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Antibiotics in the Food Chain

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Legal and Regulatory Aspects of Antibiotics in the Food Chain

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The antibiotic era, which started in earnest after the Second World War, revolutionized the treatment of infectious diseases both in human and veterinary medicine. Antibiotics, as natural microbial secondary metabolites, have existed probably as long as bacteria themselves. Likewise antibiotic resistance is an ancient phenomenon, and resistance genes have been detected in environmental samples dating back to millions of years.

The massive use of antibiotics during the last decennia, however, has caused a selective pressure for the spread of resistances. Consequently, we are facing a situation, in which the resistant microorganisms form again a real threat to public health. The food chain has been identified as one of the sources of resistant bacteria and transmissible resistance genes, and regulatory measures have been adopted to contain this threat, particularly in the European Union (EU).

The therapeutic veterinary use of antibiotics in the EU is regulated mainly by Directive 2001/82/EC, which lays down the authorization procedure for veterinary medical products. Directive 96/23/EC deals with the residues of veterinary drugs, including antibiotics, in animals and animal products. The annual sales of veterinary antimicrobial agents is followed by the member states and the European Medicines Agency (EMA).

In addition to therapeutic use, antibiotics have been used at subclinical doses as growth promoters in animal production scale since early 50'ies. This practice has resulted in the documented increase of resistances in animal associated bacteria. In Europe, particularly the use of avoparcin, a glycopeptide antibiotic related to a clinically important antibiotic vancomycin, caused concern because of the occurrence of Vancomycin Resistant Enterococci (VRE) in the food chain. This led to the ban of avoparcin use, first in certain member states, and subsequently in the EU in 1997. Subsequently, in 2006, the use of antibiotics as growth promoters has been totally prohibited in the EU.

The European ban of the growth promoter use of antibiotics has not led to negative consequences in animal production, and decreasing trends of antibiotic resistances associated with animal products have been recorded.

In other parts of the world, especially in the USA, the use of antibiotics as growth promoters has continued. However, the Food and Drug Administration of the USA has recommended judicious use of medically important antimicrobials in food producing animals, and certain legislative initiatives for a tighter policy have been made.

In addition to reducing the direct use of antimicrobials in the food chain, the European policy has been also to reduce the risk of the spread of antibiotic resistance genes themselves by controlling their presence in microbial feed additives or as marker genes in genetically modified plants.

The responsible use of antibiotics is essential to preserve their therapeutic value. The European experience shows that there are viable alternatives to controversial practices like the growth promoter use.

Use of Antibiotics in Animal Production

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Antibiotics belong to the most successful therapeutic agents for treating infectious diseases in animal and man. In farm animal production application of antibiotics has contributed significantly to a better health status of the animals and helped to improve welfare both of single animals as well as of flocks or herds by avoiding disease. However, the increasing use of antibiotics in recent decades in livestock production gave cause for several concerns. First, the chronic and often massive exposure of the animals to antibiotics includes a certain amount of risk regarding selection of resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or extended spectrum β -lactamase (ESBL)-producing bacteria and can promote the development of resistance reservoirs. Secondly, exhaust air (including dust) and manure from housed stock treated with antibiotics can carry considerable amounts of antibiotic substances and resistant micro-organisms into the environment where they can be transmitted to nearby residents or neighbouring farms. Third, after treatment residues of antibiotics in products like meat, eggs and milk may reach the consumer. Therefore guidelines were developed for the prudent use of antibiotics giving criteria for selection of the most appropriate antibiotic preparation, dosage and duration of therapy for a necessary treatment. At the same time new and more efficient types of antibiotics were developed in order to have alternative antibiotics available if a bacteria species developed resistance. About 8000 antibiotic substances are known today of which approximately 80 are used in more than 2700 different preparations licensed for human and veterinary medicine. From some field surveys it is known that tetracyclines were the most frequently used substances in pigs and cattle, in pigs followed by β -lactams, in cattle by the trimethoprim/sulfonamide group. Most antibiotics are administered orally. In an investigation it was found that about ninety-two percent of all applications to pigs were oral applications. In cattle, the fraction of drugs applied orally was 75.3%, followed by 17.3% parenteral and 7.4% local applications. A real estimate of the amount of antibiotic substances which are administered to an animal or a population is not simple to perform because of different concentrations of active substance in the preparation, the age of the animal or its weight. Therefore it is preferred practice to use the number of daily doses which are estimated by dividing the free substance by the product of the dosage recommended in the summary of product characteristics (in mg/kg animal weight; respecting the administration route) as well as the average animal weight per species and animal age group. This method helps to compare quantitative data from different sources although not all countries are using the same approach yet. An actual publication of the German Federal Office for Consumer Protection and Food Safety (BVL) reports that about 1,734 tons of antibiotics were delivered from pharmaceutical industry and retailers to veterinarians in Germany in the year 2011. The largest part were tetracyclines (576 tons) followed by amino-penicillin (505 tons). Further substances were fluoroquinolones (8 tons) and cephalosporines of the 3rd and 4th generation (3.8 tons). A recent European study revealed that the relative proportion of the various classes of antibacterial agents sold varied considerably from 18 to 188 mg/kg (expressed in mg antibacterial drugs sold/kg biomass of slaughtered pigs, poultry and cattle). The wide variations of these data indicate the need for further harmonised in-depth analyses in Europe. The single on farm monitoring approach as proposed in Germany seems to be rather accurate but may be costly to perform.

Novel Developments in Rapid Analysis of Antibiotics

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Antimicrobial residues still play an important role in food industry. Problems associated with residues of antiinfective substances in dairy products and other foods of animal origin include the risk of adverse health effects after consumption, increased resistance of pathogenic bacteria towards antibiotics and inhibition of starter cultures used in dairy production. In order to ensure the safety and quality of food, a number of regulatory authorities have decreed maximum residue levels (MRLs) for antibiotics. To comply with these regulations, a broad range of microbiological and receptor-/enzyme-based tests for the detection of antimicrobial residues in food of animal origin have been described.

During the last decade a major focus of commercial developments was on rapid screening tests for different classes of antimicrobials either as receptor-type assays or immunoassays. They permit the specific detection of certain groups of substances in a relatively short period of time and are usually designed for field analysis, e.g. tank milk analysis. In addition, these assays are easy to handle and generally show sufficient sensitivity. Still, they only allow detection of a limited number of residues and usually do not permit quantification.

Besides these assays, a number of biosensors for the rapid detection of antibiotics have been developed. Although most of these sensors offer good performance, they allow only the use of a limited number of antibodies or receptor proteins and can detect only a limited number of antibiotics. The most recent development is a fully automated and rapid antibody-based biosensor, which quantitatively determines antibiotic residues in milk. This newly developed microarray system is designed for the parallel analysis of 13 different antibiotics in milk within six minutes by applying an indirect competitive chemiluminescence microarray immunoassay and allows multiple analyses by using a regenerable microarray chip.

Multi Antibiotic Residue Detection – Status quo and Challenges

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Veterinary drugs are important for conventional and – to a smaller extend - also for ecological animal breeding to optimise animal health. The compounds are applied to prevent or treat diseases or to promote growth. Depending on the specific compound and its application the use of veterinary drugs is regulated by international and national legislation and either banned with zero tolerance or permitted up to specific concentration levels in the final food product. Abuse of veterinary drugs and consequently its residues in food become an increasing problem due to economic pressure on farmers and changes in breeding practices. Public perception and the growing awareness of this issue have raised the interest among regulators to check for such residues. As an example shrimp and fish are most often reported in the rapid alert system of the EU, the RASFF.

In total the analytical determination is a huge challenge, since there is a high variety in structure and chemical properties of the compounds, furthermore the protein rich commodities are extremely difficult matrices leading in time consuming and cost effective procedures in the laboratory.

At the moment a broad range of analytical methods is in use to cover the whole spectrum of relevant analytes; differentiating screening and confirmatory analytical technologies such as biosensors, immunoassays and liquid chromatography coupled with mass spectrometry. Methods have to be extensively validated to be used in accordance to the requirements of the Commission Regulation (EU) No 37/2010 with regard to the MRLs.

Within this presentation on the one hand a confirmatory method for the simultaneous determination of about 100 veterinary drug residues (ivermectines, benzimidazoles, quinolones, nitromidazoles, β -lactams, macrolides, triphenylmethan dyes, sulfonamides, tetracyclines) will be presented. This method has been developed and validated complying with the guidelines for the implementation of decision 2002/657/EC for the application in routine analysis of meat and fish samples. On the other hand, it will be discussed if high resolution mass spectrometry technology could be a fast and effective alternative used as detection mode for fast compound screening. Since the analytical costs nowadays are the main factor limiting the possibilities of industry self-control and official monitoring programs, there is an enormous need to reduce the laboratory effort. Both methods based on a quick and easy sample preparation. Whereas the triple quadrupole method impressed by its robustness, precision and sensitivity, calculation and result validation is time consuming. The higher selectivity of HR-LC-MS and application of large spectral libraries for analytes identification and confirmation could be beneficial when the mode is changed from time consuming quantification into verification at the relevant concentration level.

New Approaches for Detection of Antibiotics in Food

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Traditionally antibiotics residues in food are detected by the use of microbiological screening techniques and identified/quantified by the use of physical chemical techniques like LC-DAD or MS(/MS). The main aim to detect antibiotics in food is testing if maximum residue levels (MRLs) are not exceeded. Nowadays, next to the exposure of the consumer the problem of microbial resistance makes it necessary to detect a diversity of antibiotic residues at different levels of interest in different food commodities. Furthermore, the food producers and sometimes the retailers will test their own products at different times during food processing so called 'in- field' or 'on-line' testing. This increases the need for easy to perform, cost-efficient, multi-analyte high quality screening and identification/quantification methods.

For screening analyses effect based assays – biological binding assays – are developed based on techniques like (imaging)SPR and bead-based approaches like 'Luminex' using antibodies, transport proteins and receptors. Interesting examples are the iSPR approach or the 'Luminex- flow cytometry' approach for the simultaneous detection of different classes of antibiotics in milk and the use of a luminescent bacterial biosensor for the detection of tetracyclines in poultry muscle. The use of iSPR techniques makes miniaturisation - hand-held devices - possible.

For identification/quantification the new methods are based on innovative instrumental techniques e.g. UPLC-high resolution (HR)MS. These so called full scan techniques makes it possible to detected more than hundred antibiotics in one single analysis. However the main advantage of full scan is that no a priori hypothesis about the presence of certain antibiotics is required. The HR full-scan data permit the testing of any a posteriori hypothesis by extracting any desired exact (accurate) mass chromatogram. Furthermore the accurate-mass determination and calculated elemental composition data can be used for structure elucidation. In this way new antibiotic compounds - tested positive in an effect based assay - can be identified.

All new approaches of detection of antibiotics in food have in common that they are quick, 'multi-analyte' and very sensitive even ng/kg levels are detected.

Antibiotic Residues in Milk and Milk Products

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A suitable distinction in reasons for the administration of antibiotics to lactating animals is: udder infections, dry cow therapy and other reasons. Beta-lactam antibiotics (penicillins and cephalosporines) are still the most relevant substances in all categories. Aminoglycosides, macrolides, sulfonamides and tetracyclines are applied to a lesser extent.

In practice, control of antibiotics (and other veterinary drugs) in the dairy chain and the avoidance of residues in milk and milk products is based on two pillars:

- the promotion of a prudent use of antibiotics on dairy farms;
- the detection of contaminated batches as early as possible in the dairy chain: cow – farm bulk milk tank – milk collection truck – silo at the dairy plant – dairy products.

Key elements in a prudent use on dairy farms are traditionally:

- the education of dairy farmers and veterinarians and continuous communication with them;
- keeping animals healthy through hygienic measures and prevention;
- the canalization of antibiotics and their administration;
- a ban on administration of antibiotics through feed or water;
- the restriction to use only approved antibiotics according to prescription;
- a full and correct registration of all animal treatments;
- the marking of treated animals;
- keeping to the prescribed withholding times.

The issue around growing antimicrobial resistance has recently resulted in more concrete additional demands to milk producers. For instance, in the Netherlands it is now required that:

- dairy farmers have a farm health plan and act accordingly;
- dairy farmers have a plan for the treatment of the major animal health problems on their farms;
- veterinarians have a proven QA record;
- data on antibiotic treatment are provided to a national database system;
- 3rd and 4th generation cephalosporines are no longer administered to animals.

With regard to the detection of antibiotics in milk and dairy products for both quality control as well as regulatory purposes, an extensive box of analytical tools is available with:

- microbiological test methods;
- (rapid) immunochemical tests;
- instrumental methods (i.e. LC-MS/MS)

Each analytical tool has its own strong point and limitations. The purpose of testing at each spot in the dairy chain is leading for the choice of the appropriate analytical strategy. Some consequences and still existing challenges in light of existing requirements will be outlined.

Antibiotic Residues in Meat and Meat Products and Honey

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Veterinary drugs are frequently used in large amounts in intensive livestock farming. Approximately 800 tons of antibiotics were used in Germany in 2011. During the legislation process of a veterinary drug, studies have to be performed in order to evaluate the pharmacological behavior in food producing animals. Derived from excretion kinetics, withdrawal periods are calculated and Maximum Residue Limits (MRLs) are established to protect the consumer from toxicological relevant residues in food of animal origin. However, there is currently no protocol available for the establishment of MRLs for bee treatments.

Employing analytical screening techniques like the microbiological 3-plate test, hundred thousands of food samples are investigated each year in Germany and the EU. Furthermore, sophisticated analytical techniques, in most cases gas or liquid chromatography combined with mass spectrometry, allow the confirmation of positive findings in screening tests as well as for the search for illegal substances in food. All analytical methods must be performed according to EU Commission Decision 2002/657.

Although antibiotics are frequently used, the number of positive findings for antibiotics in meat, meat products and honey is low. In Germany, positive results of the microbiological 3-plate test were between 0.12 and 0.33 % from 2001 to 2010. In detail, 664 out of 263.970 samples were tested positive in the microbiological 3-plate test (0.25 %) in 2010. The application of confirmatory methods to these positive samples demonstrated, that 263 samples (0.1 %) contained one or more residues above the MRLs. Two out of three positive samples contained tetracyclines and this substance class was detected in nearly each of the suspected samples (see table 1).

Substance group	Confirmed above MRL	Detected below MRL
Tetracyclines	178	645
Fluorchinolones	83	61
Penicillines	65	79
Aminoglycosides	62	53
Sulfonamides	53	55

Table 1: Results of the National Residue Control Program in Germany 2010 (Ref.: BVL, Jahresbericht 2010 zum Nationalen Rückstandskontrollplan (NRKP)).

In conclusion, current residue data illustrate, that the number of positive findings in meat, meat products and honey in Germany is still at a low level. A toxicological risk for the consumer cannot be derived from these investigations. However, as often as possible, the use of veterinary drugs in intensive livestock farming should be reduced.

Antibiotic Residues in Fish: Necessity, Cause and Effect of Treatment

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Fish in aquaculture may be subject to a wide range of diseases, some of which are of infectious nature mediated by vira, fungi, bacteria, or by internal and external parasites.

More than 100 bacterial species have been associated with diseases in freshwater and marine fish farming. World-wide, the greatest economic losses have been caused by *Aeromonas hydrophila*, *A. salmonicida*, *Flavobacterium sp.*, *Flexibacter sp.*, *Tenacibaculum spp.*, *Edwardsiella tarda*, *Photobacterium damsela* subspecies *piscicida* (*Pasteurella piscicida*), *Renibacterium salmoninarum*, *Streptococcus sp.* *Vibrio anguillarum* and *Yersinia ruckeri*. The most severe bacterial diseases encountered in temperate water aquaculture have been furunculosis (*Aeromonas salmonicida*), cold water vibriosis (*Vibrio salmonicida*), vibriosis (*Listonella anguillarum*, former *Vibrio anguillarum*), yersiniosis (*Yersinia ruckeri*), *Francisella noatunensis*, *Piscirickettsia salmonis* and several external infections mediated by bacteria as *Moritella viscosa* (former *Vibrio viscosus*), *Flavobacterium sp.* and *Flexibacter sp.*

For infectious diseases where a treatment is available, veterinary drugs may be administered by baths or by inclusion in the feed. Even though vaccines have been developed for prevention of several diseases caused by fish pathogenic bacteria, application of antibiotics is still a common practice during disease outbreaks. The global application of veterinary drugs for prophylactic purposes is not known. However, one could argue that antibiotic treatments in aquaculture during an outbreak is predominantly prophylactic, since the non-diseased fish will eat considerably more medicated feed compared to diseased fish.

The use of antibiotics included in the feed remains largely unrestricted in aquaculture in several countries with high and growing aquaculture production. Information on types and amounts of therapeutic agents used in aquaculture throughout the world is not easily obtainable, since only a few nations provide reliable, detailed and accessible statistics on consumption of these drugs e.g. Denmark (VetStat), Scotland and Norway. As an example, Norway has provided statistics from aquaculture since the late eighties (updated statistics at <http://www.fhi.no/>). In 2011 the following amounts, measured as pure substance, of *per oral* antibacterial agents were used in Norway: oxolinic acid 212 kg, florfenicol 336 kg and oxytetracycline 1 kg. This adds up to 549 kg and gives a consumption of 0.6 g/ton fish produced. In Chile, which is the largest aquaculture nation in the Americas, the consumption of antibiotics reached approximately 385 metric tonnes in 2008, equalling 560 g antibiotics/ ton produced fish.

Systems should be enforced to ensure that residues of approved therapeutic agents in unacceptable concentrations, or illegal drugs are not found in food products intended for consumption or animals. Of special importance in the current EU legislation is Directive 96/23/EC that demands analytical examination of food producing animals at a frequency of at least one sample pr 100 ton. In accordance with this directive Norway have from 1998 until 2011 examined samples from a total of 70 000 farmed fish. In this material one pooled sample of five fish tested positive for chloramphenicol in a concentration of 0.3 ng/g. Otherwise no residues of illegal drugs have been detected (LOD ranging from 0.2 to 50.0 ng/g) and no residues above MRL were detected for legal drugs.

In conclusion, the application of antibiotics in world aquaculture is largely undocumented and unregulated, giving the possibility of unacceptable residues in products and contributing to the general selection of resistant bacteria.

Uptake of Tetracycline Antibiotics into Cereals

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Antibiotics may reach plants in the following way: by contaminated organic fertilizer (e.g. liquid manure) or water for irrigation and contaminated soil. Conditions for the uptake of different antibiotics into plants were studied for soybeans, alfalfa roots, rice and other cereals (Boonsaner and Hawker 2010 and 2012, Eggen et al. 2011, Kong et al. 2007, Lillenberg et al. 2010). Some of the studies indicate an accumulation in the roots without translocation, whereas others report detection of antibiotics in further plant tissues beside roots. Our initial investigations revealed an uptake of chlortetracycline (CTC) - a widely used veterinary antibiotic substance - from nutrient solution into wheat roots and leaves (Grote et al. 2007). Not much is documented about the potential for uptake of tetracyclines into cereal seeds although considerable amounts of tetracycline residues ($\sim 100 \mu\text{g kg}^{-1}$) were detected in a screening study of cereal seeds grown under usual agricultural practice (Freitag et al. 2008).

In the present work we investigated the uptake of CTC into wheat plants and the translocation of CTC up to the seeds. Wheat plants were cultivated in hydroponic nutrient solution using vermiculite as solid support for roots under controlled conditions in a growth chamber. CTC was added in two different concentrations to the nutrient solution at a certain point of plant growth. Ripe kernels were harvested, weighted and immediately frozen. After milling, homogenised whole meal wheat flour was extracted and extracts were analysed for occurrence of CTC and related substances using an established chromatographic method in combination with tandem mass spectrometry.

The finding of appreciable CTC concentrations in wheat kernels, originating from our controlled growth culture experiment, strongly supports the hypothesis that wheat plants took up CTC via the roots and also transferred CTC into the seeds. A higher CTC concentration in the nutrient solution led to higher amounts of tetracycline residues in roots, leaves and the kernels. CTC was metabolised during uptake to more highly polar substances, which might have a different risk associated with than the original chemical agent CTC.

The second aim of the study was to investigate the contamination rate with tetracycline residues of German wheat and rye samples obtained from usual agricultural practice. TC-residues were detected in 31 (48%) wheat and rye samples (n=64) from North Rhine-Westphalia and Lower Saxony of harvest 2009 and 2010 at a concentration range between 0.1 to $5 \mu\text{g kg}^{-1}$. After fifteen months storage time the concentrations of residues were reduced to 95 % of their original level.

Further research has to be done to confirm the occurrence and quantity of antibiotics in cereal grains. The question arises if small amounts of antibiotic residues in food and feed influence the initiation of resistant bacteria in animals or humans or have other effects e.g. on the metabolic system in plants, animals or humans.

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Antibiotic Residues in Vegetables

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Are raw vegetables healthy and safe? The question is legitimate, as studies reveal a path of human exposure to veterinary antibiotics through plant-derived food [1]. A potentially large source of antibiotics and resistant bacteria is livestock waste, which is distributed on fields as fertilizer. The role of vegetables - cultivated by conventional or organic methods - as reservoir and carrier of antibiotic contaminants as well as antibiotic (multi-resistant) pathogenic bacteria [2], requires further investigations, to assess the risk to human health and possible consequences with regard to food safety.

At first, the incorporation of antibiotics into lettuce was estimated by growing the plants under hydroponic conditions, where no sequestration by the soil can occur [1]. The plants were exposed to non-labelled and ³H-labelled sulphonamide and tetracycline. Data obtained by LC/MS-analyses and liquid scintillation counting corroborate the assumption that intact plants can take up substantial amounts of antibiotics by the roots. Subsequently these antibiotics are translocated and distributed in our plant organs. As a consequence, the potential of frequently consumed vegetables such as leek, green and red cabbage and carrots for uptake of highly prescribed veterinary drugs was also tested [3]. For this purpose sulfadiazine, enrofloxacin (ENR), tetracycline (TC), chlortetracycline (CTC), monensin or amoxicillin were added separately to nutrient solutions (2.5 – 5.0 µmol/L of each). The active compounds and conversion products were quantified in various organs of leek and cabbage (e.g. roots, young and old leaves). Depending on the type of antibiotic, vegetable species and plant organ, the visible effects of the drugs on plants were greatly different (bleaching of leaf sections, yellowing, lesions). The most severe plant damaging effects were caused by ENR. The concentrations of antibiotic residues determined in the plant organs comprised several orders of magnitude ranging from µg/kg to mg/kg of fresh weight (fw). For example, in the edible parts of carrots 6.0 mg/kg fw CTC and 4.1 mg/kg fw ENR were found. The roots contained up to 70.5 mg/kg fw CTC and 72.8 mg/kg fw ENR, respectively. Leek as well as green and red cabbage showed a high potential for uptake of chlortetracycline and, in particular, for enrofloxacin, which was partially metabolised in the plants to ciprofloxacin. The leaves of red cabbage contained 0.21 mg/kg fw CTC and 14.6 mg/kg fw ENR.

In addition, a field experiment was conducted to obtain further information on factors influencing the bioavailability of soil-bound antibiotics. Red cabbage was grown on experimental plots that were fertilized with manure, to which different amounts of CTC and ENR had been added. At harvest time the edible parts of the cabbage showed levels of 9.2 to 16.9 µg/kg fw ENR. However, no evidence was found for the uptake of CTC, which might be partly an effect of its strong sorption to soil matrix. Furthermore, samples were taken at various stages of the production of canned red cabbage and assayed for antibiotics. In fact, traces of tetracycline (16.4 - 19.2 µg/kg fw) were found in deliveries of freshly harvested red cabbage, grown conventionally, but not in the marketable canned vegetable [4].

It cannot be excluded to date that low (subinhibitory) levels of antibiotics in vegetables can contribute to the risk of developing bacterial antibiotic resistance. Therefore defined field experiments are carried out at the present, to take into account both, the uptake of drugs used in animal husbandry by vegetable from manured soil and possible influences on the frequency of resistance transfer. The spreading resistance of extended-spectrum-β-lactamase (ESBL)-producing bacteria (e.g. *Escherichia coli*) from animal reservoirs, such as pigs and poultry production, via food is a matter of concern in the BMBF-research project RESET („Resistance in Enterobacteriaceae“, www.reset-verbund.de). In particular, the resistance of foodborne pathogenic *Enterobacteriaceae* against β-lactams (e.g. amoxicillin, ceftiofur) and fluoroquinolones (e.g., enrofloxacin and ciprofloxacin) are taken into account.

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Antibiotic Residues in Vegetables

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Transfer of Antibiotic Resistances in a Model Gut System

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The human gut microbiota, consisting of up to several hundred different species and 10^{14} bacterial cells, is assumed to serve as a reservoir of antibiotic resistance (ABR) genes. The gut ecosystem is daily confronted with new incoming bacteria, mainly via ingested food which can contain bacteria carrying transferable ABR genes. Particularly fermented food can harbor high concentrations of *Enterococcus* strains. Enterococci are highly prone to acquire and transmit ABR genes and are considered to be a turn-table in the dissemination of ABR genes. However, enterococci belong to the regular gut microbiota of humans and animals and must be regarded as contaminants of food products but also as nosocomial infectious agents. We investigated the hypothesis that resistant bacteria such as enterococci can transmit their ABR genes, most probably by conjugation, to pathogenic or commensal bacteria of the human gut microbiota in a continuous colonic fermentation system.

As a prerequisite tool, a suitable ABR donor strain had to be constructed to circumvent the high background of ABR genes in gut samples. Therefore, the conjugative multiresistance 50-kb plasmid pRE25 from an *Enterococcus faecalis* food isolate was adapted to be suitable as model plasmid for conjugation experiments. Plasmid pRE25 was genetically marked by the integration of *tet(M)* and two short random sequences. This marker plasmid, named pRE25*, was then transferred to *E. faecalis* CG110/*gfp*, a strain tagged in the chromosome with a *gfp* gene. The constructed strain, designated *E. faecalis* CG110/*gfp*/pRE25*, must be unique and therefore applicable as donor strain in horizontal ABR gene transfer experiments in gut fermentations.

The continuous colonic fermentation system (300 ml) was consisting of a stabilized high-density bacterial ecosystem from infant feces immobilized to gellan-xanthan beads. The horizontal gene transfer (HGT) potential of pRE25* was assessed in a first fermentation. For that, *E. faecalis* donor strain CG110/*gfp*/pRE25* was co-immobilized with the human pathogen *Listeria monocytogenes* 10403S and infant feces. Plating of effluent samples during the 8-day fermentation revealed that plasmid pRE25* was transferred to *L. monocytogenes* at a frequency of 2.8×10^{-4} transconjugants per donor, despite the presence of competing microbiota. A second fermentation should reveal whether a conjugal transfer of our model plasmid occurs to commensal colonic bacteria. The donor strain CG110/*gfp*/pRE25* was co-immobilized with infant feces but without a defined targeted recipient. Monitoring the conjugational behavior of pRE25* during the 16-days fermentation was performed by a qPCR approach assessing the ratio of pRE25* to the *gfp* gene. The pRE25*/*gfp* ratio increased by 60% from day 1 to day 16, a clear indication for transfer of pRE25* to commensal fecal bacteria. Transconjugants were isolated on selective media and revealed that pRE25* was transferred to the opportunistic pathogen *Enterococcus avium*.

We concluded from our study that HGT from *E. faecalis* to pathogenic and commensal bacteria can occur in the presence of competing fecal microbiota in colonic fermentations. Since this model mimics the microbial environment of high density and diversity in the human gut, *in vivo* HGT events might take place and contribute to ABR transfer from food-relevant to commensal bacteria.

Molecular Mechanisms of Antibiotic Resistances

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Resistance to antimicrobial agents can be subdivided into two basic types of resistance, intrinsic resistance and acquired resistance. Intrinsic resistance, also known as primary resistance, describes a status of general insensitivity of bacteria to a specific antimicrobial agent or class of agents. This is commonly due to the lack of target structures for certain antimicrobial agents (e.g. β -lactam resistance in cell wall-free bacteria) or their inaccessibility in specific bacteria. Intrinsic resistance is a genus- or species-specific property of bacteria. In contrast, acquired resistance is a strain-specific property which can be due to the acquisition of resistance genes or mutational alteration of chromosomal target genes. Mutations that up-regulate the expression of multidrug transporter systems may also fall into the category acquired resistance. Three different basic types of resistance mechanisms can be differentiated: (1) reduced intracellular accumulation of antimicrobial agents (2) enzymatic inactivation of antimicrobial agents and (3) modification of the cellular target sites. **Reduced intracellular accumulation** can be achieved by decreased influx due to permeability barriers or loss/downregulation of porins; but also by increased efflux of antimicrobial agents via specific transporters or multidrug transporters. **Enzymatic inactivation** is due to either disintegration or chemical modification of the antimicrobial agents. **Modification of the cellular target sites** can be based on mutation, chemical modification, or protection of the target sites, but also on over-expression of susceptible targets or the replacement of susceptible target structures by functionally homologous resistant ones. Examples of genes specifying the different resistance mechanisms will be given for two groups of microorganisms relevant to the food chain: the Gram-negative *Escherichia coli*–*Salmonella enterica* and the Gram-positive *Staphylococcus aureus*. Moreover, examples for the association of resistance genes with mobile genetic elements, that are essential for their dissemination via horizontal gene transfer, will be shown. This will help to understand the accumulation of different resistance genes – and consequently different resistance mechanisms – in the same bacterial strain.

Antibiotic Effects on Promotion of Antibiotic Resistance

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There is a general worry about the huge amount of antimicrobials used as growth promoters in livestock and its relationship with the development of antibiotic resistance. The use of antibiotics in animal husbandry selects for resistance determinants that can spread by different ways to human pathogens. The exposure of bacteria to antibacterial agents results in the selection of pre-existing resistant variants that survive the challenge. Also, many antibiotics increase mutation and recombination rates in bacteria, increasing the probability of evolution to resistance, even to non-related antibiotics. Moreover, antibiotics can promote the acquisition of DNA sequences from other organisms via horizontal gene transfer (HGT). HGT plays a major role in pathogen evolution allowing bacteria to share resistance genes. Therefore, it seems clear that the use of antibiotics in livestock can be, if not properly regulated, a dangerous source of antibiotic resistance determinants for human pathogens. Finally, animal food is routinely complemented not only with antibiotics, but other supplements to improve growth. Some of these supplements, considered as safe compounds, may help bacteria to acquire antibiotic resistance.

Livestock Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA)

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Livestock associated *Staphylococcus aureus* (LA-MRSA) are mainly represented by clonal complex CC398 of this species. It obviously evolved from human adapted methicillin susceptible *S.aureus* (MSSA) of this clonal complex (1).

Although having its main reservoir as MRSA in livestock such as pigs, poultry, LA-MRSA CC398 has no pronounced host specificity and can colonize and cause infections in other animals such as horses and dogs and also in humans (2). In conventional farming systems nasal colonization of the animals and of humans occupationally exposed to them is frequent (up to 86%). It was not found in pigs raised in an alternative system (3). Dissemination to humans beyond animal contacts is obviously rare (~ 4% of humans living on farms but without occupational exposition [4]). Furthermore, dissemination seems also possible by contaminated meat, in particular of broiler chicken carcasses (~30% of thawing liquid highly contaminated).

Nasal colonization with LA-MRSA of humans at hospital admission is found in 0,08 – 0,2 % for Germany in general (with MRSA altogether 0,78% - 2%). This is, however, higher in areas with a high density of livestock farms such as in the North West of North Rhine Westphalia.

LA-MRSA CC398 is no less pathogenic for humans as *S.aureus* in general. It accounts for ~ 15% of MRSA from deep seated infections of skin and soft tissue in the community (5).

When introduced into the hospital it can cause postoperative wound infections and even septicemia.

Different from hospital associated MRSA, LA-MRSA has obviously only limited capacity to spread in the nosocomial setting so far (proportion of ~ 1.8 % among MRSA from nosocomial infections, the proportion among MRSA from blood cultures is ~ 1% (5)). Livestock farmers are included in the group of patients at risk for which a screening at hospital admission is recommended (6).

Although LA-MRSA nearly always exhibit phenotypes of multiresistance to a number of antibiotics, there are still sufficient treatment options left (5). As transferrable resistance to linezolid (mediated by *cfr* encoded rRNA methylase) originated in coagulase negative staphylococci from farm animals, emergence of this resistance trait in LA-MRSA and further spread to HA-MRSA needs particular attention (7).

LA-MRSA came as an MSSA from humans and adapted to animals, the way back by future acquisition of transferrable mobile genetic elements containing immune evasion and/or virulence genes needs to be detected in time.

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Antibiotic Resistance Emerging along the Food Chain, for Example MRSA and ESBL

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Resistance has accompanied and diminished the success of the antimicrobials from the very first year - 1940 - of the installation of penicillin into therapeutical practice. For over three decades, the discovery of new lead substances could compensate for the loss of efficacy. After about 1975 (i) new antimicrobials appeared at ever lower rates, (ii) were chemical derivatives of known antibiotics rather than new molecular structures, and (iii) resistance factors tended to be disseminated at an ever increasing pace through entering mobile genetic elements such as phages, plasmids, transposons, integrons etc. Assembly of several resistance factors soon gave rise to transferable multiple antimicrobial resistance and started to cause the first really serious problems in the hospital environment where the selection pressure promoted the dominance of resistant variants over susceptible ones. This is documented in the literature by countless studies on nosocomial outbreaks caused by an astonishingly moderate number of species of usually opportunistic pathogens from roughly 1985 to the present.

From around the years 2000-2005, a new and very significant trend started to arise: the flash-over of resistant opportunistic pathogens to the general human public. In the literature this caused a new terminology to be coined: community-acquired (CA) (multi)-resistant infectious agents versus healthcare-acquired (HA) ones. This trend manifested itself most clearly with respect to two - nowadays very famous - multi-resistant agents, (i) methicillin-resistant *Staphylococcus aureus* (MRSA à CA-MRSA) among the Gram-positive bacteria, and (ii) extended-spectrum β -lactamase producing *Enterobacteriaceae*, particularly *Escherichia coli* (ESBL-E. coli à CA-ESBL-E. coli) among the Gram-negative rods. This is one reason why this presentation focuses on these two resistance mechanisms. The second reason is the fact that both mechanisms compromise β -lactam antibiotics. This is extremely worrisome owing to the fact that worldwide roughly two thirds of all antimicrobial therapeutics prescribed to humans belong to the β -lactams.

The intriguing trend from HA- to CA-infectious agents calls for explanations and counter-measures. The Institute for Food Safety and Hygiene of the University of Zürich committed itself to the search for MRSA and ESBL-*E. coli* along the food chain and in the environment, in order to provide (i) scientific insight into the ways of dissemination and the dynamics of CA-pathogens, and (ii) basic knowledge for the rational design of urgently needed efficient counter measures.

Antibiotic Resistance in Fermentation Organisms and Probiotics

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It is estimated that approximately one-quarter of the global food production involves microbial fermentation processes, primarily using lactic acid bacteria (LAB). As natural food contaminants or as deliberately added starter cultures, various *Lactobacillus* species are omnipresent in the food chain. Together with *Bifidobacterium* spp., lactobacilli are also the most important bacterial group incorporated in probiotics and thus hold huge economic importance in the functional food business.

Despite the long history of safe use of (non-enterococcal) LAB for human consumption, regulatory agencies such as the European Food Safety Authority (EFSA; <http://www.efsa.europa.eu/>) have started to demand from producers and distributors of starter cultures and probiotics to scientifically document this safety. Reporting of the antibiotic susceptibility profile is one of the most important issues in such a safety dossier because (i) lactobacilli and bifidobacteria may reach levels of several millions per gram in fermented foods consumed on a daily basis worldwide, (ii) being adapted to both intestinal and food production ecosystems, both bacterial groups are regularly exposed to antibiotic residues potentially triggering the development of antibiotic resistance (AR) traits, and (iii) lactobacilli in particular are genomically very flexible in exchanging mobile elements possibly harboring AR genes.

Triggered by the complete lack of knowledge on the presence and distribution of AR in non-enterococcal LAB, the EU projects PROSAFE (2002-2005) and ACE-ART (2004-2006) were instrumental to develop new antibiotic susceptibility test protocols, recommend epidemiological cut-off levels for MIC data interpretation, inventorize the most common AR genes and test the potential for in-vitro, in-situ and in-vivo AR gene transfer. A part of this work has meanwhile been officialized in the ISO 10932:2010 standard, which has become an important tool for both industry and regulators in safety assessments of new strains and cultures already in use.

In the slipstream of both EU projects a greater scientific awareness has been generated on this topic, as witnessed by the fact that AR data on starter culture and probiotic LAB from other continents such as Asia are now increasingly being published. Also, in support of conventional MIC determinations, it is nowadays becoming more and more common to screen whole-genome sequences of industrially important or promising LAB strains for the presence of AR genes. Provided that currently available genomic reference frameworks are complete and comprehensive enough, this approach theoretically allows to predict all potential AR-related problems as part of the safety assessment procedure.

Antibiotic Resistance of Contaminants of the Smear Cheese Surface Flora

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Smear cheeses such as Limburg, Tilsit and Gruyère are surface ripened cheeses which are covered by a layer of various yeasts and bacteria. The smear microflora is difficult to control: traditionally, by “old-young” smearing, the smear is transferred from mature to green cheeses, with the obvious risk that undesirable contaminants (enterobacteria, enterococci, pseudomonads, moulds or pathogens) are disseminated in the whole factory. Today, a broad spectrum of specialised bacterial surface cultures can be used to minimize concentrations of these contaminants and thus improve food safety. Up to date, culture research was clearly focussed on surface flora optimisation in order to obtain Listeria-free cheeses. However, early studies performed at the ETH Zurich (Burri, 1999; Gianotti, 1999) showed that drug resistant enterobacteria and enterococci, present on the surface of practically all smear cheeses, could be an important factor for the observed emergence and spread of antibiotic resistant microorganisms - the food chain could serve as a reservoir and disseminator. In 2 PhD theses, Burri (1999) and Gianotti (1999) showed that multiple antibiotic resistance was common among strains of enterococci and enterobacteria isolated from Swiss and French smear cheeses, a rather dramatic finding. This stimulated the interest of our institute to initiate a survey of smear cheeses produced in 4 European countries. Enterobacteria and enterococci were isolated from the smear cheese surface and analysed for antibiotic resistance with an annual repetition between years 2000 and 2005. In these studies, results described by Burri and Gianotti were essentially confirmed. In the presentation, most common antibiotic resistance markers, differences between countries, changes of resistance profiles over the 5-year time period and stability of multiple antibiotic resistance properties in gram-negative and gram-positive strains during subculturing are discussed.

Animal Health – Potential Approaches to Reduce Antibiotic Consumption in Food Production

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Whereas in the early years of the availability of sulphonamides and antibiotics, only life-threatening infections of humans were treated, but soon more and more application areas were added: less harmful infections in humans, bacterial diseases in animals, more and more non-fatal diseases in humans up to the treatment of just “annoying” infections such as common colds, growth promotion (“non-therapeutic use”) in food animals, and the routine prophylactic and “metaphylactic” use in large scale food animal production units. This development of expanding the use of antimicrobial substances is the core of the actual criticism with the present use of antibiotics in food animals.

The most important step in guiding medical and veterinary users of antimicrobial substances as treatment of bacterial infections was the development of the concept of the “prudent use of antibiotics”, which is defined as applying antimicrobials in a way that leads to the highest possible health effect in humans or animals and to the lowest possible resistance in the bacteria that are exposed to the compound.

However, these guidelines for veterinarians are not sufficient enough to lower the antibiotic use, but a growing number of scientific papers on the huge variability of the amount of antimicrobials used in food animals tells us that the animal-health awareness of farmers and their management skills, more than the veterinarian, determine the health status of the herd or flock in question, which in turn, determines the necessary amount of antibiotics applied or prescribed by the veterinarian.

Concluding, the paper speculates that there will be no significant reduction in the amount of antimicrobials used in food animals, unless farmers and veterinarians find new approaches to investing money in the improvement of the health of herds and flocks, i.e. paying veterinary services for maintaining the animals’ health rather than for curing their diseases.

Subinhibitory Doses of Natural Substances Modulate Bacterial Virulence Factors

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Data accumulated from decades of research in antibiotics led to the generalization assumption that antimicrobial substances, even at subinhibitory doses, should necessarily reduce bacterial virulence and their environmental fitness. Natural antimicrobial substances are plant secondary metabolites which possess mild antimicrobial activity when compared to antibiotics and most of them target few cell components. Their antimicrobial properties, as well as their classification as GRAS make them good candidates for replacing both antibiotics and food preservatives. Though extended research has been done regarding the antimicrobial properties of these substances, little attention has been given to the effect of exposure to subinhibitory (SI) concentrations of these substances and its affect on bacterial virulence factors. We show that exposing different pathogenic bacteria to SI levels of natural substances increase bacterial virulence determinants. Adapting *S. aureus* strains to Epigallocatechin gallate (EGCG) caused an increase in resistance to UV irradiation, hemolysin secretion and biofilm formation. Exposing *P. aeruginosa* strains to SI levels of menthol, limonene or thymol caused an increase in the secretion of siderophores, pyocyanine, elastase and exopolysacchride in addition to an increase in biofilm formation and motility. Exposing *P. carotovorum* (a plant pathogen) strains to SI levels of menthol, limonene or thymol vapor caused an increase in the production plant cell wall degrading enzymes and thus increased the necrotic damage done by these bacteria, as well as increasing biofilm formation and motility. Since both the human body and food systems are complex and diverse environments, bacteria are likely to encounter SI doses of natural substances if these are used as antibiotics or food preservatives. Our observations raise concerns over the potential of utilization of natural antimicrobials in therapy and as preservatives in that exposure to SI doses of these substances may enhance bacteria virulence.

Significance and Challenges of Monitoring Programmes for Antimicrobial Resistance - Experiences From DANMAP

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The objectives of DANMAP are: 1) To monitor the consumption of antimicrobial agents for food animals and humans, 2) to monitor the occurrence of antimicrobial resistance in bacteria isolated from food animals, food of animal origin and humans, 3) to study associations between antimicrobial consumption and antimicrobial resistance, and 4) to identify routes of transmission and areas for further research studies.

DANMAP, was established in 1995 on the initiative of the Danish Ministry of Health and the Danish Ministry of Food, Agriculture and Fisheries, as a coordinated national surveillance and research programme for antimicrobial consumption and antimicrobial resistance in bacteria from animals, food and humans. The participants are Statens Serum Institut (SSI), the National Veterinary Institute and the National Food Institute.

The monitoring of antimicrobial resistance is based on three categories of bacteria: Human and animal pathogens, zoonotic bacteria, and indicator bacteria. Human and animal pathogens are included because these cause infections and they reflect primarily resistance caused by use of antimicrobial agents in the respective reservoirs. Zoonotic bacteria are included because they can develop resistance in the animal reservoir, which may subsequently compromise treatment effect when causing infection in humans. Indicator bacteria are included due to their ubiquitous nature in animals, food and humans and their ability to readily develop antimicrobial resistance in response to selective pressure in both reservoirs. The surveillance methods have developed over the year and consists now of phenotypic testing of antimicrobial resistance supplemented with genotypic testing and bacterial typing techniques.

Human consumption data are obtained from SSI (former done by the Danish Medicines Agency). The SSI has the legal responsibility for monitoring the consumption of all human medicinal products. Since 2001, animal consumption data have been obtained from the VetStat database. In Denmark, all therapeutic drugs are prescription-only and VetStat collects data on all medicines prescribed by veterinarians for use in animals.

DANMAP measured and documented the effect of the avoparcin ban, and data showed that the ban had a substantial effect on lowering the occurrence of resistant Enterococci from broiler chickens. And later also the discontinuation of the use of growth promoters in pig production showed an efficient reduction in resistant Enterococci. Today, the focus has changed from antimicrobial growth promoters to antimicrobials critical for human therapy. In June 2010, the Danish Meat Association launched a voluntary programme discontinuing the use of cephalosporin for a two-year period in pigs. As a result, cephalosporin consumption in pig production approximates zero and a significant decrease in cephalosporinase (ESC) producing *E. coli* from slaughter pigs has been observed. The Effect of the 'Yellow card system' that targets farms with the highest consumption are followed and has so far led to an increase in the consumption for pigs of 30% in 2011. Another focus area has been on resistance in imported and Danish meat and especially on the presence of ESC producing *E. coli* in broiler meat including Danish broiler meat despite no use of cephalosporins in the Danish broiler production for more than ten years. The DANMAP report is available at www.DANMAP.org.

Role of Probiotics and Prebiotics in Animal Feeding to Reduce Antibiotic Consumption

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The development of antimicrobial resistance and the transference of antibiotic resistance genes from animal to human microbiota have emerged as a major problem in medicine. Indeed, pathogenic bacteria are proving to exhibit increasing degrees of Multi Drug Resistance (MDR), presumably in response to the common use of antibiotics. One of the principal cause of the widespread of MDR in bacteria has been scientifically strongly associated with the food chain. Indeed, for years farmers have applied low doses of antibiotics in animal feeding, since it was discovered that animals grew stronger and faster; on the other hand bacteria were developing genetic strategies to overcome this hurdle, expanding and spreading antibiotic resistance. In EU antibiotic growth promoters (AGP) have been recently banned because their use has been related to an increase in risks to human. Unfortunately, the plague has been diffused (each year in the EU alone, over 25000 people die from infections caused by MDR bacteria) and now the problem of MDR is considered a treat to mankind. One of the future researches, addressed to control and to prevent MDR, should focus on the gut microbiota activities to improve the understanding of the interactions with specific intestinal diseases, and on the mechanisms which allow the establishment of a proper equilibrium between different species in a microbial community. We should consider that in a microbial community, a stronger group is overtaking another for the competition of substrates. Consequently, we could induce and improve the growth of a given beneficial species in order to contrast pathogenic species. In particular, we could fight MDR and contrast pathogens with beneficial microbes and their growth promoters, as probiotics and prebiotics, respectively.

Indeed, probiotic bacteria are known to reduce populations of many different entero-pathogens at cell surface both *in vitro* and *in vivo*. By means of ecological strategies, probiotics bacteria can overcome pathogens mainly by: competition, competitive exclusion and displacement, through known mechanisms such as the prevention of adhesion to binding sites, the production of bacteriocins and the variations of ecological factors (e.g. pH decrease). Moreover, the addition of selected prebiotics is recognized to enhance the probiotic community already resident in the gut and can strengthen its mucosal colonization to the disadvantage of pathogens and opportunistic microbes. Based even on our research experience made *in vitro* and *in vivo*, we have recently conducted works that are reflecting the suitability of probiotics to contrast pathogens, which will hence results in an alternative or a reduction for antibiotic use and misuse. For example, based on intestinal cell models we have demonstrated that selected *Bifidobacterium* species can reduce the adhesion of *Campylobacter* species, or that the supplementation in the feed of husbandry piglets of a symbiotic formula of probiotic and prebiotic can constitute for a prolonged period a protection from some pathogen bacteria. Moreover, the substitution of probiotics to AGP, resulted even in a gain weight and general better condition of the animals. Thus, both *in vivo* and *in vitro* the reduction of the number of cells of pathogens by probiotics competition is an assured fact by means of molecular analysis.

Different research groups have demonstrated the ability of some probiotics species on limiting the growth of different Vancomycin-Resistant Enterococci (VRE). If we consider that the hallmark of antibiotic resistance in the food chain is the vancomycin resistance (VR), we can stress out the role of probiotics. For a long time vancomycin-like antibiotics were the most used AGP, and currently VR pathogens represent a new menace. These bacteria may remain on the carcasses of animals after slaughter and on foods and then contaminate human. In addition, since in human medicine vancomycin is considered a last line antibiotic, the road to find and block VR pathogens results seriously harder and much more expensive. The capability of probiotics to contrast VR pathogens could be considered as the crushing prove that probiotics can contribute to antibiotics withdrawal in the food chain.

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