence allows the bacterial protein synthesis to continue. The genes coding for this property in bacteria are designated *erm* (erythromycin resistance rRNA methylase). Various different *erm*-genes have been identified. They present a substantial sequence diversity but studies on their evolutionary relationship suggest that they are of ancient presence in at least *Streptomyces* spp, gram-positive cocci and *Bacillus licheniformis* (Arthur *et al.*, 1987). However, transfer of *erm* genes occurs under natural conditions to bacteria which are phylogenetically remote from the above mentioned bacteria, such as *Escherichia coli* (Brisson Noel *et al.*, 1988). This transfer is believed to be a recent event although the genes are already disseminated in enterobacteria.

Transfer of *erm*-genes has been demonstrated experimentally in numerous studies. Poyart-Salmeron and co-workers (1990) showed by way of *in vitro* mating experiments the conjugative transfer of *erm*B, carried on plasmid pIP811 together with other resistance determinants, between among others *Listeria monocytogenes* and *E. faecalis*. Doucet-Populaire and co-workers (1991) demonstrated transfer *in vitro* and in an *in vivo* model of transposon *Tn*1545, harbouring *erm*B, *aph*A3' (kanamycin resistance) and *tet*M (tetracycline resistance) between *E. faecalis* and *L. monocytogenes*. McConnell and co-workers (1991) demonstrated transfer of plasmid pAMβ1, carrying *erm*B, from *Lactobacillus reuteri* to *E. faecalis in vivo*.

Inducible or constitutive expression

Expression of *erm*-genes can be inducible or constitutive. When constitutively expressed, the methylase encoded for by the *erm*-gene will always be produced. On the other hand, when inducibly expressed, the methylase is only produced when the gene is activated by an inducer. Various macrolides may function as inducers, depending on the class of *erm* gene and bacterial host.

The type of expression is related to the class of *erm* gene but depends on a regulatory gene sequence upstream from the methylase gene sequence itself (the messenger). The mechanisms of resistance by target modification and induction of this resistance has been described by Weisblum (Weisblum, 1985; Weisblum, 1995a; Weisblum, 1995b; see figures).

The figures in this report is only available in the printed version

Figure E.II. Induction of the *erm*C gene (Adapted from Weisblum, 1995a)

The entire gene consists of a leader peptide and a series of inverted complementary repeat sequences that can redistribute and assume alternative double stranded conformations, followed by the methylase gene sequence. In the presence of a macrolide the ribosomes are inhibited ("stalled"). Activation of the messenger depends on the degree to which ribosomes, during translation of the leader peptide, are inhibited by the macrolide and thereby disrupt the secondary structure in a certain part of the control region. The result of inverted repeat sequence redistribution is the unmasking of a ribosome binding site for synthesis of the methylase. The inductive capability of different macrolides is dependent on their capability of stalling the ribosome at the right step in the translation of the leader peptide. This varies between different *erm* genes and between different bacterial species, but 14-membered macrolides are generally the best inducers. Inducibly resistant strains can convert to constitutively resistant ones by deletions or single nucleotide changes in the regulatory gene, either in the leader peptide or in the inverted repeat sequences.

Enterococci and streptococci

In enterococci and streptococci (formerly group D streptococci), MLS$_B$ resistance can be expressed either inducibly or constitutively. However, in some cases all MLS$_B$ antibiotics can act as inducers which explains the diversity of resistance phenotypes coded for by similar genes. Thus, in the case of MLS$_B$ resistance, phenotypic characteristics do not necessarily provide a basis for conclusions on genotype. Reports from Japan and France have shown a trend from predominantly inducible resistance to predominantly constitutive resistance in staphylococci whereas in British and German reports inducible type still dominate (Jenssen *et al.*, 1987). A non-inducing macrolide or streptogramin would be expected to select for constitutively expressed resistance, since mutation to the constitutive form would be the only way bacteria harbouring inducible *erm*-genes could survive exposure to a non-inducer.

Investigations of Swedish isolates from humans of *Streptococcus pyogenes* revealed that the resistance determinant was present on an *erm*B-carrying conjugative plasmid, possibly originating from plasmid pAMb1 of *E. faecalis* (Schalen *et al.*, 1995). In an Italian study on the same species, in addition to the MLS$_B$ type mediated by *erm*-genes, a second phenotype, possibly due to an efflux-mechanism, was reported (Coranaglia *et al.*, 1996).

Streptococcus suis, originally described as a cause of outbreaks of arthritis and meningitis in piglets is today more commonly associated with bronchopneumonia in weaners and fatteners. In a study from USA (Stuart *et al.*, 1992) a substantial increase in resistance to the MLS antibacterials in *S. suis* was demonstrated when compared to a similar study undertaken previously. Further, the study showed that resistant isolates were capable of transmitting resistance by conjugation possibly mediated by a transposon similar to *Tn*916. Wasteson and co-workers (1994) investigated a collection of Norwegian isolates and demonstrated MLS-resistance in *S. suis* as determined by *erm*C and/or *erm*B genes and furthermore, that the resistance genes could be transmitted from *S. suis* to *E. faecalis.*

Enterococci are known to frequently exchange resistance genes, not only with other enterococci but also with other bacterial genera. Transfer of resistance genes between enterococci and staphylococci appears to take place in nature, maybe mediated by broad host range plasmids (Bonafede *et al.*, 1997).

Staphylococci

In staphylococci harbouring *erm*C or *erm*A genes, inducible resistance is triggered by 14- and 15-membered macrolides only. *In vitro*, those strains are resistant to 14- and 15- membered macrolides. As long as the induction persists (presence of, for instance, erythromycin) the methylase will be produced and the bacterial cell will be resistant to all MLS_B antibiotics. The 16-membered macrolides, lincosamides and streptogramin B cannot induce expression of the gene and will, when tested separately, remain active *in vitro*. When expressed constitutively in staphylococci, the *erm*-genes confer cross-resistance to all MLS_B. No information has been found on whether staphylococci constitutively expressing MLS_B resistance due to mutations are likely to mutate back to the inducibly expressed form of resistance in the absence of a selective pressure for the constitutive variety, i.e. the presence of a 16-membered macrolide. Experimental data indicate that the inactive structure of the inducibly expressed resistance gene is energetically favoured (Horinouchi and Weisblum, 1981), so a mutation back to this state would not be totally unexpected. Moreover, it is not known whether constitutively expressed *erm* genes would still be constitutively expressed after transfer into a new host bacterium. As a comparison, transfer experiments with enterococcal strains expressing *van*B constitutively have shown that the resulting transconjugants can be either of constitutive or of inducible type (Hayden *et al.*, 1997).

Eady and co-workers (1993) investigated the distribution of *erm*A, *erm*B and *erm*C in staphylococci from human and animal sources. In addition, the presence of *msr*A was studied. One hundred and seventy-two human isolates of coagulase-negative staphylococci from dialysis patients, blood cultures and untreated acne patients were analysed, along with 33 isolates of coagulase-negative staphylococci from pigs and 16 isolates of *Staphylococcus intermedius* from dogs.

Table E.II. Number of staphylococcal strains from various sources harbouring macrolide-streptogramin-resistance genes. Data from Eady and co-workers (1993)

Gene	Swine	Dogs	Humans
*erm*A	0	1	14
*erm*B	7	11	0
*erm*C	21	1	89
*msr*A	2	1	70

Fourteen of the human isolates, but none of the isolates from pigs, and only one isolate from a dog harboured *erm*A. The *erm*B gene was only found in animal isolates. The *erm*C gene was the most common among human and pig isolates, while this gene was only found in one of the dog isolates. The

*msr*A gene was only found in 2 pig isolates and one dog isolate, but in 70 human isolates. Several isolates included in the study harboured more than one of the resistance determinants studied. All of the pig strains, half of the dog strains and 13 of the human strains expressed MLS$_B$ resistance constitutively. This illustrates the fact that expression of resistance genes cannot be used to predict type of resistance determinant, and that some *erm* genes circulate in both human and animal populations. Since pigs, as opposed to dogs, are not exposed to 14-membered macrolides, neither for therapy nor growth promotion, only 16-membered macrolides, the constitutive expression of *erm* genes in isolates from these animals is not surprising. Dogs, like people, may be given 14-membered macrolides for therapeutic purposes and would therefore be expected to harbour a much higher proportion of isolates with inducibly expressed *erm*-genes.

Westh and co-workers (1996) investigated the presence of *erm*A and *erm*C in human isolates of *Staphylococcus aureus* and coagulase negative staphylococci from Denmark. They found that the *erm*A gene was solely responsible for erythromycin resistance in strains isolated before 1971, but had since then gradually disappeared. Today the *erm*C determinant is responsible for 72% of erythromycin resistance in Danish *S.aureus*, according to the authors. Both constitutive and inducible expression was noticed, with the inducible form being the most common. No *erm*A genes were found in coagulase-negative staphylococci and in paired isolates, i.e. isolates of *S.aureus* and coagulase-negative staphylococci originating from the same sample, the same *erm* gene was only found in both species in 4 out of 15 pairs. This study illustrates the variability over time in the presence of different *erm* genes, a fact that emphasises the importance of identifying the genes involved in the resistance patterns studied.

Clostridia

The hitherto described genes encoding for macrolide resistance in clostridia belong to the *erm* family. The most common resistance determinant seems to be *erm*Q which has been found in isolates from pigs and humans from a wide geographical range (Berryman *et al.*, 1994). A second, apparently less prevalent gene, the *erm*B-*erm*AM (also called *erm*BP) gene, has also been described in *C. perfringens* (Daube *et al.*, 1992; Devriese *et al.*, 1993; Berryman and Rood, 1995). Genes belonging to this subgroup have earlier been described in numerous bacterial genera, both gram-positive and gram-negative, indicating that they are readily transferred. Sequencing of the *C. perfringens erm*B determinant and its flanking regions by Berryman and Rood (1995) revealed a close similarity to the corresponding determinant on plasmids from *E. faecalis* (pAMb1) and *Streptococcus agalactiae* (pIP501) which are both of the conjugative, broad host range type. It was therefore

postulated that the *C. perfringens erm*BP determinant was derived from an enterococcal or streptococcal determinant. The gene *erm*B is carried by transposon Tn917, often residing on a self transmissible, broad host range plasmid. This gene is widespread in human and animal isolates of enterococci as well as in other bacteria (Rollins *et al.*, 1985; LeBlanc *et al.*, 1986).

E.4.2   Enzymatic inactivation

Unlike target modification, which causes resistance to structurally distinct antibiotics, enzymatic inactivation confers resistance only to structurally related drugs.

Macrolide modifying enzymes have been described in lactobacilli of animal origin (Dutta and Devriese, 1981; Arthur *et al.*, 1987) and in *Streptomyces* spp. (Arthur *et al.*, 1987), but the genes in question have not been described (Arthur *et al.*, 1987). Inactivation of erythromycin by production of erythromycin esterases or phosphotransferases has been described in human enterobacteria isolated from patients undergoing treatment with erythromycin (Arthur *et al.*, 1987). Two types of esterases (I and II) are encoded for by the genes *ere*A and *ere*B, respectively (Arthur *et al.*, 1987; Leclercq and Courvalin, 1991b).

Resistance to streptogramin antibiotics, caused by the modification of both components, was described in the 70s, in *S.aureus* (Le Goffic *et al.*, 1977a; Le Goffic *et al.*, 1977b). This resistance is coded for by two genes, *sbh* and *saa,* located on a large plasmid. Most of the strains are also resistant to low levels of lincosamides although these antibiotics are not inactivated. Since this original description, several different enzymes and genes with similar activity have been described (Rende Fournier *et al.*, 1993; Allignet and El Solh, 1995). In staphylococci, resistance to the group A compound will always confer resistance to the mixtures of group A and B compounds. This is not the case with resistance to group B compounds where group A compounds still may remain active (Duval, 1985).

E.4.3 Active efflux

Resistance due to active efflux has been reported in staphylococci. The resistance gene *msr*A described in *Staphylococcus epidermidis* encodes a transport-related protein that confers inducible resistance to 14- and 15-membered macrolides (Leclercq and Courvalin, 1991b). The strains with this characteristic are, for unknown reasons, cross-resistant to streptogramin B antibiotics. Further, a constitutively expressed resistance of efflux-type mediated by the gene *erp*A has been reported in *S. epidermidis* (Leclercq and

Courvalin, 1991b). In this case, the streptogramins retain their effect. Active efflux of streptogramin A is mediated by the gene *vga* coding for an ATP-binding protein probably involved in the active transport of the compound. This gene has been found in *S. aureus* and *S. epidermidis* (Allignet *et al.*, 1992; Rende Fournier *et al.*, 1993).

E.4.4   Co-transfer of resistance genes

Co-transfer of vancomycin resistance and erythromycin resistance, from *Enterococcus faecalis* to *Staphylococcus aureus* has been shown to occur both *in vitro* and *in vivo* , in a mouse model (Noble *et al.*, 1992). Others have reached similar results. Leclercq (1989) achieved co-transfer of vancomycin resistance and *erm*AM, located on the plasmid plP819, between different enterococcal species and from enterococci to streptococci and *Listeria monocytogenes*. Further, the transfer of pheromone-responsive plasmids harbouring virulence- and resistance factors (among others *erm*B on Tn*917*) between strains of *E. faecalis* in an animal model has been demonstrated (Huycke *et al.*, 1992).

E.4.5   Exposure to AFA and resistance

Evaluating resistance data between different countries or different regions, is difficult without information about the degree of exposure to various antibacterials in various regions. Little information is available concerning statistics on the quantities of MLS antibacterials used for different purposes in different countries. Moreover, differences between countries in climate, animal husbandry and animal health, frustrate comparisons. However, Denmark and Sweden are, in these respects, similar enough for a comparison to be attempted. Recent statistics from Denmark and Sweden are presented in table E.VI. The statistics on human consumption are only available as the sum of macrolides and lincosamides. An estimate of the proportion of macrolides in this group was made based on substance-specified figures from Sweden 1994 (Naturvårdsverket, 1996). The same figures were also used to estimate an average DDD of 1.7g.

Table E.VI. Usage of MLS antibacterials in 1995 in Sweden and Denmark expressed as kg active substance (Apoteksbolaget, 1996; DANMAP, 1997)

Country	Tylosin	Spiramycin	Macrolides (total)	Virginiamycin
Denmark				
animal therapy			9500	
feed additives	52275	507	52782	2590
human therapy			6500[1]	
Sweden				
animal therapy	1238	565	1803	575
human therapy			6100[1]	

[1] Estimated as described above

   In the debate concerning the contribution of MLS antibacterials used as growth promoters to development of resistance, it has often been assumed that their therapeutic use dominate the selective pressure. The figures from Denmark show, however, that the amount of macrolides used for growth promotion exceeds the therapeutic use in animals by more than 5 times. Comparing the amounts used for therapy in Sweden and Denmark, the differences can be attributed to different sizes of animal populations. Thus, it is a reasonable assumption that the observed differences in prevalence of MLS resistance between enterococci isolated from Danish and Swedish animal sources (see table E.II) are largely explained by the use of MLS antibacterials, mainly tylosin, for growth promotion.

   In a paper by Lacey (Lacey, 1988) data on macrolide consumption in humans (erythromycin) and animals (tylosin) during 1986 in the UK are presented. The quantities used in animals and man are about the same, 47 000 and 51 000 kg active substance, respectively. However, the majority of animals (pigs) would have received a low level of antibiotic for a long time, whereas the humans would have received a high level for a short time. Thus, more individuals were exposed for a longer time period in the animal population, as compared to the human population.

E.5 Effects on specific animal diseases

Virginiamycin may be used for prevention of necrotic enteritis (NE) in poultry. In a large study, Jansson and co-workers (1992) found that virginiamycin given in feed at 20 ppm effectively prevented the experimentally induced NE. Another study by Elwinger and Teglöf (1991) showed similar results; virginiamycin at 20 ppm in feed notably reduced mortality (p<0.001) of chickens in necrotic enteritis, as compared to non-medicated groups.

Stutz and co-workers (1983) showed that virginiamycin, at 55 ppm in feed, reduced the amount of *C. perfringens* in the intestines of chickens compared to non-medicated controls (p<0.05). The numbers of clostridia were inversely correlated with performance data.

MLS antibacterials are still considered to be alternatives in the therapy and prevention of swine dysentery (Allen *et al.*, 1992). Although tiamulin is often the drug of choice for treatment of this disease, tylosin is in many areas important for maintaining the means to control the infection.

There seems to be a good correlation between MIC values of *S. hyodysenteriae* for tylosin and therapeutic effect of this substance. Williams and Shively (1978) found that tylosin at 100-110 ppm completely prevented swine dysentery induced by a tylosin-susceptible isolate of *S. hyodysenteriae*, while it was only partly effective against the disease induced by isolates with higher MIC values for tylosin. Jacks and co-workers (Jacks *et al.*, 1986) also prevented swine dysentery in pigs experimentally infected with a tylosin-susceptible strain, by feeding tylosin at 110 ppm. When a tylosin-resistant strain was used the disease was not prevented, although mortality was lower in the tylosin-medicated group as compared to the non medicated group. A new tylosin compound, 3-acetyl-4"-isovaleryl tylosin was also tested and found to be effective at 50 ppm.

Miller and co-workers (1972) studied the effect of different levels of virginiamycin and tylosin on pigs experimentally infected with *S. hyodysenteriae*. They found a good prophylactic effect of virginiamycin at about 25 ppm but only a moderate effect of tylosin even at about 100 ppm. Antimicrobial resistance pattern of the infecting strain is not presented, but presumably it was resistant to tylosin. Williams and Shively (Williams and Shively, 1978) fed experimentally infected pigs virginiamycin at 50 to 100 ppm. Virginiamycin could not control the disease, although clinical signs were a little less common in medicated pigs than in non-medicated animals. This was supported by the observations of Rønne and co-workers (1992) who investigated the effect of 20 ppm of virginiamycin.

McOrist and co-workers (1997) evaluated the efficacy of orally administered tylosin for the prevention and treatment of experimentally induced porcine proliferative enteritis (PPE). They found that tylosin at therapeutic levels could prevent PPE in challenge exposed pigs and could also be used for treatment of previously induced PPE. Even at growth promoting levels, tylosin was effective in preventing development of PPE. Moore and Zimmermann (1996) reported successful prevention of PPE by tylosin at about 100 ppm. Fleck and Jones (1994) compared 0, 40 and 100 ppm of tylosin for the treatment and prevention of PPE. The higher level of tylosin was effective as treatment of PPE, while 40 ppm not quite prevented emergence of clinical signs.

E.6   Impact of resistance on animal and human health

E.6.1   Consequences of MLS resistance

As stated earlier, macrolides and streptogramins are important therapeutic or prophylactic substances for animals. Macrolides also play an important role in human medical therapy (Kirst and Sides, 1989). Streptogramins are not, except in some countries, yet widely used in human medicine, but due to the development and spread of antimicrobial resistance among human pathogens these compounds are expected to become more commonly used in the future (Pechere, 1992; Pechère, 1996).

From an animal health point of view resistance to MLS-antibacterials in *S. hyodysenteriae* is perhaps most alarming. Uncontrollable outbreaks of swine dysentery can lead to substantial losses and eradication of whole herds may be the only option left (Bouwkamp, 1982). *In vitro* data on resistance of this bacterium seems to be a good predictor of clinical outcome after treatment (Williams and Shively, 1978; Jacks *et al.*, 1986). Unfortunately, such data indicate that the therapeutic potential of the MLS drugs is now limited (Ronne and Szancer, 1990; Gunnarsson *et al.*, 1991; Fellström *et al.*, 1996; Molnar, 1996). Buller and Hampson (1994) claimed that the present situation presents a potential threat to the pig industry.

Bearing in mind that both macrolides and streptogramins are attractive therapeutic options for several important animal and human diseases, a further increase in resistance prevalence would be most unfortunate

E.6.2   Influence of AFA usage

The possible influence of the use of macrolides as growth promoters on the resistance of human pathogens was intensively discussed in relation to the recommendations of the Swann committee. The debate has, to a large extent, been concentrated on the possible influence on the resistance of staphylococci. Knothe (1977b) concludes, in a review on medical considerations of the use of macrolides in animal feeds, that transfer of staphylococcal strains from animals to humans is possible. However, he considers this to be of no significance since the likelihood of an animal strain actually causing disease in humans is small and the proportion of macrolide resistant strains in human medicine is low. The basis of this assumption is a review of investigations published from 1953-1971 and an accompanying paper describing the prevalence of resistance in Germany from 1968-1976 (Knothe, 1977b; Knothe, 1977a). In the latter study, prevalences of erythromycin resistance between 13 and 24 % were reported with a tendency

towards a reduction over time. In a more recent report from the same region of Germany, the prevalence of erythromycin resistance in *S. aureus* from community acquired infections was 8%, from general ward 13% and from intensive care 28% (Shah *et al.*, 1993). In some recent reports from other European countries, though, the prevalence of resistance to macrolides in staphylococci and enterococci approaches 50% (Turano *et al.*, 1994). At the time when Knothe (1977b) published the review, exchange of genes between gram-positive cocci was thought to be a rare event in natural environments.

The conclusion is thus based on the assumption that animal strains have to colonise and infect humans in order for resistance to have an impact on human health. This view is no longer predominant (see chapter 4).

Genes of the *erm*-family conferring resistance of the MLS$_B$ type have been demonstrated in both human and animal bacteria (Arthur *et al.*, 1987; Roberts and Brown, 1994; Wasteson *et al.*, 1994). This may be due either to common ancestry of the bacteria and/or to genetic exchange in more recent times.

The rarity of tylosin resistance in human pathogenic bacteria has been used as evidence that the flow of resistant organisms or their genes from animals to man is rare (Lacey, 1980; Lacey, 1981; Lacey, 1984; Lacey, 1988). In the most recent paper on this topic by Lacey (1988), the author presented five statements supporting the view that resistant bacteria in animals and humans are two separate entities;

Firstly, that animal staphylococci survive poorly in human environments. This may well be the case, but as long as resistance genes from these staphylococci (and other bacteria, such as enterococci) can be transferred to human strains, the survival of the bacteria themselves is not necessary. Moreover, other bacterial species have been found to be less host-specific.

Secondly, he stated that tylosin resistant strains may grow more slowly than sensitive cultures and would therefore disappear as the exposure to the antibiotic stops. Since use of tylosin as a growth promoter means an almost continuous exposure, this arguing seems to support the view that the use should be limited. Moreover, resistant bacteria may persist even in the absence of a selective pressure (see chapter 4). Relieving the selective pressure of antibiotic exposure in humans usually leads to a drop in the prevalence of resistance, but one cannot assume that the pool of resistance genes would disappear as long as a selective pressure is in force in animal husbandry.

Thirdly, the difficulties in transferring resistance between animal and human staphylococci were pointed out. This was supported by an earlier investigation by the same author (Lacey, 1980), showing that transfer between animal and human staphylococci can occur, but at a lower frequency and in fewer strains than between isolates of staphylococci from the same source. The low frequency of transfer in this study is not surprising, since

only phage dependent transfer is studied. Conjugative transfer is generally believed to be more common. Regarding what can be deemed a "low" transfer frequency, see chapter 4.

Fourthly, it was stated that tylosin resistance requires the presence of erythromycin to occur, a statement that is true regarding phenotypic expression of erythromycin-inducible *erm* -encoded resistance, but overlooks the lack of correlation between genotype and phenotype in MLS_B resistant staphylococci (see table E.I).

Fifthly the author proposed that the low level of tylosin used for growth promotion in pigs may be too low to select for resistance. The concentrations used for growth promotion are above the MICs of naturally susceptible bacteria (see chapter 4). Finally, the author also concluded that since there are no residues in meat, no selection pressure will be exerted in man. Acknowledging that this is true, the real concern about meat would be that it can contain microbes carrying transferable resistance genes. Besides meat, there are numerous direct and indirect contact areas between human and animal bacteria.

Lately, streptogramin resistance in animal and human bacteria has received increased attention as the streptogramin quinpristin-dalfopristin has recently been introduced for human therapy. Woodford and co-workers (1997) reported streptogramin resistance in vancomycin-resistant enterococci (VRE) isolated from raw chicken (3 isolates) and from a hospital patient (1 isolate) in the UK. The resistance trait included cross-resistance to macrolides and lincosamides and was transferable to other enterococci. The authors commented on the fact that no streptogramin is yet licensed for use in human therapy in UK, whereas virginiamycin is widely used for growth promotion in animals. A reservoir of streptogramin resistance may be present in animal bacteria. Since infections with VRE is one of the main indications for quinpristin-dalfopristin therapy, acquisition of streptogramin resistance by those organisms is most alarming. Similar observations have been reported from The Netherlands (van den Bogaard *et al.*, 1997). In this study, enerococci were isolated from healthy humans and pigs. The prevalence of streptogramin, macrolide and vancomycin resistance in human samples was 30%, 50% and 12%, respectively. The corresponding figures for the samples from pigs were 75%, 84% and 34%. The higher prevalences observed in the material from pigs indicate a higher selective pressure in this population. No information on consumption of macrolides and streptogramins in either humans or pigs in The Netherlands were given.

E.7   Other effects on the microflora

E.7.1 Salmonella

There are several studies available on the possible influence of antibacterial feed additives on colonisation of the intestines and the shedding of salmonella. At least 10 of these studies from the last decade involve virginiamycin and 3 of those also include tylosin.

   The main problem with all studies in this area, however, is the variability in study design which makes them difficult to compare and evaluate. Weaknesses that are found in several of the studies undertaken include, among other things, inadequate number of animals in the experimental groups, lack of nonmedicated control and undocumented sensitivity of the bacteriological methods employed to recover the salmonella organisms. Taken together, the design of these studies only allows for extremely large differences in outcome. Some of the studies use commercial feed and do not state whether care has been taken to ascertain that this feed contains no antimicrobials. Since antimicrobial feed additives are very common ingredients in commercial feedstuffs this may otherwise influence the outcome of the experiment. Several authors claim that their experimental models are reproducible, but any well described method is reproducible. The results obtained with the method, however, have to be reproducible too.

   Bearing in mind the weaknesses mentioned above there are some studies which are nevertheless worth further comment.

   Abou-Youssef and co-workers studied the effect of in-feed virginiamycin on experimental infection with *Salmonella* Typhimurium in chickens and swine (1979; 1983). No significant differences between medicated animals and control groups were found, when comparing prevalence and duration of salmonella shedding. However, the experimental groups were small in both studies, between 5 and 20 animals per group, which would only allow for very large differences between groups to be detected.

   In his thesis from 1981, Leuchtenberger (1981) compares the salmonella excretion of experimentally infected broilers treated with avoparcin, virginiamycin or tylosin with nonmedicated controls. In a series of experiments 2 different concentrations of antimicrobial (20 and 30 ppm virginiamycin, 50 and 100 ppm tylosin), 2 types of housing (wired cages and floor housing), 2 methods of experimental infection (direct oral inoculation and mixed in the feed) and 2 different infectious doses (10^3 or 10^4 organisms of *Salmonella* Typhimurium) were tried in various combinations. Most experiments were performed in duplicate, with groups of 20-30 animals. Sampling was performed on several occasions throughout the trials, by cloacal swabs on live birds and from the heart, liver, duodenum and caecum of killed birds at the end of the experiment. Not all birds were sampled on all

occasions, though. The author concludes that feeding avoparcin, virginiamycin or tylosin, at both levels tested, can prolong the persistence of *S.* Typhimurium infection in the intestine as well as internal organs, and significantly increases the amount of salmonella found in samples from these sites. It was also found that the duration and frequency of Salmonella excretion depends on the dose and way of infection, as well as the frequency of dosing with infectious organisms and on the housing system.

It is rather surprising that so many of the trials yield significant differences, since the sample sizes are invariably too small for any small differences to be detected. However it is only in the main experiments, where cloacal swabs were taken continuously, that large enough numerical differences were recorded to be interpreted as a strong tendency towards increased salmonella excretion in treated birds. The trials where all birds were killed and cultures made from internal organs must be regarded as inconclusive, due to too small sample sizes and too variable numerical differences in the results to be statistically significant.

In 1981 Gustafson and co-workers published a study where chicks fed avoparcin or virginiamycin or no antimicrobial were compared after receiving *S.* Typhimurium via the drinking water (Gustafson *et al.*, 1981). The authors tried to achieve a level of infection that corresponds to the level of natural infection and the administration of the salmonellae was distributed over several days. The results indicate that the feeding of virginiamycin or avoparcin led to a larger proportion of animals shedding salmonella as compared to controls during the first 3 weeks after infection. This proportion of positive birds then decreased to become lower than that in the control group at about 4 weeks post infection. However, in the samples taken from the caeca after slaughtering the birds at the end of the trial, the proportion of salmonella positive birds was highest in the virginiamycin-fed group and lowest in the control group.

The sample size in this study is comparatively large, 100 animals in each group (all animals were sampled on each sampling occasion). The bacteriological method used for isolating the inoculated salmonella from the faecal samples include selective culture on media containing nalidixic acid, as the experimental strain was resistant to nalidixic acid. This isolation procedure would, however, also yield nalidixic acid-resistant mutants of other lactose negative enteric bacteria, and one cannot be certain that the proportion of coliforms mistakenly identified as salmonellae would be the same in all experimental groups. No further identification of the presumed salmonellae isolated in this way is reported in the article. The major objection against the design of this study is, however, that all birds received monensin at 100 ppm in the diet. Since monensin has antibacterial effects, this must be regarded as a possible confounder. Thus, the study only

investigated the differences between birds fed monensin and birds fed monensin plus avoparcin or virginiamycin.

Smith and Tucker have published several studies on the influence of various antimicrobial substances on the course of salmonella infection and excretion in broiler chickens (Smith and Tucker, 1975a; Smith and Tucker, 1975b; Smith and Tucker, 1978; Smith and Green, 1980; Smith and Tucker, 1980). One study from 1975 includes both tylosin and virginiamycin (Smith and Tucker, 1975b) and one from 1978 includes tylosin (Smith and Tucker, 1978). Tylosin was given at concentrations of 10 and 100 mg/kg feed in both studies and in the study from 1975 virginiamycin was also given at concentrations of 10 and 100 mg/kg. Duplicate experiments were performed on groups of 33-45 chickens receiving either antibiotic-containing feed or control feed without antimicrobial additives. In the study from 1975, the animals were experimentally infected with 0.3 ml of a nalidixic acid-resistant strain of *Salmonella* Typhimurium at a concentration of 10^9 CFU/ml. In the study from 1978, the animals were infected through contact with experimentally infected chickens. Cloacal swabs were taken from all animals throughout the trials and caecal contents were sampled after slaughter at the end of each trial. The bacteriological methods are the same as those employed by Gustafson and co-workers (1981), i.e. culture on selective media containing nalidixic acid. The authors claim that very few faecal bacteria grow on this medium and the colonies of those that do can easily be differentiated visually from those of the infecting salmonella strain. No validation of this statement is presented.

In these two studies tylosin, at both concentrations tested, gave a higher rate and a greater amount of salmonella excretion in the birds given this diet, compared to that of the control groups. Virginiamycin, at the concentrations tested, caused only a slight increase, or no increase, in the rate and amount of salmonella excretion.

No studies addressing the issue of dose-response effects of spiramycin, tylosin or virginiamycin on salmonella colonisation have been found, nor any studies concerning effects on infectious dose.

Taken together, it appears that tylosin and virginiamycin might effect salmonella colonisation, but available studies can not provide the basis for any proper assessment. No information has been found on spiramycin in this matter.

E.7.2   Other enteric pathogens

No data on influence of macrolides on colonisation with other enteric pathogens, such as *Campylobacter* spp. or *Yersinia enterocolitica* has been found.

E.7.3   Other effects

Continuous administration of MLS antibacterials to animals might interfere with disease surveillance. Kempf and co-workers (1991) suggested that macrolide (spiramycin or tylosin) treatment might hinder the detection of *Mycoplasma gallisepticum* in cultures, or antibodies to this pathogen, in samples from subclinically infected chickens. Ronne (1992) reported a decrease in the isolation rate of *Serpulina hyodysenteriae* after introducing virginiamycin in the feed of pigs with clinical signs of infection.

   Such effects need to be considered for disease control based on surveillance programs, as they may substantially affect the efficiency and the benefit/cost ratio of the program.

E.8   Toxicological aspects

As spiramycin, tylosin and virginiamycin are safely used in clinical therapy, they are not expected to have any obvious toxic effects on the target species at growth promoting dosages.

E.8.1 Residues

Spiramycin and tylosin were evaluated by the Committee of Veterinary Medical Products(CVMP) in 1994 and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) 1991 (1991). In table E.VII, provisional maximum residue limits (MRL) fixed by the Commission in 1995 for spiramycin and tylosin when used as veterinary medical products are shown. Corresponding values for spiramycin when used in cattle were not given at the time, but temporary MRLs for cattle as fixed by JECFA 1991 to 50, 300 and 200µg/kg for muscle, liver and kidney. respectively.

   MRLs are based on ADI (acceptable daily intake). These limits are based on risk assessments and are set to ensure that residues in animal products do not harm the consumers of these products.

Table E.VII. Maximum residue limits of spiramycin and tylosin as veterinary medical products in foodstuffs of animal origin according to Commission regulation (EC) 1442/95

Substance	Marker residue	Animal species	MRL, µg/kg	Target tissue
Spiramycin	spiramycin	swine	600[1]	liver
			300[1]	kidney, muscle
			200[1]	fat
Tylosin	tylosin	bovine, swine, poultry	100	muscle, liver, kidney
		bovine	50	milk

[1] Provisional MRL, expired July 1, 1997.

According to a study by Green Lauridsen and co-workers (1988), usage of tylosin and virginiamycin at dosages used for growth promotion did not result in residues even if no withdrawal time is practised.

Published studies on residue aspects on spiramycin when given at growth promotion dosages have not been found. Spiramycin fed to pigs at approximately 8 times the maximum concentration permitted for growth promotion resulted in liver residues at zero withdrawal time of 10 times the MRL (FAO/WHO, 1991). Assuming a linear relationship, residues in swine liver, from spiramycin used for growth promotion, could be above MRL at zero withdrawal time (see chapter 5). The pharmacokinetics of spiramycin are not linear in swine (Sutter *et al.*, 1992), which could result in higher residue levels than what might be expected. Similar experiments in poultry, with similar assumptions of a linear relationship, indicates that in poultry the MRL would not be reached with spiramycin at growth promotion dosages(FAO/WHO, 1991). For tylosin at growth promoting dosages, MRL is not expected to be reached (see chapter 5).

Further investigations into possible residues from spiramycin as antibacterial feed additives (AFA) in target species are therefore necessary.

E.8.2 Allergy

Allergic reactions triggered by macrolide therapy in humans are rarely reported (Descotes *et al.*, 1988; Periti *et al.*, 1993). Occupational contact dermatitis and/or asthma is, however, not unusual in farmers (especially pig farmers), feed plant workers, veterinarians and people working in the pharmaceutical industry (Hjorth and Weismann, 1973; Gollins, 1989; Lee *et al.*, 1989; Caraffini *et al.*, 1994; Danese *et al.*, 1994). Hypersensitivity to tylosin and/or spiramycin, indicating a certain but not absolute cross-reactivity, has been reported (Hjorth and Weismann, 1973). Airborne antigen is thought to be the main cause of these reactions.

There is no information available on the prevalence of hypersensitivity to virginiamycin, but there can be no doubt that both tylosin and spiramycin are capable of functioning as potent antigens and that people continuously exposed to dust containing these substances may become allergic. Frequent use of macrolides in animal feed may therefore be regarded as a professional hazard for farmers and other people in contact with this feed.

E.8.3   Other immunological effects

An apparent reduction of the response to vaccination following administration of macrolides has been noted in two studies. In a study by Hassan (1990), chickens, vaccinated against Newcastle disease virus (NDV), were challenged with NDV and subsequently treated with spiramycin. This resulted in a twice as high mortality in treated and vaccinated birds as compared to non-treated, vaccinated birds. The spiramycin was given as a subcutaneous injection at a high dose, which must be regarded as a rather extreme challenge.

In one study by Vahl (1985), virginiamycin was given in the feed at a concentration of 20 mg/kg in a floorpen experiment. Immunological response to vaccination with NDV, in the form of antibody production, was measured. The NDV titers in the group receiving virginiamycin were depressed as compared to the non-treated group. In a corresponding experiment with caged birds the results were inconclusive. No challenge experiments were made.

No explanations of the mechanisms behind these findings have been provided by the authors. No other, similar reports have been found. In many countries the control of NDV infections depends on vaccination programs. In view of this, these observations certainly merit further investigation.

E.9   Environmental effects

Like other AFA, if the use of spiramycin, tylosin or virginiamycin reduces the amount of feed consumed per kg weight gain in the target animal, they would also be expected to reduce nitrogen output per kg weight gain.

The effect of tylosin and tylosin fermentation wastes on microbial activity in soil has been investigated (Bewick, 1978). Tylosin from waste was detected in leachates and after 10 weeks 20-32% had been leached from the soil. Addition of pure tylosin to potting compost in concentrations expected from fertilising soil with the wastes (2, 20 or 40 tonnes/ha) resulted in a decrease in microbial respiration for 5-7 weeks after the addition of the antibiotic. In an experiment by Jagnow (1978), the degradation of spiramycin added at 10 ppm to chicken manure was investigated. After 4 weeks, 70% of

the added spiramycin had been degraded. However, when the manure was mixed with soil in the ratio 1:3, degradation of spiramycin was complete within a week. Gavalchin and Katz (1994) studied the degradation of tylosin and erythromycin in sandy loam from a non-agricultural area mixed with chicken faeces at different temperatures. Sterile soil-faeces mixtures were used as controls. At 20 and 30°C, inactivation of tylosin occurred rapidly and after 5 days the detection limit was reached. At 4°C however, 40% of the initial concentration was still present after 30 days of incubation. No degradation of either antibiotic occurred in the sterile controls. This indicates that the degradation process is due to microbial degradation of the antibiotic, probably by enzymatic inactivation. In the experiment, non-agricultural soil was used and the antibiotics were mixed in faeces from non-medicated birds. As the prevalence of bacteria carrying resistance genes coding for macrolide degrading enzymes can be expected to be higher in both faeces from animals given the substance in question and in agricultural soil previously fertilised with macrolide containing manure, the degradation process can be expected to be more efficient. However, during the degradation period the microbial respiration is expected to be lowered as observed by Bewick (Bewick, 1978), presumably due to impairment of sensitive microbiota.

E.10   Summary comments

Spiramycin, tylosin and virginiamycin all belong to important antibacterial classes. Increased resistance to spiramycin, tylosin and virginiamycin would hamper the therapeutic use of substances from these classes in both animals and humans. Exposure of bacteria to spiramycin, virginiamycin and tylosin selects for resistant strains, usually carrying one or several transmissible resistance determinants. In order not to further diminish their therapeutic value, these substances should be restricted to therapeutic use. Spiramycin, tylosin and virginiamycin are potent allergens, and may as such represent an occupational hazard for farmers and feedmill workers.

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Annex F: Carbadox and olaquindox

F.1 Introduction

Carbadox and olaquindox belong to the group of quinoxaline-di-N-dioxide derivatives, or quinoxalines. This group consists of synthetically produced substances with activity against anaerobic bacteria but also against certain gram-negative facultative anaerobes such as salmonellae (Prescott and Baggot, 1993). Carbadox is also active against gram-positive bacteria such as staphylococci (Devriese, 1980). The antibacterial activity of the quinoxalines is markedly improved under anaerobic conditions (Prescott and Baggot, 1993). Quinoxalines that are presently approved as antibacterial feed additives (AFA) in the European Union are carbadox and olaquindox. Other substances of this class are quindoxin and cyadox.

   After oral administration, olaquindox and carbadox are rapidly absorbed and are extensively metabolised (FAO/WHO, 1991; FAO/WHO, 1994). The concentration profiles of the active substances in the gut show a gradual decline from the stomach to the colon where the concentrations are below detectable levels (Baars *et al.*, 1988; de Graaf *et al.*, 1988; Spierenburg *et al.*, 1988c).

   The quinoxalines are not used for in human medicine. Carbadox and olaquindox are approved as feed additives for swine for growth promoting purposes at dosages ranging from 20 to 50 ppm and 50 to 100 ppm respectively. Quinoxalines are also used at similar or higher dosages for prevention of intestinal infections (see F.7).

   The use of quinoxalines has been questioned for toxicological reasons, mainly due to their genotoxic and/or carcinogenic potential (Anonymous, 1995), they are also known to have toxic effects on the target species (Prescott and Baggot, 1993).

F.2 Mode of action and resistance mechanisms

As mentioned above, the activity of the quinoxalines is markedly improved under anaerobic conditions. The mode of action of the quinoxalines on bacteria was investigated by Suter and co-workers (1978). The authors showed that the antibacterial effect of quindoxin was always accompanied by a reduction of the drug. Such a reduction only occurred under anaerobic conditions, and generated free radicals. Based on a series of experiments, the authors suggested that radicals produced during the intracellular reduction of

The figures in this report is only available in the printed version

Figure F.I. Tentative sketch of carbadox, olaquindox, nalidixic acid and enrofloxacin

the parent substance or some decomposition products thereof damage DNA without being bound to this target. As a consequence, DNA synthesis ceases, breakdown of chromosome ensues and the bacterial cell dies.

The quinoxalines are somewhat structurally related to the quinolones (see figure F.1) which also exert their antibacterial activity by interfering with the bacterial DNA synthesis. The quinolones inhibit the activity of bacterial topoisomerases, i.e. enzymes controlling the supercoiling of DNA, converting relaxed covalently closed circular DNA to a superhelical form by an energy dependent strand breakage and resealing process (Maxwell, 1997). Although there are still unclarities as to the precise bactericidal mechanism of the quinolones, it is thought that when the gyrase is inhibited, resealing following breakage is prevented and DNA is exposed to and degraded by exonucleases.

Resistance to quinolones develop through alterations of the target of the drug and to some extent through altered permeability or increased efflux.

As the information on mechanisms of action of the quinoxalines predates the discovery of bacterial gyrases, possible similarities with the precise mechanism of action of the quinolones cannot be determined. No information on the molecular background of observed quinoxaline resistance, nor on cross-resistance between quinoxalines and other DNA-active drugs such as quinolones has been found. It is likely, however, that were resistance due to altered permeability or active efflux to occur, this would confer cross-resistance to at least the other quinoxalines.

F.3   Development of resistance

An experimental study on germ-free mice fed carbadox, olaquindox, flavomycin and chlortetracycline was reported by Corpet (1984). The mice were inoculated with intestinal microflora from 4 piglets, and the drugs were given at dosages corresponding to those used for growth promotion in livestock. Little effect on coliform resistance against olaquindox and carbadox was observed, nor was there any effect of the quinoxalines on tetracycline resistance.

A prospective study to monitor the development of olaquindox resistance in coliforms following the introduction of olaquindox as a feed additive was conducted in commercial farms in Suffolk by Linton and co-workers (1988). Olaquindox resistance in coliforms from farms with and without use of the antibacterial are shown in table F.I. As is often the case in field studies, there were problems with sampling variability and difficulties to control the management on the farms. In spite of this, the overall results were consistent and showed an increasing level of resistance to olaquindox in coliforms from farms using olaquindox. Incidence and level of resistance increased on

neighbouring farms not using olaquindox as well, but to a lesser extent. The latter finding is not surprising as the herds were not isolated from the environment.

Table F.I. The average percentage of coliforms resistant to 50 μg/ml olaquindox in each year of the survey (from Linton *et al.*, 1988)

Year	Resistance (%)	
	Control farms	**Test farms**
1981[1]	0.00	-
1982	0.03	0.04
1983	0.12	5.63
1984	0.68	6.14

[1] Sampled before olaquindox was used in the UK

   The ecological aspects of olaquindox resistance were investigated by Hedges and Linton (1988). In a prospective study, chosen pens in four pig farms, two of which used olaquindox were sampled weekly. Coliform bacteria were isolated from the samples and biovariant along with sensitivity to olaquindox and other antibacterials was determined. The overall proportions of coliforms resistant to olaquindox (counts on selective medium divided by counts on non-selective medium) were 0 and 0.02%, respectively, for control farms and 1.3% and 6.5% for the farms using olaquindox. A transient appearance of olaquindox-resistant strains in one of the control farms coincided with a changeover in the occupancy of the pen. The weekly fluctuations in proportion of resistant strains were very marked.
   An increase in carbadox resistance in salmonellae over time was noted in a study from Kansas by Mills and Kelly (1986). The study included clinical isolates from necropsied swine in Kansas during 1980-1983. A steady increase in carbadox resistance, from 37% 1980 to 61 % 1983 was noticed. The authors note that in-feed carbadox in Kansas is labelled for prevention of swine dysentery and for treatment of salmonella infections.
   Ohmae and co-workers (1983) reported the incidence of carbadox resistance in *Eschericha coli* from cattle, pigs and chickens, collected during 1976-1980 in Japan. Carbadox resistance was only found in isolates from pigs. All isolates with a minimum inhibitory concentration (MIC) of carbadox under anaerobic conditions of >25μg/ml originated from 1 farm, where carbadox was used for prevention of swine dysentery. Baumgartner and co-workers (1985), investigating samples from the same animal species in Switzerland, found olaquindox-resistant coliforms in samples from pigs (24%) and calves (20%) but none in samples from chickens. Spanoghe and

Pohl (1987) reported a 5 times higher prevalence of carbadox-resistance in coliforms from pigs receiving carbadox, as compared to pigs not fed antibacterials, in a material collected in various European countries.

Several investigations report on a uniform sensitivity of *Serpulina* spp to carbadox (Molnar and Magyar, 1987; Walter and Kinyon, 1990; Molnar, 1996) with reported MICs generally considerably below 0.4μg/ml. Contrary to this, in a study from Canada, a MIC_{90} (minimal inhibitory concentration for 90% of the isolates) of carbadox for *Serpulina hyodysenteriae* of >6μg/ml was reported (Messier *et al.*, 1990). This figure is considerably higher than those reported by other authors, indicating an emerging resistance. In an investigation from Sweden, carbadox had a MIC of >0.1μg/ml for 3 out of 67 (4%) of the Serpulina isolates investigated. Although MICs in this range might not cause the isolates to be classified as resistant, their sensitivity seems reduced as the modal MIC in this study was 0.012μg/ml. This might indicate a gradual increase in MICs, a phenomenon which is seen when stepwise mutations are needed for full resistance. Interestingly, carbadox was not used in Sweden at the time when the isolates were collected.

Two reports show the MICs of olaquindox and carbadox for individual strains of *Serpulina* spp (Williams and Shively, 1978; Fellström *et al.*, 1996). No clear relation between MICs of carbadox and olaquindox can be noted in the data presented. In the study by Williams (1978), a good correlation between MICs obtained *in vitro* and therapeutic efficacy of respective drug in experimental infections was noted. Infection with the three strains with MICs for olaquindox of 2.5μg/ml or more resulted in considerable morbidity in spite of olaquindox treatment, while the treated pigs infected with a strain with a MIC of olaquindox of 0.3μg/ml remained healthy. The same 4 strains were uniformly sensitive to carbadox both *in vitro* and *in vivo.* Taken together, these studies indicate that strains resistant to olaquindox are not cross-resistant to carbadox. The mechanisms for the observed resistance were not investigated.

Concerning the activity of carbadox against gram-positive bacteria, few publications are available. Devriese (1980) reported on the carbadox-sensitivity of 950 isolates of *Staphylococcus aureus* from cattle, poultry and pigs. All the isolates had MIC values <1.6μg/ml and no indications of acquired resistance were found. Similarly, in an investigation of MIC of carbadox for clostridia from the same animal species, all 68 isolates had MIC values below 0.25μg/ml (Dutta *et al.*, 1983).

Indications of a simultaneous increase in minimum inhibitory concentrations for olaquindox and the quinolone enrofloxacin was recently detected in a Swedish survey (Greko, 1997). The investigated strains originated from three different cathergories of piglet producing herds; 10 herds were using olaquindox as medicated feed (160 ppm), 10 herds were using zinc oxide (2500 ppm) and 10 herds used no medication. The observed

phenotypes with respect to olaquindox and enrofloxacin sensitivity are shown in table F.II. 16 out of 61 isolates with resistance to olaquindox according to the break-points assigned had MIC values of 0.25 for enrofloxacin. This intermediary MIC value of enrofloxacin was not found in isolates sensitive to carbadox. Only two isolates had higher MIC values (0.5 µg/ml).

Table F.II. Phenotypes with respect to olaquindox and enrofloxacin sensitivity in *E.coli* isolated from piglets in herds using different regimes (Greko, 1997)

Phenotype[1]	Proportion of isolates with phenotype from sampling group (%):[1]			
	Olaquin-dox	Zinc-oxide	No medi-cation	All groups
	(n=60)[2]	(n=73)	(n=85)	(n=218)
Olaquindox[R]-Enrofloxacin[R]	0	0	1	<1
Olaquindox[R]-Enrofloxacin[I]	15	9	0	7
Olaquindox[R]-Enrofloxacin[S]	20	9	30	20
Olaquindox[S]-Enrofloxacin[R]	0	1	0	<1
Olaquindox[S]-Enrofloxacin[I]	0	0	0	0
Olaquindox[S]-Enrofloxacin[S]	65	80	68	71

[1] Determined according to MICs (µg/ml); Olaquindox[R] >32, Olaquindox[S] <64, Enrofloxacin[R] >0.25, Enrofloxacin[I] =0.25, Enrofloxacin[S] <0.25

[2] n= number of isolates

F.4   Acquisition of resistance

Omahae and co-workers (1983) investigated the transferability of carbadox resistance from resistant *E.coli* originating from one farm. The carbadox-resistance was transferable by conjugation to other strains of *E.coli*. Transfer was invariably linked to transfer of resistance to spectinomycin, streptomycin and ampicillin. The resistance determinants were shown to reside on a conjugative plasmid. No transfer to *Serpulina* spp was observed. Spanoghe and Pohl (1987) tested 61 *E.coli* strains with anaerobic MIC values for carbadox of >8µg/ml for their ability to transfer resistance (including carbadox). The experiments were successful in 55 and 69% of the attempts when two *E.coli* strains were used as recipients. Transfer of carbadox resistance to salmonellae was achieved only in 10% of the experiments. Transfer of carbadox resistance was constantly associated with co-transfer of

resistance to at least one other antibacterial. The most frequently linked transfer was carbadox-streptomycin-tetracycline resistance. In contrast, in similar experiments reported by Baumgartner and co-workers (1985), transfer was successful from only 3 out of 64 strains tested and no co-transfer was observed.

Linton and co-workers (1988) demonstrated transfer of olaquindox-resistance from 6 out of 12 *E. coli* strains to a laboratory strain of *E. coli*. Transfer of resistance did not correlate to the appearence of a plasmid nor was it linked to transfer or other resistance traits. The evidence therefore suggests that the gene(s) conferring olaquindox resistance were located on the chromosome.

An apparent suppression of coliforms carrying R-plasmids has been reported for carbadox and olaquindox (Gedek, 1979) but no mechanism explaining this was provided. Quinolones and novobiocin, inhibitors of DNA gyrase, have been shown to eliminate plasmids *in vitro* both by inhibiting their replication (Uhlin and Nordström, 1985) and their transfer (Weisser and Wiedemann, 1987).

No information on the precise genetic nature of either carbadox or olaquindox resistance has been found. Plasmid mediated resistance to nalidixic acid, a quinolone, was reported from Bangladesh in 1987 (Munshi *et al.*, 1987). This was later found to be due to a mutation of the recipient strain. Possibly, the transferred plasmid induced mutations leading to nalidixic acid resistance (Ahmed, cit. by Courvalin, 1990). Although speculative, a similar phenomenon could explain why the transconjugant strains in the experiments cited above had markedly lower MICs than those of the donor strains (Ohmae *et al.*, 1983; Baumgartner *et al.*, 1985). If stepwise mutations are necessary to reach higher MICs, single or two-step mutations induced by a transfered gene element might result in the comparatively lower MICs observed in the recipients.

F.5   Effects on specific animal diseases

The preventive effect of the quinoxalines, especially carbadox, against certain intestinal diseases in animals, especially swine dysentery, is well documented.

Studies on the pharmacokinetics of the quinoxalines have shown that the intestinal concentrations gradually decline from the stomach to the colon (Baars *et al.*, 1988; de Graaf *et al.*, 1988; Spierenburg *et al.*, 1988c). As swine dysentery is primarily a disease of the lower intestine, a preventive rather than a therapeutic effect is to be expected.

Williams and Babcock (1978) investigated presence and development of carbadox-resistance *in vitro* in *S. hyodysenteriae,* and prevention of experimentally induced infection by carbadox. No carbadox-resistant strains

were found and carbadox at 5 ppm above maximum growth promoting level was found to be effective in preventing swine dysentery. Similar results were obtained by Williams and Shively (1978), who found no carbadox-resistance in isolates of *S. hyodysenteriae* and managed to prevent swine dysentery by carbadox at, or slightly above, growth promoting levels. Jenkins and Froe (1985) prevented experimentally induced swine dysentery by feeding carbadox at growth promoting levels to pigs. In a study by Jacks and co-workers (1986), carbadox was fed at 5 ppm above growth promoting level and was found to successfully prevent swine dysentery.

Raynaud and co-workers (1980a; 1980b) developed a swine dysentery model for evaluation of drug prophylaxis. Using this model carbadox was found to effectively prevent the outbreak of swine dysentery. Olaquindox only prevented the disease during the medication period, but not post-medication. It is not clear what dosages were used in this experiment.

Taylor and Davey (1980) found that carbadox at maximum growth promoting levels prevented the onset of swine dysentery and eliminated *S. hyodysenteriae* infection in experimentally infected pigs. Rainier and co-workers (1980b) investigated therapeutic effects and prevention of the carrier state in pigs experimentally infected with *S. hyodysenteriae*. Carbadox at 5 ppm above permitted growth promoting levels effectively eliminated the infectious agent from the host, thereby curing the disease without leaving any asymptomatic carriers. Similar results were shown in a similar study by the same authors (Rainier *et al.*, 1980a). Biehl and co-workers (1984) used the same dosage of carbadox and were able to successfully treat pigs experimentally infected with *S. hyodysenteriae*.

In a large scale trial in commercial pig herds in France (Raynaud and Bretheau, 1973), 50 ppm carbadox was found to be sufficient for both prophylaxis and treatment of swine dysentery.

As mentioned under F.3, olaquindox at growth promoting levels completely prevented swine dysentery induced by an olaquindox-susceptible strain (Williams and Shively, 1978). When olaquindox-resistant strains were used as challenge, the disease was not prevented, but morbidity was lower than in the non-medicated control group. Davis and Libke (1976) prevented clinical signs of swine dysentery in experimentally infected pigs by dosages of olaquindox higher than what is permitted for growth promotion.

These experimental results have been confirmed by various field studies. In a study from Pfizer Technical Information Service (Anonymous, 1980), carbadox at the maximum growth promoting dosage totally prevented occurrence of swine dysentery. Hunneman (1980) reported a notable decrease in outbreaks of swine dysentery in an area when carbadox was introduced as a feed additive in local swine herds. Molnar (1987) reported an attempt to eradicate swine dysentery from swine herds by the aid of carbadox at growth promoting levels. The eradication program failed, but on an

individual basis, medication was successful both as a preventive and therapeutic measure. No carbadox-resistant *Serpulina* spp. were isolated. Colibacillosis and salmonellosis were also successfully prevented in the medicated herds. Olson (1986) reported successful eradication of swine dysentery with in-feed carbadox at 5 ppm above the maximum growth promoting level. Wood (1987) also reported successful eradication of swine dysentery on a farm with in-feed carbadox. The dosage used in this case was not reported.

Some information is also available concerning the preventive effect of quinoxalines for other diarrhoeal conditions of pigs. Bertschinger (1976) found that olaquindox at low dosages (50 ppm) in feed was effective for prevention of experimentally induced *E. coli*-diarrhoea in piglets. Holmgren (1994) found that prophylactic treatment with 122 ppm or 173 ppm olaquindox in commercial swine herds significantly (p<0.01) reduced the incidence of post-weaning diarrhoea in all but one of the herds investigated. From the herd where olaquindox had no prophylactic effect, *E. coli* resistant to olaquindox were isolated. Troutt and co-workers (1974) found that carbadox at the maximum growth promoting concentration reduced clinical signs and intestinal lesions in pigs experimentally infected with *Salmonella* Cholerasuis. Winkelman and Hawkins (1996) evaluated carbadox for the control of proliferative enteropathy in swine. Carbadox at growth promoting levels was found to reduce clinical signs and pathological lesions in pigs experimentally infected with *Lawsonia intracellularis* (the causative agent of porcine proliferative enteritis).

Stutz and co-workers (1984) showed that carbadox at 55 ppm significantly (p<0.05) reduced the amount of *C. perfringens* (associated with necrotic enteritis) in the intestines of chickens. Carbadox is not used in poultry, so this may be of limited interest, but it is clear that carbadox is effective against clostridia, both *in vivo* and in *vitro*.

F.6 Impact of resistance on animal and human health

Carbadox, and to some extent olaquindox, are used for prevention or therapy in veterinary medicine. Neither of those, nor related substances, are used in human medicine. As no information on possible cross-resistance to the modern quinolones, which are valuable drugs in human and animal medicine, is available, the impact of an emergence of resistance cannot be fully assessed.

Quinoxalines are valuable for prevention of swine dysentery and weaning diarrhoea. Development of resistance in *Serpulina* spp. to carbadox or olaquindox would entail reduced effectiveness of strategies employing

quinoxalines for prevention of this disease. Few drugs are available for this purpose. However, available data indicate that the development of resistance to quinoxalines is slow.

F.7   Other effects on the microflora

The influence of carbadox treatment on salmonellosis was investigated by Troutt (1974). When fed at 55 ppm to pigs experimentally infected with *Salmonella* Choleraesuis, carbadox diminished the clinical signs and reduced the extent and severity of lesions. Development of resistance in salmonella towards carbadox has been documented (Mills and Kelly, 1986). No investigations concerning possible effects of quinoxalines on infections with resistant strains of *Salmonella* spp. have been found.

   No information on the influence of quinoxalines on other food-borne pathogens such as *Campylobacter* spp. or *Yersinia enterocolitica* has been found.

F.8   Toxicological aspects

F.8.1   Target species

A series of investigations concerning adrenal toxicity of quinoxalines, particularly carbadox, has been published. Field observations of intoxications in pigs fed high doses of carbadox (up to 150 ppm) were confirmed by an experimental study where 150 ppm carbadox was given to weaned pigs for up to 10 weeks (van der Molen *et al.*, 1985). Clinical signs of dehydration and impaired growth were observed from 3 weeks and onwards. Histopathological examination revealed atrophy of the glomerular zone of the adrenal glands.

   In a subsequent experiment, pigs fed carbadox at doses between 25 and 200 ppm were investigated (van der Molen *et al.*, 1986). The results confirmed the observation of adrenal damage as lowered plasma-aldosterone concentrations and corresponding changes in sodium and potassium in blood samples were observed for all doses. The magnitude of the effects were dose dependent.

   Further investigations into the pathomorphological changes showed that after 10 weeks of carbadox at 25 ppm or more, damage to the cells of the zona glomerulosa of the adrenal gland could be observed histologically (van der Molen, 1988). Again, the effects were dose and time dependent. Only two animals per dosage group were examined histologically.

The hormonal changes induced by carbadox were further investigated (van der Molen *et al.*, 1989). After 9 weeks, the levels of plasma renin were higher in all groups fed carbadox than in the control group.

Taken together, these experiments show that carbadox supresses the mineral-corticoid secretion from the adrenal gland, inducing secondary hormonal changes in the renin-angiotensin system. These observations explain the clinical picture observed. The dose-dependent inhibition of the aldosterone production of porcine adrenal cells was confirmed by *in vitro* experiments (Spierenburg *et al.*, 1988a; Spierenburg *et al.*, 1988b; Jager *et al.*, 1994).

In a study comparing the effects of carbadox, cyadox and olaquindox similar toxic effects were noted. For olaquindox, adrenal toxcicity was observed, although less pronounced, at dosages of 100 ppm or more (Nabuurs *et al.*, 1990). Again, the effects were dose- and time dependent. The clinical signs observed were also dose-related, ranging from mild to severe. At 50 ppm of carbadox or 100 ppm of olaquindox, mild effects in the form of increased faecal dryness were observed. Other signs included dehydration (urine drinking), decreased abdominal volume and lowered haematocrit values. Further, changes in hair quality, with hair becoming longer and withered, irritable behaviour and a decrease in feed intake and weight gain could sometimes be observed. It should be noted that also in other respects, there is a difference in activity between the substances, and carbadox is permitted in feed at a concentration of 50 ppm, whereas olaquindox is used at 100 ppm.

In the *in vivo* studies cited above, the growth-rate of the medicated animals was either equal to or reduced compared to the control group. This is in conflict with the reported growth promoting effect of these substances. It is possible that in a field situation, the preventive effects of the quinoxalines against common enteric diseases act as confounders, i.e. if the (sub)clinical diseases negatively affect the growth-rate.

Accidental overdosing of olaquindox (Köfer *et al.*, 1990; Stockhofe-Zurwieden *et al.*, 1991) and carbadox (Power *et al.*, 1989) has been reported to cause death and severe adrenal damage in piglets.

As the main early sign of intoxication with quinoxalines, i.e. dry faeces, may be mistaken for recovery from enteric disease, mild intoxications are expected to be overlooked by farmers and farm workers. Further, as the symptoms are diffuse, a definite diagnosis might be difficult to reach.

F.8.2   Adverse effects in humans

Carbadox

In a study where radiolabelled carbadox was fed to swine at 55 ppm for 5 consecutive days, total residues 30 days after withdrawal were around 5, 74 and 15 µg/kg in muscle, liver and kidney, respectively. After 70 days, the measured concentrations in liver were 13µg/kg (FAO/WHO, 1990).

Carbadox is rapidly metabolised to, among other metabolites, quinoxaline-2-carboxylic acid (QCA) and desoxycarbadox. In a pig study on residues of carbadox and its metabolites, feeding of 50 ppm carbadox did not result in any detectable residues after a withdrawal time of one day (Baars *et al.*, 1991). Desoxycarbadox was measurable in liver for 14 days (detection limit 1µg/kg). Liver samples yielded residues of QCA above 30 µg/kg in liver and kidney for 4 to 5 weeks. In an experiment by Rutalj (1996), QCA was still present at around 10 µg/kg at 62 days after cessation of feeding of carbadox at 50 ppm.

Detection of QCA is dependent on the extraction method used, and it has been suggested that this is due to other unknown intermediate metabolites in the pathway of carbadox to QCA (Baars *et al.*, 1991). QCA, being the major residual metabolite, has been suggested as a marker substance for residue studies (FAO/WHO, 1990).

Long term toxicity studies on carbadox in rats have shown dose-related increases of benign and malignant liver tumors.

Positive results were reported in 14 out of 15 mammalian and non-mammalian genotoxicity studies (FAO/WHO, 1990), clearly indicating a genotoxic potential of carbadox.

Chronic administration of the meatabolite desoxycarbadox to rats has resulted in an increase of liver tumour incidence in all dosage groups (FAO/WHO, 1990; FAO/WHO, 1991). Most tests for genotoxicity have produced negative results, but positive findings were recorded in the cell transformation test and in Ames test using liver cells pre-treated with polychlorinated biphenyls.

With respect to QCA, no studies indicate reasons to suspect carcinogenic or genotoxic properties (FAO/WHO, 1990; FAO/WHO, 1991)

In the 36th report of JECFA (FAO/WHO, 1990) it was concluded that an ADI could not be established, due to the carcinogenic and genotoxic nature of carbadox and some of its metabolites. MRLs set in 1990 (FAO/WHO, 1990) for QCA as marker substance were apparently based on the detection limit of the analytical method.

Olaquindox

Olaquindox is rapidly absorbed from the gut and mainly excreted via urine. No bound residues appear to be present in tissue (FAO/WHO, 1995). In a study where radiolabelled olaquindox was given to pigs as a single oral dose of 2 mg/kg body weight, <1, 2 and 1 µg/kg of total residues were detected in muscle, liver and kidney, respectively after 28 days. (FAO/WHO, 1990). At doses corresponding to recommended feed inclusion doses (2.5 mg/kg body weight) and a 28 days withdrawal period, olaquindox residues were below 5µg/kg in muscle and below 10µg/kg in kidney (FAO/WHO, 1995). The substance is extensively metabolised the animal. The metabolites found vary between tissues and between animal species (FAO/WHO, 1995). In pigs given 60 ppm olaquindox in the feed up to 16 weeks of age, and with a withdrawal period of 28 days, olaquindox residues were below 0.005 ppm in muscle and below 0.01 ppm in kidney (FAO/WHO, 1995). One of the metabolites, 3-methylquinoxaline-2-carboxylic acid (MQCA) has been chosen as a marker compound (FAO/WHO, 1995). Radiolabel studies indicate that the MQCA accounts for approximately 25% of the total residues.

In long-term toxicity studies in mice, an increase in the incidence of benign adrenal cortical adenomas and benign proliferative lesions in the lungs were noted at the highest dose level (54 mg/kg). There was no effect on the incidence of malignant tumours (FAO/WHO, 1990). In another long term study in rats, no increase in the incidence of tumours was noted.

The genotoxicity of olaquindox has been investigated in a range of *in vitro* and *in vivo* studies. Both positive and negative findings were reported in assays using various mammal and non-mammal systems (Nunoshiba and Nishioka, 1989; FAO/WHO, 1990).

The marker substance, MQCA, is known to be responsible for the bacterial mutagenecity of other quinoxaline derivatives (FAO/WHO, 1995). No studies specific for olaquindox are available.

An ADI could not be allocated by JECFA 1995, because of the genotoxic potential of the parent compound and the absence of specific toxicity studies on the metabolites. No MRL has been set.

Photoallergy

Carbadox, olaquindox and other quinoxalines induce both photoxicic and photoallergic mechanisms through formation of a reactive oxaziridine reacting with proteins upon exposure to light (de Vries *et al.*, 1990b).

The photoallergic properties of olaquindox has been confirmed in studies on rats (de Vries *et al.*, 1990a). Olaquindox-induced photoallergy has been described in pig breeders following airborne exposure (Schauder, 1989;

Hochsattel *et al.*, 1991; Schauder *et al.*, 1996). In a report by Schauder (1996), twelve out of 15 patients habitually mixed mineral feed containing 1000 ppm olaquindox on the farm, two of the patients only handled pellets. The average interval of exposure at onset of clinical signs for the patients who mixed feed was 2.5 years.

   The clinical symptoms are characterised by eczema worsened by sunny weather. At worst, the affected persons have to stay indoors during daytime. The reaction may be persistent and can be severely disabling (Schauder *et al.*, 1996). In spite of the experimental findings indicating similar properties of carbadox, no clinical reports have been found. Nonetheless, carbadox should be regarded as a potential photoallergen (de Vries *et al.*, 1990b).

Some comments on toxicological aspects

The quinoxalines are suspected of having carcinogenic and genotoxic properties (Cihák and Srb, 1983; Nunoshiba and Nishioka, 1989). Carcinogenic and genotoxic effects are not acceptable since the effect could occur at very low intake levels, especially if the substance in question is ingested regularly over a number of years. Farm and feedmill workers are a special risk group, frequently exposed to AFA when handling animal feed. If appropriate protection cannot be ensured, the handling of animal feed containing quinoxalines and other AFA with potentially genotoxic effects must be regarded as an occupational hazard both due to potential genotoxicity and to photoallergenicity. Althogh the precise exposure cannot be determined, a conservative approach is often recommended for genotoxic substances in order to prevent underestimation of the risks.

F.9   Environmental effects

No information on the environmental fate of quinoxalines has been found. The quinoxalines are mainly excreted via urine (FAO/WHO, 1990; FAO/WHO, 1995). They are sensitive to photodegradation. Considering the potential genotoxicity of some of these substances, not only the fate of the parent substance but also of relevant metabolites should be investigated.

F.10   Summary comments

Although transferable resistance to carbadox and olaquindox has been reported, the mechanisms and molecular biology of this or other resistance phenomena are still unclear. The precise target in the bacterium of the quinoxalines or their metabolites is still unknown. The development of resistance in important animal pathogens such as *Serpulina* spp. seems slow.

No information is available concerning cross-resistance to the most modern class of antibacterials, the quinolones. Therefore, the impact of a possible emergence of resistance in enteric bacteria cannot be fully assessed.

The preventive effects of carbadox and olaquindox against enteric diseases of pigs is well documented.

Olaquindox and carbadox have several unwanted properties related to toxicology. Both substances have toxic effects on the adrenal glands of exposed animals at the dosages used for growth promotion. Profound disturbances in the stereoid balance of the animals, resulting in clinical signs of dehydration has been reported.

The quinoxalines and some of their metabolites are, or are suspected to be, genotoxic. Olaquindox is a well known photoallergen and according to *in vitro* data, carbadox shares this property. Therefore, exposure to quinoxalines must be regarded as an occupational hazard.

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Annex G: Buget sheets to 3.5

G.1   Budget sheets for piglet production

(SLU INFO - OMRÅDESKALKYLER)

Basic assumptions

Herd size 50 sows, a sow produces 20 piglets per year, pigs are 100 % Scan-H piglets, 5 weeks to weaning, piglets sold at 25 kg (at 85 days of age), purchased feed, the feed for gilts until farrowing included, new buildings and interest rate 7%, results per sow

  Assumptions on the effect from the use of antibiotics as additives in animal feed on growth 6.8%, feed conversion 4.6%, and reduction of mortality 0.6%.

Gross margins (from table G.I)

	Without AFA	With AFA	Gain
Gross margin (TB 1) = Income minus variable costs	3092	3281	189
Gross margin (TB 2) = income minus variable and capital costs	1376	1581	205
Gross margin (TB 3) = income minus variable, capital and fixed costs	-7345	-7140	205

Table G.I Budget sheet for piglet production

Income	Unit	Quan-tity	Price/unit		Added quan-tity	Price SEK	Added benefit	Sum add. be-nefit
Piglets	n.	20	454	9080	0.12	454	54	
Premium	SEK	20	19.00	380	0.12	19.00	2	
Meat from culled sows	n	0.50	1076	538				
Total income				9998			10054	56
Variable costs								
Replacement	n	0.5	2000	1000				
Sow feed	kg	1289	1.70	2191				
Piglet feed	kg	938	2.16	2026	55	2.16	93	
Straw, bedding	kg	400	0.35	140				
Electricity	kWh	710	0.45	320				
Health control fee	SEK			58				
Sow production control fee	SEK			150				
Cost of boar	SEK			351				
Insurance	n			70				
Miscellaneous	SEK			600				
Additional vet costs if AB prohibited		20	2.00				40	
Sum variable costs				6906			6773	189
Buildings, maintenance	SEK	73200	2.1%	1537				
Costs of animal capital	SEK	1538	7%	108				
Costs of operational capital	SEK	1009	7%	71	14	7%	1	
Savings due to shorter period to 25 kg					14	1.06	15	
Sum of variable and capital costs				8622			8473	205
Buildings, maintenance and depreciation	SEK	73200	8.3%	6076				
Labour	hours	23	115.00	2645				
Sum of variable, capital and fixed costs				17343			17194	205

G.2   Budget sheets for pig meat production

(SLU INFOS OMRÅDES KALKYLER)

Assumptions

Herd size 288 pigs per batch, purchased feed, dry feeding, live weight at slaughter 107 kg, slaughter weight 73% of live weight, new buildings, interest rate 7%, 2.7 batches per year, and feed conversion 2.8 kg feed per kg weight gain, results per slaughter hog

   Assumptions on the effect of antibiotics used as additives in animal feed on growth 1.86%, and feed conversion 1.65%.

Gross margin per pig (from table G.II)

	Without AFA	With AFA	Gain
Gross margin (TB 1) = income minus variable costs	155	162	7
Gross margin (TB 2) = Income minus capital and variable costs	62	71	9
Gross margin (TB 3) = Income minus variable, capital and fixed costs	-267	-253	14

Gains in a farm with 500 pigs per batch

	Without AFA	With AFA	Gain
Pigs produced per year	1350	1375	
Gross margin TB1 (SEK)	209250	227750	13500
Gross margin TB2 (SEK)	83700	97625	13925
Gross margin TB3 (SEK)	-360450	-323360	12575

Table G.II. Budget sheet for pig meat production

Per slaughter pig	Unit	Quantity	Price	SEK	Added quantity	Price	Added bene-fit	Sum of added benefit
Income								
Meat kg	kg	78	13.80	1076				
Delivery premium	SEK			26				
				1102			1102	0
Variable costs								
Piglets costs 25 kg	n	1.00	454.00	454				
Delivery fee	SEK			39				
Feeding stuff	kg	229.60	1.76	404	3.8	1.76	7	
Service fee	SEK			3				
Energy	SEK			24				
Mortality (2 %)	SEK	627	2.0%	13				
Miscellaneous	SEK			10				
Sum variable costs				947			940	7
Capital costs								
Buildings maintenance	SEK	3407	2.1%	72	64	2.1%	1.3	
Cost of animal capital	SEK	183	7%	13	4	7%	0.3	
Cost of operational capital	SEK	111	7%	8	2	7%	0.1	
Sum variable & capital costs				1040			1031	9
Capital costs								
Buildings depreciation and interest payment	SEK	3407	8.3%	283	63	8.3%	5	
Labour	hours	0.4	115.00	46				
Sum variable, capital and fixed costs				1369			1355	14

G.3 Budget sheets for egg production

(SLU INFOS OMRÅDES KALKYLER)

Assumptions

Batch size 10000 layers, production period 66 weeks, age at start in flock 16 weeks, and at slaughter 80 weeks, purchased feed, feed consumption 2.25 per kg eggs.

New buildings, caged birds, three story cages, annual egg production 20 kg per introduced young bird and interest rate is 7%, results per 100 hens

Assumptions on the effect of antibiotics used as additives in animal feed on egg performance 1.63%, and feed conversion 1.32%.

Gross margins (from table G.III)

	Without AFA	With AFA	Gain
TB1 = Income minus variable costs	4372	4535	163
TB 2 = Income minus variable and capital costs	3570	3913	163
TB 3 = Income minus variable, capital and fixed costs	-31	132	163

Table G.III. Budget sheet for egg production

	Unit	Quantity	Price	SEK	Added quantity	Price	Added benefit	Sum added benefits
Income								
Eggs	kg	1564	9.30	14545	21	9.30	195	
Meat for slaughter	n	69	1.38	95				
Quality deductions	kg	1564	-0.53	-829	21	-0.53	-11	
Washing costs	kg	126	-0.25	-32	2	-0.25	-1	
Sum income				13779			13962	183
Variable costs								
Replacement birds	n	79	33.00	2607				
Growing feed	kg	79	1.75	138				
Laying feed	kg	3519	1.76	6193	-9	1.76	-15	
Misc. costs	SEK	1564	0.30	469	21	0.30	-6	
Sum variable costs				9407			9427	163
Capital costs								
Buildings maintenance	SEK	22000	2.1%	462				
Cost of animal capital	SEK	1351	7%	95				
Operational capital	SEK	922	7%	65	1	7%	0	
Sum variable and capital costs				10029			10049	163
Capital costs								
Buildings, depreciation and interest payments	SEK	22000	8.3%	1826				
Labour	hours	17	115	1955				
Sum variable, capital and fixed costs				13810			13830	163

G.4 Budget sheets for poultry meat production

(SLU INFOS OMRÅDES KALKYLER)

Assumptions

Flock (batch) size 80000, weight at slaughter 1.65 kg, new buildings, purchased feed, batches per year 6.75, annual production 540000 broilers, number of day old chicks per 1000 broilers slaughtered 1050, feed conversion 2.85 kg feed per kg broiler, interest rate 7% and age at slaughter 36 days, the results in a farm producing 80000 birds per batch (expected values).

  Assumptions on the effect of antibiotics used as additives in animal feed on growth 2.09%, and on feed conversion 1.47%.

Gross margins (from table G.IV)

	Without AFA	With AFA	Gain
TB 1 = Income minus variable costs	988616	1065977	77361
TB 2 = Income minus variable and capital costs	909608	988677	79069
TB 3 = Income minus variable, capital and fixed costs	-61132	26890	88022

Table G.IV. Budget sheet for poultry meat production

	Unit	Quantity	Price	SEK	Added quantity	Price	SEK	Net benefita
Income								
Broilers	kg	891000	7.42	6611220	16500	7.42	122430	
Quality penalty	SEK	6611220	-1%	-66112	122430	-1%	-1224	
Sum income				6545108			6666314	121206
Variable costs								
Day old chicks	n	567000	2.88	1632960	-10500	2.88	-30240	
Chicken feed	kg	1563300	2.13	3329829	-5544	2.13	-11809	
Electricity	kWh	194940	0.45	87723	39910	0.45	-1796	
Oil	SEK	631		34070				
Straw, bedding	kg	50	1.00	27000				
Insurance	SEK	157		84780				
Misc. costs	SEK	99		53460				
Sum variable costs				5556492			5600337	77361
Capital costs								
Buildings, maintenance	SEK	5720000	1.3%	74360	124348	1.3%	1617	
Cost of operational capital	SEK	66406	7%	4638	1298	7%	91	
Sum variable and capital costs				5635500			5677637	79069
Capital costs								
Buildings depreciation and interest rate	SEK	5720000	7.2%	411840	124348	7.2%	8953	
Labour	hours	4860	115.00	558900				
Sum variable, capital and fixed costs				6606240			6639424	88022