

**FINAL PROGRAMME  
ABSTRACTS OF LECTURES & POSTERS**

The International  
Debate Conference  
for the Feed & Food Chain

# **Antimicrobial Growth Promoters: Worldwide Ban on the Horizon?**

31 January – 1 February 2005

Including the mini-symposium  
*Recent advances in the analysis  
of AGPs and related products*

## **Advisory Committee**

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# Welcome!

Dear participant,

We are pleased to present you the lecture and poster abstracts of **Antimicrobial Growth Promoters: Worldwide Ban on the Horizon?** - the international debate conference for the feed & food chain.

With the ban of AGPs in the European Union in sight and a general shift towards a search for alternative products and strategies to fill the gap, the conference strives to bring together the knowledge and experience of the stakeholders in the feed and food chain. **Antimicrobial Growth Promoters: Worldwide Ban on the Horizon?** - the international debate conference for the feed & food chain - reflects the state of AGPs and alternatives today and discusses the way forward.

Join the experts at informative sessions, participate in the satellite mini-symposium, and take part in the panel discussion. We wish you an active and fruitful meeting!

On behalf of the Advisory Committee,

Dr. Daniel Barug  
Organising Committee

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Key to the abstracts of lectures and posters:

- abstracts of lectures, mini-symposium and posters are grouped separately;
- the lectures and mini-symposium presentations are grouped according to the daily programme; and
- the posters are according to theme.

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# PROGRAMME

Monday 31 January 2005

08.30 Opening

Dr. Herman B.W.M. Koëter  
Deputy Director, European Food Safety Authority

## Session 1:

### Setting the context for debate

Chair: Dr. Shabbir Simjee  
Elanco Animal Health, UK

08.45 *Current use of antimicrobial growth promoters in food animals: the benefits*  
Dr. Stephen W. Page  
Advanced Veterinary Therapeutics, Australia

09.15 *Use of antimicrobial growth promoters in food animals: the risks outweigh the benefits*  
Prof.dr. Henrik C. Wegener  
Danish Institute for Food and Veterinary Research, Denmark

09.45 *Antimicrobial growth promoters: consumer concerns and demands*  
Prof.dr. Lucas Reijnders  
University of Amsterdam, Institute for Biodiversity and Ecosystem Dynamics /  
Expertise Centre for Sustainable Development (IBED/ECDO), the Netherlands

10.15 Coffee/tea break and exhibition

**Monday 31 January 2005**

**Session 2:**

**AGPs – regulatory aspects and international developments**

Chair: Dr. Pierre-André Geraert  
Adisseo, France

- 10.45 *Phasing out antibiotic feed additives in the EU: worldwide relevance for animal food production*  
Dr. Andrew Chesson  
University of Aberdeen, School of Biological Sciences, UK / European Food Safety Authority (EFSA), Panel on additives and products or substances used in animal feed (FEEDAP)
- 11.15 *The US government's strategy on antimicrobial growth promoters*  
Ms. Dr. Paula J. Fedorka-Cray  
U.S. Department of Agriculture, Agricultural Research Service, Antimicrobial Resistance Research Unit (USDA-ARS-RRC), USA
- 11.45 *Non-human usage of antimicrobials: recent developments at FAO/WHO/OIE*  
Ms. Dr. Hilde Kruse  
National Veterinary Institute, Norwegian Zoonosis Centre, Norway
- 12.15 Buffet lunch and exhibition

**Session 3:**

**AGPs – risk assessment**

Chair: Dr. Niels J. Kjeldsen  
The National Committee for Pig Production, Denmark

- 13.30 *The use of risk analysis as applicable to antimicrobial growth promoters*  
Dr. Jacques F. Acar  
Pierre et Marie Curie Université, France
- 14.00 *Antimicrobial growth promoters: to ban or not to ban?*  
Ms. Dr. Emma L. Snary  
Veterinary Laboratories Agency, Centre for Epidemiology and Risk Analysis, UK
- 14.30 *Analysing the impacts of antibiotic growth promoters on human health: the case of virginiamycin*  
Dr. Louis A. Cox  
Cox Associates, USA
- 15.00 Coffee/tea break and exhibition

**Monday 31 January 2005**

**Session 4:**

**AGPs – use/non-use practice**

Chair: Prof.dr. Martin W.A. Verstegen  
Wageningen University, the Netherlands

15.30 *Terminated use of antimicrobial growth promoters: effects on pig welfare and productivity*

Dr. Niels J. Kjeldsen  
The National Committee for Pig Production, Denmark

16.00 *The AGP use/non-use situation in poultry production*

Prof.dr. Martin Wierup  
Swedish University of Agricultural Sciences (SLU), Sweden

16.30 *The antibiotic feed additive situation in veal calves*

Jacques L. de Groot, M.Sc.  
VanDrie Group, the Netherlands

**Session 5:**

**Modes of action – from AGPs to replacements**

Chair: Dr. Andreas Kocher  
Alltech, Ireland

17.00 *Molecular basis for AGP effects in animals*

Ms. Dr. Margie D. Lee  
University of Georgia, Poultry Diagnostic and Research Center, USA

17.30 *Rational development of novel microbial modulators*

Dr. Juha H.A. Apajalahti  
Alimetrix, Finland

18.00 *The use of a dynamic in vitro model of the gastrointestinal tract (TIM) in studying replacements for AGPs*

Dr. Rob Havenaar  
TNO Quality of Life, the Netherlands

**18.30 – 19.30**

**Poster presentations at the Scientific Café**

20.30 Conference dinner

**Monday 31 January 2005**

**Mini-symposium:**

**Recent advances in the analysis of AGPs and related products**

Chair: Dr. Jacob de Jong  
RIKILT-Institute of Food Safety, the Netherlands

15.30 *Rapid detection of many illegal antibiotics in feed, a way forward for the control in animal husbandry: the Feedstuffs-RADIUS project (EU project 'Rapid antibiotic detection for illegal and unlicensed substances in animal feedingstuffs')*

Dr. Chris Elliott  
Queen's University Belfast, UK

16.00 *Towards a control strategy for banned antibiotics and growth promoters in feed: the SIMBAG-FEED project (EU project 'Screening and identification methods for official control of banned antibiotics and growth promoters in feedingstuffs')*

Dr. Jacob de Jong  
RIKILT-Institute of Food Safety, Wageningen University and Research Centre,  
the Netherlands

16.30 *Novel approaches for the determination of probiotics in feed in the context of official control*

Ms. Dr. Renata G.K. Leuschner  
EC-DG JRC Institute for Reference Materials and Measurements (IRMM), Belgium /  
Central Science Laboratory, Department of Environment and Rural Affairs, UK

17.00 *Pitfalls and challenges for the official control of enzymes in feed*

Dr. Roger Ziebal  
DGCCRF, Laboratoire de Rennes, France

17.30 *Review of the possibilities to detect herbal active principles and prebiotics in feed*

Dr. Gianfranco Brambilla  
Istituto Superiore di Sanità, Toxicological Chemistry Unit, Italy

18.00 *New authorisation of feed additives in the EU: the role of the Community Reference Laboratory and the network of European Laboratories*

Dr. Christoph von Holst  
EC-DG JRC Institute for Reference Materials and Measurements (IRMM), Belgium

**18.30 – 19.30**

**Poster presentations at the Scientific Café**

20.30 Conference dinner

Tuesday 1 February 2005

**Session 6:**

**AGPs – alternatives of the present and future**

Chair: Dr. Arie K. Kies  
DSM, the Netherlands

08.30 *Nutritional solutions beyond AGPs*  
Dr. Milan Hruby  
Danisco Animal Nutrition, UK

08.55 *The likely benefits of feed enzymes in AGP free diets*  
Dr. Michael R. Bedford  
Zymetrics, UK

09.20 *Interfacing gut health and nutrition: the use of pre- and probiotics to maximise growth performance*  
Dr. Andreas Kocher  
Alltech, Ireland

09.45 *Acidification of diets as alternative*  
Mr. Gerd Diebold  
BASF AG, Germany

10.10 *Are herbs, botanicals and other related substances adequate replacers of AGPs?*  
Prof.dr. Caspar Wenk  
ETH Zurich, Institute of Animal Sciences, Switzerland

10.35 Coffee/tea break and exhibition

11.00 *Bacteriophage: a safe and natural alternative to AGPs*  
Dr. William E. Huff  
U.S. Department of Agriculture, Agricultural Research Service, Poultry Production and Product Safety Research Unit (USDA-ARS-PPPSR), USA

11.30 *Intestinal genomics for the evaluation of alternatives to AGPs: current situation and perspectives*  
Dr. Theo A. Niewold  
Animal Sciences Group of Wageningen University and Research Centre,  
the Netherlands

12.00 *Sense and non-sense of innovative approaches to replace AGPs*  
Prof.dr. Bruno Goddeeris  
K.U. Leuven, Laboratory of Physiology and Immunology / Ghent University,  
Laboratory of Immunology, Belgium

12.30 *Setting and meeting standards for the efficient replacement of pronutrient antibiotics in poultry and pig nutrition*  
Prof.dr. Gordon D. Rosen  
Pronutrient Services, UK

13.00 Buffet lunch and exhibition

## Tuesday 1 February 2005

### Session 7:

#### Panel discussion – where are we now, where are we going?

14.00-16.00 Brief presentations and panel discussion

##### *Moderator*

Prof.dr. Martin W.A. Verstegen  
Wageningen University, the Netherlands

##### *Panel members*

Asko Haarasilta, M.Sc. – Member of the European Feed Manufacturers Association (FEFAC)  
Praesidium, Belgium / Director R&D, Suomen Rehu Oy, Finland

Dr. Philippe Becquet – Operational Team member, European Federation of Animal Feed Additive Manufacturers (FEFANA), Brussels, Belgium / Regulatory Affairs Manager Europe-Middle East-Africa, DSM Nutritional Products Europe, Switzerland

Dr. Willem Penning – Head of Unit Animal Nutrition, Directorate Food Safety: production and distribution chain, The European Commission Health and Consumer Protection Directorate-General, Brussels, Belgium

Ms. Dr. Paula J. Fedorka-Cray – Research Leader, Antimicrobial Resistance Research Unit, Agricultural Research Service, U.S. Department of Agriculture, USA

Prof.dr. Gordon D. Rosen – Pronutrient Services, UK

##### *Purpose and organisation of the panel discussion*

During this two-day conference the present and future of antimicrobial growth promoters have been discussed from different viewpoints, from benefits to consumer concerns, from risk analysis to international and regulatory developments, from use to alternative strategies and products, etc. But, what is the take home message? The purpose of this session is to discuss the issues, practices and perspectives of use/non-use of antimicrobial growth promoters, and to inform the participants of the way forward. During the first part of the discussion the panelists representing the various stakeholders will give brief presentations (15 minutes each) concluding on:

- What do we know about the effects of banning antimicrobial growth promoters?
- What is the consequence of banning or not banning?
- What do we do next and how do we do it?
- And how do we know it will actually achieve anything?

During the second part of the discussion questions from the participants will be answered.

#### **16.00 Closing lecture**

**Appropriate use of AGPs - oxymoron or opportunity?**  
**Dr. Stephen W. Page, Advanced Veterinary Therapeutics, Australia**

#### **16.30 End of conference**

# LECTURES

## Current use of antimicrobial growth promoters in food animals: the benefits

Stephen W. Page

Advanced Veterinary Therapeutics, Australia

It is widely acknowledged that the inclusion of antibiotic growth promoters in the diet of livestock increases growth rates. However, the range and diversity of benefits arising from the supplementation of diets of animals with these tools of livestock production is not well known. This presentation seeks to provide a broad review of published information on the benefits of use of those antibiotics as in-feed growth promoters.

Table 1. Antibiotic growth promoters.

Compound		Approved species (Australia 2004)			
Generic name	Class	Cattle	Sheep	Pigs	Poultry
Bambermycin	Glycolipid	+		+	+
Lasalocid	Ionophore	+			+
Monensin	Ionophore	+			+
Narasin	Ionophore	+			+
Salinomycin	Ionophore	+		+	+
Kitasamycin	Macrolide			+	
Oleandomycin	Macrolide	+			
Tylosin	Macrolide	+		+	+
Avilamycin	Orthosomycin				+
Bacitracin	Polypeptide				+
Virginiamycin	Streptogramin	+	+	+	+

The era of antibiotic growth promotion commenced in 1946 with the recognition of substantial growth responses to the inclusion of streptomycin in the feed of chickens. In 1949 it was shown that pigs and chickens consuming a diet supplemented with dried mycelial mass recovered from the fermentation of *Streptomyces aureofaciens* significantly improved daily gain in bodyweight. At a time when livestock management was changing rapidly from low performance, high morbidity free range farming to more controlled and intensive husbandry, and when post-war demands on increased food production were high, the discovery of an unexpected means of accelerating growth was received with enormous interest and enthusiasm, not only by scientists but the general public. In April 1950 this serendipitous event was front-page news across the globe from New York to London.

A large number of studies were conducted from the time of discovery, peaking in the mid-fifties, and still continuing at a high rate. While the antibiotics of interest for growth promotion in the 1950s included a number also used for treatment of animals and humans, in response to the recommendations of the Swann committee in 1969, antibiotics for livestock growth promotion were restricted in many countries, including Australia, to those with limited or no human therapeutic uses. Reflecting the age and history of use of the available antibiotic growth promoters, only salinomycin and its methyl analogue narasin, both polyether ionophores, a class with no human indications, have been discovered and introduced since

the Swann report was completed.

The benefits of the antibiotic growth promoters arise from their principal mode of action, which is directed at manipulation of the microbial flora of the intestinal tract in most species and the rumen of ruminants. Results of this interaction with the organisms of the gut comprise improved digestion, metabolism and absorption of an array of essential nutrients, including energy, protein, amino acids, minerals and vitamins. In addition and as a result of enhanced utilisation of their diets, supplemented animals require less feed and produce less waste. The benefits can be broadly categorised into environmental, performance improvement, and disease control benefits, those associated with the prevention of metabolic and fermentation disorders, as well as a set of other related benefits. The principal advantages arising from the use of the antibiotic growth promoters are summarised in Table 2.

In the past substantial interest surrounded the discovery of growth promotion because of the need to accelerate the expansion of global food production. Current population growth forecasts continue to motivate massive augmentation of the global food supply. Coupled with this overriding nutritional imperative for human health and welfare is concern that animal health and the environment may be adversely impacted. Consequently, the sensible and intelligent application of antibiotic growth promoters to modern food production programs offers a number of significant advantages. Increasing the efficiency of nutrient utilisation decreases the demands for feed intake. Increased efficiency also leads to reduced waste and decreased environmental impact. Maintenance of stable fermentation within the rumen, small intestine and hindgut of ruminants can lessen the likelihood of metabolic disorders in high producing animals. Finally, by improving immune status and preventing important enteric diseases, animal health and welfare can be maintained at a high standard.

While antibiotic growth promoters were considered applicable in all stages of animal production in decades past, they now occupy a more well defined and important place for use in situations where responses are most likely to be greatest. For example, high producing dairy cattle are at increased risk of ketosis, a serious metabolic disease associated with ill health and significant falls in milk production. The adverse effects of ketosis can be attenuated by appropriate use of monensin. Feedlot cattle on high carbohydrate diets are highly susceptible to rumenitis and hepatic abscessation. Judicious use of tylosin and virginiamycin can significantly reduce the adverse consequences of this affliction. Feeding ruminants in times of drought can be very challenging as livestock managers attempt to meet nutritional needs economically and efficiently. Use of selected compounds can increase feeding options substantially, improving animal health, decreasing labour and reducing feed costs. Transmissible antibiotic resistance can reduce treatment options in infected animals. Use of those antibiotic growth promoters that interfere with successful transmission of resistance provides the potential to enhance therapeutic success. Adverse environmental loading with greenhouse gases (particularly methane and nitrous oxide) and the nutrients nitrogen and phosphorus can also be reduced significantly by appropriate use of these products.

There have been significant advances in the enhancement of existing technology and development of many new approaches to efficient livestock production. Noteworthy improvements in housing, environment, nutrition and genetic selection have been introduced. Rather than replacing the use of antibiotic growth promotants recent advances in livestock production allow the refined and focused use of these agents which continue to offer additional and important environmental, health and production advantages.

Optimal realisation of the potential benefits of supplementation with antibiotic growth promoters is dependent on their judicious selection and prudent use as a component of integrated livestock management.

Table 2. Summary of benefits of antibiotic growth promoters.

Benefit	Avilamycin	Bacitracin	Bambermycin	Lasalocid	Monensin	Narasin	Salinomycin	Kitasamycin	Oleandomycin	Tylosin	Virginiamycin
<b>Environmental benefits</b>											
Reduced methane emission (primarily ruminants)			+	+	+	+	+			+	+
Reduced nitrogen excretion (all species)	+	+	+	+	+	+	+			+	+
Reduced phosphorus output (all species)				+	+	+	+				+
<b>Performance improvements</b>											
Increased rate of bodyweight gain	+	+	+	+	+	+	+	+	+	+	+
Lower feed requirements for each unit of gain	+	+	+	+	+	+	+	+	+	+	+
Improved carcass yield	+		+								
Improved sow performance							+				+
Improved piglet survival and growth							+				+
Increased dairy cow milk production				+	+						+
Increased wool growth			+								+
<b>Disease control</b>											
Necrotic enteritis in poultry	+	+		+	+	+	+	+			+
Clostridial enteritis in pigs							+				+
Porcine proliferative enteropathy	+	+			+		+			+	+
Swine dysentery							+				+
Acute pneumonia in cattle				+	+	+	+				
Coccidiosis in calves and sheep				+	+	+	+				
Toxoplasmosis in ewes					+						
<b>Prevention of metabolic and fermentative disorders</b>											
Decreased lactic acidosis				+	+	+	+				+
Decreased laminitis				+	+	+	+				+
Decreased ketosis					+						
Decreased ruminal bloat				+	+						
<b>Other benefits</b>											
Protein sparing	+	+	+	+	+	+	+			+	+
Energy sparing	+	+	+	+	+	+	+			+	+
Improved mineral absorption				+	+						+
Improved heat tolerance	+			+	+	+	+				+
Decreased boar taint		+									+
Reduction in antibiotic resistance and its transfer		+	+								
Improved immune status		+								+	
Drier litter and reduced foot problems in broilers	+										+
Decreased fly survival in cattle faeces				+	+						

# Use of antimicrobial growth promoters in food animals: the risks outweigh the benefits

Henrik C. Wegener

Danish Institute for Food and Veterinary Research, Denmark

The growth promoting properties of antimicrobials for farm animals were discovered in the 1940's and their use became common agricultural practice in Europe during the late fifties and sixties. Many of the trials claiming to document a wide range of beneficial effects are consequently several decades old. Today agricultural practice has undergone many changes and the animals raised are also genetically considerably different from those produced four to five decades ago.

In the European Union antimicrobial growth promoters (AGPs) are regulated separately from veterinary medicines (including medicated feed). Antimicrobials used for growth promotion are considered feed additives, alongside vitamins and minerals, and regulated through Council Directive 70/524, with amendments. The Directive specifically states that the use of an AGP must not (i) *adversely affect human or animal health*, (ii) *at the levels used treatment or prevention of disease is excluded*; this condition does not apply to coccidiostats, and 3) for serious reasons concerning human or animal health *its use must not be restricted to medical or veterinary purposes*.

In Europe, considering the nature of the legislation, the primary effects claimed for the AGPs were improved feed utilisation and/or improved growth rates. The effects of AGPs were ascribed to a number of not entirely well defined mechanisms, such as enhanced digestibility of feed, less competition for nutrients between the animal and the gut microflora, and other mechanisms. While not included in the official claims, reduction of pathogenic microorganisms and prevention of disease was widely recognised as an important secondary effect.

In Europe, all veterinary antimicrobials, including medicated feed, are available on veterinary prescription only. Thus a licensed professional has to ascertain the need for the use of therapeutic antimicrobials and decide the appropriate therapy. In most European countries routine prophylactic use is prohibited, however the national legislations, as well as the use of antimicrobials for disease prevention, probably varies considerably.

A number of countries have completely discontinued the use of antimicrobials for growth promotion. This has created an opportunity to investigate the effects of the AGPs *in vivo* as changes in growth rates, feed efficiency, and other parameters after the discontinuation, compared to the period before the discontinuation, might be attributable to the AGPs.

The Swedish Parliament banned the use of AGPs in Sweden in 1986. For poultry a transition period of two years were approved to investigate the most appropriate way of dealing with necrotic enteritis (NE), a clostridial infection of poultry. It was concluded that construction and climate of stables, hygiene, management and feed composition all contributed to the occurrence of NE, and that coccidiostats of the ionophore type prevented NE. Currently NE is not a problem in Swedish broiler production. This is mainly attributed to the changes in feed composition and the continued use of ionophore coccidiostats prescribed as medicated feed. Broiler productivity has increased, in part due to genetic improvement. In pig production problems with weaning diarrhoea emerged after the withdrawal of olaquinox. These problems have been addressed by changes in management, feeding, hygiene, sectioning and by zinc supplementation of piglet feed and use of medicated feed in some herds. The

ban on AGPs did not create any problems for the production of growing and finishing pigs in Sweden [1].

The termination of the use of AGPs did not result in increasing problems in relation to weaning diarrhoea in pig production in Finland; however, in 14% of herds the use of antimicrobials was increased. Production parameters, (mortality, age at weaning, pigs weaned per sow/year) were not affected by the withdrawal. The absence of problems was attributed to effective information about correct weaning of piglets of the farmers prior to the termination, and possibly, the gradual withdrawal of the different AGPs since the mid 90'ies, which had enabled the producers to gradually adjust their production-practices [2].

In Switzerland the banning of AGPs did not result in over-all increased use of prescribed veterinary antimicrobials. The pattern of use did however; indicate an increase in treatments associated with management of diarrhoeal diseases. The number of pigs and the volume of production increased during the observation period, indicating that production parameters were not substantially affected [3].

The country in which the consequences of the termination of the use of AGPs have been most extensively studied is Denmark. The results have been summarised in an independent scientific review from the World Health Organization [4]. In broiler production the termination has had no significant effects on productivity, animal health and use of prescribed antimicrobials, and the effect on feed efficiency was entirely compensated by the cost of AGPs [5]. The absence of problems with NE is in part attributed to the continued use of ionophore antimicrobials as coccidiostats. In pig production, problems with weaning diarrhoea appear to have increased in some herds. Several different non-antimicrobial approaches have been investigated and found to be partly effective in preventing these diarrhoeal problems, however no single strategy seems to be entirely efficient. Thus, in some herds an increase in the use of prescribed antimicrobials has been observed. No problems have been observed in the production of growing and finishing pigs. The productivity of Danish pig production has improved throughout the transition period.

In the USA, Engster et al. [6] recently reported only very small benefits for growth promoters in broiler chicken. The study was on 7 million chickens (152 epidemiological units) on Purdue farms and included control groups. It showed no significant difference in mortality and weight gain without AGPs, and a 0.016 change in feed conversion (less than 1% deterioration, similar as DK). There were no outbreaks of necrotic enteritis, dermatitis or dysbacteriosis. Also no other factors were really affected either. This paper shows that AGPs make little difference, none to animal welfare and almost no economic gain, in commercial farms in the USA.

Based on recent studies the major, if not only benefit from the use of AGPs in modern intensive food animal production is that of disease prevention in weaning pigs. Other effects appear to be very minor or non-existent. Thus, the concept of growth promotion could easily be eliminated and substituted with what it really is – prophylactic use of antimicrobials. In the EU, a veterinarian must prescribe the use of antimicrobials for disease control and veterinary antimicrobials must be approved under the directives governing veterinary medicines.

The magnitude of risk acceptable from the use of antimicrobials is determined, in part, by the magnitude of the corresponding benefits. In the context of therapeutic antimicrobial usage, we generally accept the risk of resistance emergence when comparing to the benefits of the cure of infectious diseases. In contrast, if antimicrobials are used without any benefit, or where this benefit could be achieved with the use of a substantially smaller amount of antimicrobials, the acceptance of risk is consequently much smaller – ranging from non-acceptance where the benefit is absent to low acceptance where the benefit is small.

What are the risks stemming from the use of AGPs? The use of AGPs causes the selection of antimicrobial resistant bacteria in food animals. Selection means that the number of viable resistant bacteria in the site of selection increases. Until recently, it was often postulated that the “sub-therapeutic doses” in which the AGPs were used did not cause selection. This has been shown to be wrong. The use of antimicrobials in sub-therapeutic doses results in a strong selective pressure, because the concentration of drugs at the site of action exceeds the MICs of the sensitive bacterial populations, and because of the long duration of exposure. The primary site of selection in the context of AGPs is the digestive tract of the animal, but selection also occurs in the farm environment as well as fields where manure from the animals is spread. It is not possible to accurately measure and quantify the increase in occurrence of bacterial resistance in the reservoir. Only a small proportion of the GI-tract microflora is culturable, and many of the genes conferring resistance to AGPs are unknown and consequently non-detectable. In spite of these technical limitations we have solid evidence of selection of resistance to medically important antimicrobials in animals as a consequence of AGP usage – resistance to vancomycin and Synercid® are two examples of this.

Transmission of resistant bacteria from animals to humans either by direct contact or by the foodborne route is well documented, and the causation of disease in humans by pathogenic bacteria of animal origin is equally well proven. In fact, of the 1,415 microorganisms known to cause disease in humans, over 60% are zoonotic, i.e. transmitted from animals to humans.

The current medical treatment problems that are potentially attributable to animal use of antimicrobials are infections caused by resistant enterococci, streptococci, *Salmonella*, *Campylobacter*, *E. coli*, etc. It is not possible to determine the exact current and potential future magnitude of the public health impact of animal use of antimicrobials compared to medical use. This is in part due to the complexity of the epidemiology of resistance: (i) antimicrobials do not only select for resistance to themselves, but also to a wide range of other antimicrobials by the mechanism of co-selection and thus the use of one medically unimportant antimicrobial can cause increase in the occurrence of other antimicrobials that are medically important; and (ii) we cannot predict which antimicrobials will be medically important in the future. When avoparcin and virginiamycin were approved for growth promotion they were not medically important, this happened later due to increasing problems of resistance and technological developments. What we can observe is that the same resistance genes are flowing freely between animal and human bacteria confirming that the animal and human bacterial reservoirs are overlapping.

Antimicrobial resistance is increasing in human pathogenic bacteria because of animal and medical uses of antimicrobials. We must strive to preserve the power of the antibiotics, by using them prudently. Prudent use means using them when needed and when the benefits outweigh the risks. In the case of AGPs the very minor benefits do not outweigh the current and potential future risks of resistance.

In the case of the EU, disease prophylactic actions were not part of the conditions for European approval. Thus, AGPs had no benefits that were in agreement with the regulation under which they were approved and consequently a zero-risk tolerance should be applied.

## References

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# Antimicrobial growth promoters: consumer concerns and demands

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The use of antimicrobial growth promoters is closely linked to the emergence of intensive animal husbandry ("bio-industry"). This type of animal husbandry is a longstanding matter for unease among consumers. In the last decades, impacts of bio-industry on animal welfare, human health and the environment regularly emerged as concerns, especially for consumers in western industrialised countries. The discussion on antimicrobial growth promoters is thus part of a wider debate about the practices of intensive animal husbandry. It ties in with concerns about animal welfare as growth promoters have their greatest effect when animal management is suboptimal. Concerns about the impact of antimicrobial growth promoters on the development of antibiotic resistance and thereby on health have been with us since the 1960s and unease about residues in animal produce at least since the 1970s. Environmental concerns regarding the impact of growth promoters on organisms in soils and surface water are relatively recent.

Consumer concerns are now backed up by a considerable amount of scientific evidence [e.g. 1-10]. In recent decades quite a number of consumer, animal welfare and environmental non-governmental organisations (NGOs) have expressed themselves in favour of a ban on antimicrobial growth promoters. They did so mainly in view of the negative aspects of using antimicrobial growth promoters and also because they felt that alternatives such as improved management practices, wider use of vaccines and the application of pre- and probiotics are preferable. Some NGOs have stated that a ban would entail small or negligible added cost, a position that is backed up now by evidence [e.g. 3].

A Dutch proverb states that the consumer is king. Actual practice has been rather different. The uneasiness of NGOs about antibiotic growth promoters has been greatly increased in the past decades by the neglect of consumer concerns on the part of industry. For a long time little has been done with the sensible recommendations of the Swann report on the use of antibiotics in animal husbandry that appeared in 1969 [11]. Proposals for a ban on specific growth promoters were as a rule opposed by industry issuing warnings of soaring prices, manure mountains and sick animals that failed to materialise. Such a stand of the industry was not conducive to building informed consumer support. This helps to explain why, when in the European Union apparent neglect in the handling of mad cow disease undermined trust in both regulators and those involved in animal husbandry in general, the traditional balance of power that greatly favoured the use of antimicrobial growth promoters could not be maintained. The European Union now aims at a ban on antimicrobial growth promoters and this is in line with preferences of the average European consumer. As it is known by now that the scientific evidence that underlies such preferences is solid and that effects on costs will be very limited [1-10], a worldwide ban seems justified.

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# Phasing out antibiotic feed additives in the EU: worldwide relevance for animal food production

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The removal of antibiotics for growth promotion purposes within the European Community is a risk management decision. Like all risk management decisions it is based on a scientific assessment of risk but modified by other issues including a risk-benefit analysis, and by societal, financial and ethical considerations. While there continues to be debate about the extent of the risk represented by the reservoir of antibiotic resistance determinants found among the bacteria of the animal digestive tract and the consequences this has for the use of antibiotic of clinical and veterinary importance, that resistance determinants can spread from animals is in little doubt.

The spread of vancomycin resistant enterococci, generated as a result of the use of avoparcin as a growth promoter, through the food chain to the human population is now well documented [1,2]. Many similar situations appear to be emerging. For example, as the prevalence of multiresistant *Salmonella* Newport in cattle in the US increase so do the number of infections attributed to the Newport strain in humans. Similarly, there is in many parts of the world including the US a clear co-incidence in the emergence of fluoroquinolone-resistant *Salmonella* and *Campylobacter* among broilers isolates with human isolates of *Salmonella*, *Campylobacter* and *E. coli* resistant to ciprofloxacin [3,4]. Only those countries in which the use of fluoroquinolones is not approved for use in food-producing animals (e.g. Australia) or where their use is discouraged, have yet to encounter serious problems with the use of ciprofloxacin in humans. This difference is evident even within the European community where resistance to fluoroquinolones in poultry can vary from being virtually undetectable in birds grown in the Nordic countries to nearly 50% of isolates of *Campylobacter jejuni* in other Member States.

Risk managers throughout the world are thus faced with:

- the universal acceptance that the use and misuse of antibiotics have led to the spread of antibiotic resistance determinants throughout bacterial populations to an extent which threatens the capacity to deal with many diseases;
- a consensus amongst risk assessors that the reservoirs of resistance found in food-producing animals contribute to the problem.

This view has been adopted by various international bodies such as the World Veterinary Association [5] and the WHO [6], who have responded by calling for the universal withdrawal of antibiotics for growth promotion. The immediate response from risk managers in many parts of the world, including the FDA in the United States, was that the recommendation for wholesale withdrawal is too general. It was argued for example, that the risk associated with the use of bacitracin could not be equated to that of the macrolides. In addition a full risk assessment should take account of all uses of antibiotics in animal production (therapeutic, prophylactic and growth promotion) to determine where prudence could be best introduced. Although these and similar arguments were raised in the EU ultimately they fell foul of the “precautionary principle” increasingly governing the European risk managers approach to consumer safety. Underlying all of these views is the concern that withdrawal would see a substantial loss of production and profitability and an inevitable increase in morbidity and mortality. Welfare issues and environmental concerns following poorer nutrient capture are also introduced as

part of this equation.

There have been numerous comparisons made of the production of food animals in the presence and absence of growth promoters. Figures generated vary considerably depending on target species and production system but usually imply percentage losses in productivity in the low teens. These figures have often been introduced into risk-benefit equations without further analysis, ignoring the fact that a vacuum is usually filled by alternatives.

The only real experience of sufficient magnitude to judge the impact of a withdrawal comes from Denmark, and even here interpretation can be coloured by expectations. Denmark voluntarily abandoned the use of antibiotics for growth promotion in advance of the requirements EU regulations. The 100 tonnes plus of antibiotics used in 1996 for growth promotion, fell to zero by 2000. Initially, many of the anticipated adverse consequences for animal health and welfare did occur, particularly the increase in mortality associated with enteric infections [7,8] and this was accompanied by a concomitant increase in the therapeutic use of antibiotics [9]. However the national production in pigmeat in Denmark has continued to increase with no obvious dip caused by antibiotic removal as has the efficiency of production [6]. There remains a small increase in overall mortality figures for pigs due to an increase in *E. coli* diarrhoea and/or *Lawsonia intracellularis* infections. Danish farmers responded to the ban in a variety of ways, by improvements to general husbandry, by the increased use of zinc in pigfeed and by the greater use of so-called alternatives (e.g. organic acids in pigs, enzymes in poultry).

The Danish “experiment” has demonstrated that antibiotics used for growth promotion can be withdrawn without a catastrophic loss of productivity or profitability, at least within an internal market. In addition the overt aims of the withdrawal have been met in part. Levels of resistance to some antibiotics (macrolides, avoparcin/vancomycin) have fallen dramatically in the bacterial flora of Danish livestock although this has yet to be seen in the local human population [10]. The question remains as to how far the Danish experience can and should be extrapolated to other parts of the world. Denmark may mirror production method encountered in many parts of northern Europe and northern America, but is not representative of the southern parts of Europe and is far removed from the agriculture practised in the tropics. Here the different patterns of disease, climatic conditions and methods of husbandry will require different solutions to any withdrawal and needs of local populations will present very different risk-benefit analyses.

The societal benefits of therapeutic antimicrobial in animal use are assumed and unchallenged and have not been subject to economic analysis. It has been argued by those yet to control antimicrobial use for growth promotion that to ensure optimal risk management options for controlling the spread of antimicrobial resistance, analyses of non-human uses for antibiotics should segregate benefits and costs in terms of feed efficiency, disease prophylaxis and therapy on a drug and animal specific basis. This was not done in Europe and will certainly not be done retrospectively. In addition to being seen to take action in response to the very real concerns about preserving the effectiveness of antibiotics for human clinical use, European risk managers were responding to more fundamental concerns about animal production systems and the quality of food produced by intensive methods. In areas of the world in which such concerns are not as prevalent, risk managers may find different solutions.

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# The US government's strategy on antimicrobial growth promoters

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The development of antimicrobial resistance has become a global problem. Resistance emerged shortly after introduction of the penicillins and has since been observed for all antimicrobial drug classes. Although decreased bacterial susceptibility could adversely affect clinical outcome, it has been observed that susceptibility to antimicrobials is not shared equally among bacterial species, or even between different strains of the same bacterial species.

Antimicrobial drugs are used throughout the world in animals, humans, and on plants. They are an important and critical component in treating disease in human (and animal) medicine. The extent to which antimicrobial resistance currently impacts human health is not known, but there is increasing global pressure to protect the effectiveness of antimicrobials by limiting antimicrobial use. Although the development of resistance has been largely attributed to overuse and abuse of antimicrobials in human medicine, agricultural uses have also contributed to the problem.

While all uses of antimicrobial drugs are under review, significant attention has been focused on antimicrobial use in animals. Antimicrobials are used in animal production for treatment of disease, for prophylactic use in prevention of disease, and to enhance growth and performance. Low levels, well below therapeutic concentrations, are typically used for growth promotion; antimicrobials are typically placed in animal feeds and all animals within the production unit have access to this feed. Although the development of resistance varies between bacterial species, the rate and extent of the development of resistance among bacteria common to the animal production environment is unknown. However, resistance will develop among some bacterial species and strains, even at the low drug concentration.

The extent to which the use of antimicrobials for growth promotion impacts human and animal health is widely debated. The correlation between use and resistance, particularly among zoonotic bacteria, has provided the impetus for some groups and individuals to propose limiting or banning antimicrobials from use, particularly as growth promotants. In Europe, bans based on the precautionary principle against subtherapeutic use of feed grade antimicrobials are already in place. In the U.S., no official ban is in place, although the issue evokes passionate debate.

While the U.S. government has no official policy on growth promotant use, the Food and Drug Administration (FDA) has developed a guidance for industry on antimicrobials. Guidance Document #152, Evaluating the Safety of Antimicrobial New Animal Drugs with Regard to Their Microbiological Effects on Bacteria of Human Health Concern, focuses on the drugs themselves, which includes antimicrobials used for growth promotion. GD#152 regulates the labelling and marketing of antimicrobials for use in animals and addresses both the pre-approval assessment of microbial safety and the re-evaluation of currently approved antimicrobials. The FDA has completed one risk assessment on virginiamycin (an antimicrobial used for growth promotion in animals) and its impact on resistance to Synercid, which is used in human medicine. Evaluation of several other antimicrobials used for growth promotion is ongoing.

The increased scrutiny and global pressure to develop strategies to expand the lifespan of antimicrobials are producing an immediate need for more scientific data. The U.S. Centers for Disease Control and Prevention (CDC), the U.S. Food and Drug Administration (FDA), the U.S. Department of Agriculture (USDA) and many other government agencies involved in promotion and regulation of health activities around the world are vigorously engaged in developing programs intended to monitor for the emergence of antimicrobial resistance and to decrease use of antimicrobial drugs where possible.

# **Non-human usage of antimicrobials: recent developments at FAO/WHO/OIE**

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Antimicrobial agents are essential drugs for human and animal health and welfare. Non-human antimicrobial usage includes usage in food animals, including aquaculture, companion animals and horticulture to treat or prevent disease, and also usage in food animals to promote growth. The types of antimicrobials used are frequently the same as, or closely related to, antimicrobials used in humans. It is well acknowledged that antimicrobial usage can select for antimicrobial resistant microorganisms and further promote the spread of resistant bacteria and resistance genes. Today, antimicrobial resistance has become a global public and animal health problem that is impacted by both human and non-human antimicrobial usage, and the resultant development and spread of antimicrobial resistance. Importantly, resistance neither respects phylogenetical, geographical nor ecological borders. As a consequence, containment of antimicrobial resistance relies on a holistic and international approach that takes into account the complexity and multi-dimensionality of the problem. Interdisciplinary national and international co-operation is essential. Therefore, since 1997, the World Health Organization (WHO), FAO (Food and Agriculture Organization) and Organization International des Epizooties (OIE) have organised a number of consultations to address the issues related to antimicrobial use at different steps of the food chain, such as the emergence of resistant pathogens and the associated public health problems.

Considering that antimicrobial usage and resistance is a multi-factorial problem and thus requires a multidisciplinary approach, the Executive Committee of the Codex Alimentarius Commission in its 53rd session in 2003, recommended that FAO, WHO and OIE should give consideration to convening a multidisciplinary expert consultation. All issues of antimicrobials in agriculture and veterinary use (including aquaculture) should be considered and the role played by antimicrobials as essential human and veterinary medicines should be taken into account. It was agreed that the issues raised by several Committees required a more general and multidisciplinary and multi-agency response.

As a response, FAO, WHO and OIE convened jointly a two-step approach consisting of two expert workshops addressing non-human antimicrobial usage and antimicrobial resistance. The first workshop, which was held in December 2003 in Geneva, conducted a scientific assessment of antimicrobial resistance risks arising from all non-human uses of antimicrobials in animals (including aquaculture) and plants, based on available scientific information. The second workshop, which was held in March 2004 in Oslo, Norway, considered the broad range of possible risk management options for antimicrobial resistance from non-human usage of antimicrobials. In particular, it focused on potential directions of future Codex, FAO, OIE and WHO work in this area, in order to prevent and minimise antimicrobial resistance at the global level.

## **Outcome of joint FAO/OIE/WHO expert workshop on non-human antimicrobial usage and antimicrobial resistance: scientific assessment, Geneva, Switzerland, December 1-5, 2003**

The expert workshop, which consisted of independent scientist with relevant expertise, concluded that there is clear evidence of adverse human health consequences due to resistant organisms resulting from non-human usage of antimicrobials. These consequences include infections that would not have otherwise occurred, increased frequency of treatment

failures (in some cases death) and increased severity of infections, as documented for instance by fluoroquinolone resistant human *Salmonella* infections. Evidence shows that the amount and pattern of non-human usage of antimicrobials impact on the occurrence of resistant bacteria in animals and on food commodities and thereby human exposure to these resistant bacteria. The foodborne route is the major transmission pathway for resistant bacteria and resistance genes from food animals to humans, but other routes of transmission exist. There is much less data available on the public health impact of antimicrobial usage in aquaculture, horticulture and companion animals.

The consequences of antimicrobial resistance are particularly severe when pathogens are resistant to antimicrobials critically important in humans. Therefore, the expert workshop recommended that an expert clinical medical group appointed by WHO defines which antimicrobials are considered critically important in humans.

The expert workshop concluded that surveillance of non-human usage of antimicrobials and surveillance of antimicrobial resistance in food and animals is important for the identification of resistance problems and as a basis for choosing and evaluating interventions to limit the development and spread of resistance at all levels.

Several recent attempts to quantify the magnitude of related health impacts in the human population have been made. Estimates vary widely from small to large, depending on the organism and antimicrobial of interest, and are accompanied by considerable uncertainty.

The expert workshop concluded that residues of antimicrobials in foods, under present regulatory regimes, represents a significantly less important human health risk than the risk related to antimicrobial resistant bacteria in food.

Risk assessment approaches that adequately address the broad range of potential human health impacts need to be further developed with a view towards enabling efficient risk management of antimicrobial resistance in the international arena. These approaches may need to be elaborated in the FAO/WHO Expert body for microbiological risk assessment (JEMRA) in interaction with the codex Committee on Food Hygiene (CCFH). OIE is invited to continue its work on risk assessment in co-ordination with FAO and WHO.

The expert workshop recommended that the Codex Alimentarius Commission, where appropriate in collaboration with OIE, takes co-ordinated steps to define a more efficient management system for these risks focusing on the microbiological nature of the hazards.

The workshop recommended specifically to:

- Establish a national surveillance programme on the non-human usage of antimicrobial agents
- Establish a national surveillance programme on antimicrobial resistance in bacteria from food and animals
- Implement strategies to prevent the transmission of resistant bacteria from animals to humans through the food production chain
- Implement WHO Global Principles for the Containment of Antimicrobial Resistance in Animals intended for Foods and follow OIE Guidelines on Responsible and Prudent Antimicrobial Use
- Implement specific management strategies to prevent the emergence and dissemination of bacteria resistant to critically important antimicrobial agents for people
- Implement the risk assessment approaches that are needed to support selection of risk management options.
- Enhance the capacity of countries, particularly developing countries, to conduct surveillance of antimicrobial use and resistance, to implement intervention strategies to contain antimicrobial resistance and to implement risk assessment approaches to support selection of risk management options.

## **Outcome of joint FAO/OIE/WHO expert workshop on non-human antimicrobial usage and antimicrobial resistance: management options, Oslo, Norway, March 15-18, 2004**

Managing human health risks from non-human usage of antimicrobials and the resulting antimicrobial resistant bacteria requires national and international interdisciplinary co-operation. The human, animal and plant sectors all have a shared responsibility and role in efforts to prevent and minimise antimicrobial resistance selection pressures for both human and non human use of antimicrobials. Based on the outcome of the 1<sup>st</sup> Expert Workshop in Geneva, as well as other relevant input (e.g. reports of previous WHO and OIE workshops), the 2<sup>nd</sup> Expert Workshop in Oslo considered the broad range of possible risk management options for antimicrobial resistance from non-human usage of antimicrobials. In particular, it focused on potential directions of future Codex, FAO, OIE and WHO work in this area, in order to prevent and minimise antimicrobial resistance at the global level. To ensure that the conclusions of the 2<sup>nd</sup> Expert Workshop reflected the perspectives of affected parties, the major stakeholder groups (e.g., pharmaceutical industry, farmers, food processors, consumers, regulatory agencies, and veterinarians) participated in the meeting.

Among the important conclusions were the following:

- The risks associated with non-human antimicrobial use and antimicrobial resistance should be part of the human safety assessment. The concept of “thresholds of resistance” should be pursued as a tool for risk management. A range of risk management actions should be triggered if these thresholds are exceeded.
- The concept of “critically important” classes of antimicrobials for humans should be developed by WHO with a view to enabling specific resistance-preventive actions for these antimicrobials in the context of non-human use. A similar list of “critically important” classes of antimicrobials should be pursued by OIE.
- Through stringent implementation of good agricultural practices, including good animal husbandry and good veterinary practices, it is possible to reduce the necessity for antimicrobials.
- The need for governments and all stakeholders rapidly to implement the WHO Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food and the OIE Guidelines on Antimicrobial Resistance.
- There is need for capacity building, networking and co-ordination to facilitate the implementation of surveillance programmes in various countries, particularly developing countries. FAO, WHO and OIE should take a leading role in this.
- A Codex/OIE Task Force should be established to develop risk management options for antimicrobial resistance related to non-human use of antimicrobials. Risk management should include a risk assessment policy for JEMRA, introducing considerations related to antimicrobial resistance within the existing framework for microbiological risk assessment. Risk communication and transparency are critical to the achievement of effective risk management. Moreover, the *International Code of Practice: General Principles of Food Hygiene* should be reviewed to take antimicrobial resistance into account.

### **Antimicrobial growth promoters**

Both workshops referred to above endorsed the WHO Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food (Report of a WHO consultation with the participation of FAO and OIE, Geneva, 2000). It is stated in Principle 18 that: “Use of antimicrobial growth promoters that belong to classes of antimicrobial agents used (or submitted for approval) in humans and animals should be terminated or rapidly phased-out in the absence of risk-based evaluations. The termination or phasing-out should be accomplished preferably by voluntary programmes of food animal producers, but by

legislation if necessary". Principle 19 states that: "Risk-based evaluations of all antimicrobial growth promoters should be continued. Characterisation of the risk may include consideration of the present and potential future importance of the drug to human medicine, its selection of resistance, the potential exposure to humans from resistant bacteria from food animals, as well as other appropriate scientific factors".

# Antimicrobial growth promoters: to ban or not to ban?

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The use of antibiotics for animal growth promotion has been controversial because of the potential transfer of antibiotic resistance from animals to humans. Such transfer could have severe public health implications in that treatment failures could result. To evaluate policy options for the streptogramin-class of antibiotics virginiamycin, an animal growth promoter, and quinupristin/dalfopristin, a human antibiotic, a risk assessment approach was adopted [1].

Under the assumption that resistance transfer is possible, models project a wide range of outcomes depending mainly on the basic reproductive number ( $R_0$ ) that determines the potential for person-to-person transmission. Counter-intuitively, the benefits of a ban on virginiamycin were highest for intermediate values of  $R_0$ , and lower for extremely high or low values of  $R_0$ .

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# Analysing the impacts of antibiotic growth promoters on human health: the case of virginiamycin

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There is widespread concern that continued use of antibiotics in food animals might increase risk of antibiotic-resistant bacterial illnesses and compromise antibiotic treatment effectiveness in human patients. However, insufficient use of animal antibiotics may also increase pathogen loads in retail meats, human illnesses, patients-per-year treated with antibiotics, and emergence of antibiotic resistance among humans. Risk assessment modelling can be used to quantify and compare these competing risks and to help identify risk management policies that protect human health. Quantitative health risk assessment reveals that continued use of antibiotics in poultry may prevent many more human illnesses than it causes and reduce the need to treat human patients with antibiotics. We summarise quantitative risk assessment results for continued use vs. discontinued use of virginiamycin (VM), and use this as a case study to discuss strengths and limitations of quantitative risk analysis and alternatives such as qualitative risk rating approaches and “precautionary” approaches that recommend banning or restricting animal antibiotic uses without explicitly considering the probable human health impacts of doing so.

## Background

A common regulatory concern in the U.S. and worldwide is that continued use of fluoroquinolones, macrolides, streptogramins, and other antibiotics in food animals might increase the risk of antibiotic-resistant bacterial illnesses, especially campylobacteriosis, salmonellosis, and streptogramin-resistant, vancomycin-resistant *Enterococcus faecium* infections in human patients with compromised immune systems [1]. A less frequently assessed threat is that insufficient use of animal antibiotics may lead to increased microbial loads in food animal products and increased human illnesses, resulting in increased need to treat patients with antibiotics and hence more rapid spread of antibiotic resistance in human populations. To balance such conflicting concerns, quantitative risk models are essential. This paper reviews quantitative human health risk assessment results for virginiamycin (VM). It discusses when such model results can be trusted and used to build confidence in and improve the quality of regulatory decisions by increasing the probability of desired health outcomes.

## Health risk assessment framework

*Health risk assessment* estimates the health risks to individuals, groups (e.g., old, young, or immuno-compromised), and entire populations from exposures to hazards and from decisions or activities that create them. Health risks describe the frequencies and severities of adverse health effects caused by exposures. They can be expressed in units of expected adverse health effects per capita-year. Population risks are found by summing individual risks over all individuals in the population and are expressed in units of expected cases per year in different illness severity categories, e.g., mild, moderate, severe, and fatal [e.g., 2]. The main goal of applied risk assessment is to produce information to improve risk management decisions by identifying causal relations between alternative risk management decisions and their probable total human health consequences (including health benefits, if any, as well as risks) and by identifying those decisions that make preferred outcomes more likely.

A well-conducted risk analysis enables stakeholders to participate more effectively in risk

management deliberations and to communicate questions and concerns more clearly and concisely than would otherwise be possible. It does so by providing the relevant information needed to determine probable consequences of proposed actions; by showing how sensitive these predicted consequences are to specific uncertainties and assumptions in the analysis; and by communicating this information clearly and unambiguously. To these ends, it is best to avoid vague, meaningless, or subjective labels and descriptions of risk and instead to provide quantitative data-based risk estimates and uncertainty estimates where possible.

The expected number of adverse human health consequences per year in each severity category can be estimated, to a useful first approximation, as the product of an *exposure factor* giving the expected number of contaminated meals ingested per year; and a *consequence factor* giving the expected number of illness-days (or QALYs lost) by severity category from each contaminated meal ingested. If enough data are available, different degrees of contamination may be distinguished, and the product *risk = exposure factor x consequence factor* can then be calculated and summed for multiple contamination categories. More generally, the “*risk = exposures-per-year x clinical-consequences-per-exposure*” framework can be applied to each type of exposure (e.g., susceptible, resistant, and cross-resistant bacteria) and each distinct at-risk sub-population and health consequence of interest, and resulting illness-days or QALYs lost per year can be summed. Finally, depending on the available data sources, the exposure and consequence factors can be further decomposed into products of sub-factors. For example, the risk model:

*Risk = (change in animal drug use) x (exposure factor) x (unit risk factor) x (consequence factor)*

is useful when an exposure factor giving the ratio of contaminated-servings-per-year ingested to fraction of flocks treated with an antibiotic can be estimated and when a unit risk factor (giving probability of illness per contaminated serving ingested) and a separate consequence factor (giving expected illness-days or QALYs lost per illness) can be identified and estimated from data. Such risks must be summed over multiple paths (i.e., drugs, resistant and susceptible bacteria, sub-populations, and distinct health consequences) that transmit effects of changes in animal drug use to human health consequences. We refer to this as the Rapid Risk Rating Technique (RRRT).

### **Data sources, methods and results of risk assessment modelling**

The risk assessment framework outlined above has been implemented using various modelling strategies to obtain and organise the required data. *Farm-to-fork models* model the changes in microbial loads flowing from farm animals through transportation, slaughter, processing, storage, wholesale and retail, preparation, and cooking. Insufficient data and excessive combinatorial complexity of possible changes can defeat attempts to simulate accurately the physical details of processes and changes in microbial loads at each stage. However, Monte-Carlo based statistical conditioning (which estimates the conditional frequency distribution of microbial loads leaving each stage by conditioning on the load leaving the closest previous stage for which data are available) provides a sound, practical alternative to detailed simulation of physical processes and changes. It makes unnecessary any attempts to model in the absence of relevant data, while taking advantage of available microbiological sampling data. Farm-to-fork models may be combined with *population dynamics models* [3] to study the probable impacts of exposures to foodborne bacteria on susceptible and resistant bacterial illnesses in human populations.

As an alternative to farm-to-fork modelling, it is often more practical and useful to carry out risk assessment using what might be called *clinic-to-farm modelling* [4]. This starts with a total number of adverse health responses or illnesses observed per year and apportions it into fractions that are estimated to be caused by various sources, including any animal

antibiotic uses of interest. The fraction of illnesses per year that could be prevented by different risk management interventions and the clinical consequences of such a change are estimated and used to evaluate alternative risk management options. An advantage of this approach is that it can often exploit available genotyping and microbiological data as well as epidemiological data to estimate the exposure and consequence factors needed to quantify risk.

A basic quantitative risk estimate of the human health risks from withdrawing a specific drug with human-use analogues from use in chickens can be conducted using the following clinic-to-farm RRRT template based on a product of empirically derived factors:

*Preventable risk due to resistance caused by continued animal antibiotic use = (total cases per year) x (fraction of cases treated with the human drug of interest, e.g., Synercid™) x (assumed treatment failure rate per resistant case treated with the human drug of interest) x (excess QALYS lost or illness-days caused per case of treatment failure) x (fraction of treated cases with resistance caused by use of animal antibiotic that would be prevented if the animal drug use ceased)*

Plausible order-of-magnitude numbers and upper-bound estimates for these factors and for resulting human health benefits (i.e. risk reductions) from withdrawing virginiamycin can be calculated from available data. The cases of primary concern are vanA vancomycin-resistant *E. faecium* (VREF<sub>A</sub>) infections (mainly among intensive care unit patients with compromised immune systems and multiple other infections). The number of such cases per year with quinupristin-dalfopristin (QD) resistance that might be prevented by withdrawing VM is estimated as less than 1 case per year in the U.S., and much less in Australia, these being the two countries for which we present numerical results.

Similarly, human health benefits from continued use of an animal antibiotic can be estimated from a template such as the following:

*Human health risk prevented per year by continued animal antibiotic use = (Reduction in microbial loads in ingested servings, caused by continued use) x (average illnesses per year prevented per unit reduction in microbial load ingested) x (illness-days or loss of QALYS avoided per case prevented)*

Again, the required factors can be estimated from data, if it is assumed that discontinuing VM use would increase microbial loads of pathogens (e.g., *Campylobacter*) in proportion to the increase in ill (e.g., necrotic enteritis-positive) and/or underweight birds at processing. The main results suggested by these calculations are that potential increased human health risks from more pathogens reaching consumers if VM use is terminated (e.g., 6660 estimated excess campylobacteriosis cases per year in the base case) probably far outweigh potential human health benefits (risk reductions) from reduced streptogramin-resistant VREF<sub>A</sub> infections in human patients (< 0.3 estimated excess cases per year in the base case). While lack of information about impacts of VM withdrawal on average human illnesses-per-serving of food animal meat precludes a deterministic conclusion, it appears very probable that such a withdrawal would cause many times more human illnesses than it would prevent. This qualitative conclusion appears to be robust to several scientific and modelling uncertainties.

Quantitative risk assessments are sometimes criticised on the grounds that they cannot envision or model all possibilities and causal pathways; that they are necessarily based on past data that may not represent future realities; and that they require complex webs of modelling assumptions whose validity and implications may be difficult or impossible to establish. We briefly describe how these challenges have been met in previous quantitative risk assessments using “top-down” modelling, dynamic risk models, uncertainty and sensitivity analyses, and Value of Information (Vol) concepts. Ideally, a full risk assessment

should also consider the *timing* of impacts (e.g., what fraction of the total human health benefits and risks of withdrawal are achieved within 5 years?), any additional *indirect impacts* of animal drug withdrawal due to resulting changes in physician and veterinary prescription practices and effects on other bacteria and on cross-resistance to other drugs; and longer-term impacts of withdrawal on the *population dynamics* of illnesses, drug use, and emergence of resistance. We mention how these issues can be addressed within a systems dynamics framework.

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# **Terminated use of antimicrobial growth promoters: effects on pig welfare and productivity**

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This presentation describes the impact of antimicrobial growth promoter (AGP) termination on productivity and health in pig production in Denmark.

In February 1998 the Danish swine industry voluntarily stopped the use of all AGPs in finisher production (pigs weighing more than 35 kg). Experiences collected in 62 finisher herds showed that the majority of the herds (63%) did not experience any problems such as reduced growth or increased frequency of diarrhoea. 26% of the herds experienced temporary problems, while 11% experienced permanent problems. This result was confirmed by statements of the national production record system, in which the overall development in daily gain and mortality remained unaffected by the removal of AGPs from finisher pig production.

The application of all antibiotic growth promoters for weaner pigs was voluntarily stopped as of January 2000. This reduced the consumption of antibiotics as growth promoters to zero. Since then, the use of antibiotics for therapeutic treatment increased, reflecting the increasing problems with diarrhoea seen in the weaner period (7-30 kg). These consequences were reflected also in the statements of the national production record system, in which daily gain decreased and mortality increased after the removal of AGPs from weaner feed.

The cost of production increased by approximately DDK 7.75 (1.03 Euro) per pig produced (birth to slaughter) after the removal of AGPs. The economic impact of the AGP termination on the pig producer has been highly variable. Some costs associated with modifications of the production system are difficult to measure and have not been included in the economic calculation, although they may have been substantial for some producers.

Overall, the removal of AGPs from pig production in Denmark had only significant consequences for weaner production (7-30 kg). The total consumption of antibiotics was reduced significantly from 206 tons (active component) in 1994 to 102 tons in 2003. Termination of antimicrobial growth promoters also dramatically reduced the food animal reservoir of resistance to these growth promoters, and thus to several clinically important antimicrobial agents in humans.

# The AGP use/non-use situation in poultry production

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In the European Union (EU) some Member States and individual industries have already stopped the usage of the AGPs while others are preparing for the EU- ban to come into force. Outside the EU the discussion on the usage of the AGP is going on with different approaches. In summary there seems to be a scientific agreement, as declared in different ways also by the WHO that the usage of antimicrobials to promote growth is contra productive on a long term basis and unacceptable due to the risk of the evoke of antimicrobial resistant bacterial strains that will threaten the effectiveness of available and future antimicrobial drugs in both humans and animals. Instead there is a need for a general change from continuous use of antimicrobials for growth promotion to exclusive use of targeted treatment of specific animals for therapy under veterinary prescription.

The current situation is interestingly studied through the results and experiences gained from Sweden and Denmark (approximate annual production 75 and 120 million of broilers respectively), where controlled data are available from a longer period of production without the use of AGPs (Sweden since 1986 and Denmark since 1998) which thus have provided a good base for a scientific evaluations of the ban.

Generally it is in these countries found possible to achieve competitive production result without a continuous use of AGP. A recent evaluation by WHO found that in Denmark the effects of the termination of AGPs on poultry production was small and limited to a decrease in feed efficiency (-2.3%) that was largely offset by savings in the cost of the AGPs. There were no changes in weight gain or mortality that appeared to be related to the termination of the AGPs. No overall net cost associated with productivity losses were estimated. From Sweden the experiences are similar. However, it is found that even if a stopped use of AGP generally does not need to be accompanied by increased production costs it is likely that this initially, and to some extent also permanently, is the case due to investments to optimise the management, hygiene, disease prevention and well fare necessary in the absence of AGP.

Prior to the ban of AGPs most broilers were exposed to antimicrobials during most of their lives (live span 42 days to 2 kg) while after termination the average use declined to 0.4 days in Denmark and even shorter in Sweden.

The industry prior to termination of the AGPs anticipated problems with necrotic enteritis (NE), a disease associated with *Clostridium perfringens* infection. However, NE is found to be a minor health problem largely because producers continue to use ionophores for the prophylaxis of NE and coccidiosis. Ionophores are antimicrobials approved in many countries to prevent coccidiosis, a parasitic disease that predispose broilers to NE. Ionophores may also directly suppress *Clostridium perfringens* and therefore NE. Experiments have shown that the use of vaccines instead of ionophores often resulted in outbreaks of NE. So far there seems to be no available way to replace the use of ionophores for the prevention of NE outbreaks.

# The antibiotic feed additive situation in veal calves

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During the past two decades the European veal calf could be characterised as a physiological developing animal (i.e., digestion and health). Veal calves have a monogastric (enzymatic) digestive system with a developing rumen system. The environment in the gastrointestinal tract is based on an unstable equilibrium in the micro flora and a vulnerable intestinal wall. Young cattle are showing strong physiological reactions to relatively minor changes.

Group-housed animals are characterised by a high degree of heterogeneity, also in the composition of their microflora. The daily diet is based on a large amount of liquid feed (calf milk replacer) supplemented by solid feed (silo-maize, etc.). The microflora composition created during the first few weeks after birth can only be adapted (not controlled) by these feed components.

Until the mid-nineties producers of calf milk replacer carried out a considerable amount of research into alternatives for antibiotic supplements. However, this did not result in substantial replacements.

1996	Tonnage, the Netherlands	Antibiotic feed additive
Calf milk replacer veal calves	675,000	100%
Calf milk replacer rearing calves	100,000	20%

The disappearance of various antibiotic (broad-spectrum) additives in veal calf husbandry has had obvious negative results, with an increase in pressure on animal health and well being. A report published by IKC-L (July 1998), based on a scenario study, attempted to evaluate what the financial consequences would be for veal calf husbandry. It was estimated at approx. € 22.50 per unit per year.

Both fundamental and applied practical research increased their efforts to find alternative compensating measures. However, in view of the fact that in 2004 there is insufficient knowledge of the ideal micro flora composition and insufficient knowledge of adaptation/control options, current veal calf husbandry can only mitigate part of the negative effects. This situation is the result of a great deal of empirical research (trial and error) with respect to:

- technological adjustment of ingredients;
- composition adjustment;
- the use of various additive combinations;
- non-specific control measures; and
- management measures at farm level.

Other issues of concern in veal calf husbandry include the prevention of zoonotic diseases when antibiotic additives are removed and lower yields with other producing countries.

The increased loss figures, the use of curative antibiotics, the lower growth performance, the strong impact of selectively used curative antibiotics approved by the European Union (EU), the signs on the effectiveness of antibiotic additives, still approved in Canada, banned by the EU, indicate that the road to veal calf practices without antibiotics/antibiotic additives stretches a long way into the future.

# Molecular basis for AGP effects in animals

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For more than 50 years, the performance-enhancing characteristics of antimicrobial feed additives have been utilised by the animal production industry. How these antimicrobials enhance performance is unknown, but they may change the population balance of the intestinal microbiota. The intestinal microbiota is part of a complex ecosystem that is influenced by diet, age and physiology of the animal host. This microbiota contributes to the maintenance of intestinal health and has a profound effect on the economy of animal production. But many species of the intestinal microbiota of domestic animals remain uncharacterised. Studies have shown that the cultivable species represent only about 10% of the microbial diversity in the intestine. Therefore, the population ecology of intestinal bacterial community is relatively unknown and its contribution to mucosal health and overall performance is poorly understood.

Recently, molecular techniques have been developed to characterise complex microbial ecosystems including the complex intestinal microbiota. In order to study the effects of feed additives on the bacterial communities within the intestine of broiler chickens, we used two 16S ribosomal DNA community analysis protocols: terminal restriction fragment length polymorphism (T-RFLP) analysis combined with sequencing 16S rDNA clone libraries. These analyses revealed the bacterial community structure and the distribution of bacterial species that occurred in response to AGPs and different diet formulations. Some of the bacterial species were detected as abundant members of the community regardless of diet, while others varied in distribution and correlated with a specific feed additive. In order to examine the effects of the antibiotics and feed additives, the groups were compared to the control by evaluating the composition of the communities and their diversity indices. Diets that were corn or wheat-based were found to produce similar ileal bacterial communities in the chickens while monensin, an ionophore antimicrobial, produced a bacterial community rich in clostridia. The composition of the ileal community of the AGP group was highly variable from hatch to 14-days of age but was composed of few abundant species and exhibited low diversity indices. The bacterial community of 14-day-old AGP birds was dominated by an abundance of *Escherichia coli* while the communities of older birds (21-28 days of age) were composed of primarily of *Clostridium irregularis*. These results indicate that the growth-promotants suppressed some populations of bacteria. We compared the weights of the bacterial pellets retrieved from the ileal contents and did not detect significant differences between the control and AGP group. Therefore we investigated whether the ileal community of the birds that were administered AGP consisted of a subset of the control group. Statistical analysis of the cloned libraries showed that the AGP and control groups were significantly different therefore, the antibiotics selected for a uniquely different ileal bacterial community.

This study, which evaluated the effects of different treatments on the composition of intestinal microbial community, suggests that the ileal community is very sensitive to feed additives. The bacterial community of the control group, that was fed a corn soy diet, was primarily composed of lactobacilli. However, we found that the ileal microbial communities of chickens fed growth promotants were significantly different from the control, indicating antibiotics affected some phylotypes that composed the normal ileum microbial community. The effect of the antibiotics on abundance of, lactobacilli especially *Lactobacillus acidophilus*, was more significant than on the other abundant phylotypes. The data resulting from our study indicated that the bacitracin/virginiamycin AGP formulation might not have enhanced the abundance of the commonly accepted 'beneficial' lactobacteria. Rather the antibiotics and

monensin may have selected for populations of uncharacterised intestinal symbionts that enhance mucosal health and feed conversion. Further studies evaluating the effects of these commensals may illuminate the mechanisms by which AGPs enhance performance.

# Rational development of novel microbial modulators

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In the feeding of production animals it is a common practice to amend feed with ingredients, which affect microflora of the gastrointestinal tract, and thus improve animal health and performance. The most widely used microbial modulators are those called antimicrobial growth promoters (AGPs), which are antibiotics applied at a prophylactic level. For poultry, AGPs are generally used in combination with coccidiostats, which are a group of antibiotics targeted against coccidia parasites. It is worth noting that the drugs that belong to coccidiostats are also antibacterials inhibiting gastrointestinal bacteria.

In gastrointestinal communities bacteria do not live independently of the other species, but depend on each other and their host in a many ways and with complex interactions. Indeed, bacterial communities are metabolically versatile mixtures of different bacteria whose relative abundance is regulated by environmental factors such as substrate flows, antibacterial compounds, and the structure and function of the host epithelium. As entities, bacterial communities are energetically more efficient and metabolically more flexible than the representatives of any of the single bacterial species present. This is because bacterial species specialise on different functions and provide each other with cofactors and substrates, which are essential for their existence. Also, natural competition forces out inefficient bacteria that are replaced by more efficient ones. Following this general mechanism of bacterial community evolution, site and diet specific bacterial communities evolve in the gastrointestinal tract of different animals.

The mode of action of AGPs has not been confirmed, but they are assumed to be connected with antibacterial effects. It is possible that the feed conversion efficiency of healthy animals is improved due to the reduced number of bacteria in the small intestine where majority of the host nutrient absorption takes place. The reduced number of bacteria in the proximal intestine leads to enhanced partitioning of nutrients to the host. If the animals are burdened with high environmental levels of harmful bacteria and other stress factors, antibiotics may help to prevent the development of a clinical disease, which would lead to growth suppression and mortalities. Besides nutritional and health effects, AGPs can enhance meat quality by reducing bacteria that produce metabolites with adverse effects on the carcass quality but with no actual effect on the health of the host itself (e.g. skatole production in the colon of hogs).

The presence of antibiotics in feed has been one of the major selective factors affecting the composition of microflora in the gastrointestinal tract of animals. For decades, microbes tolerating antibiotics have been enriched for while microbes sensitive to antibiotics have been selected against. The normal intestinal microflora of production animals as we know it today is the antibiotic tolerant microflora, and indeed most of our knowledge and experience on animal management leans against this background. Also, most feed companies and animal breeders have developed their products for the conditions where growth promoting antibiotics are a natural part of the management practice. Removing antibiotics from feeds has changed the selection pressures prevailing in intestinal microbial communities from those favouring antibiotic resistant microbes to the advantage of the microbes growing efficiently on feed residues in the absence of antibiotics. In fact, it is likely that following the new selection criteria we will witness the outgrowth and establishment of unforeseeable bacterial species. The outcome will be unpredictable due to the fact that most bacteria in the gastrointestinal tract of production animals represent previously unknown species and

genera, and we are unaware of the health implications of most of them. Rational, knowledge-based microbial management is challenging in the present situation, and therefore the development of novel microbial management products is often based on feeding and weighing of the animals. Today, there are diagnostic tools available that can be used for the detection and quantification of all bacterial species, and therefore future research and epidemiological studies shall reveal the significance of the newly discovered bacterial species.

Regardless of the long and wide usage of growth promoters their mode of action remains to be unknown. For the rational development of AGP-replacing microbial modulators, we should first understand how AGPs change the structure and function of the total microbial community. Since AGPs seem to have lowered the frequency of enteric disorders, it is likely that the microbial community balance obtained during the AGP use has been favourable and could be used as a guideline when developing alternative microbial management solutions. In a comparative trial with broiler chickens we studied the effect of a coccidiostat (monensin) in combinations with an AGP (bacitracin methylene disalicylate, BMG) and a therapeutic antibiotic (phenoxy methyl penicillin) on the structure of the caecal microbial community. By using 16S rDNA sequencing for the analysis of the bacterial community composition we found that the antimicrobial compounds tested had significant effects on the composition and metabolism of the intestinal bacterial community of the chicken. The advantage of this method is that with the sequence information obtained, rapid and quantitative culture independent assays can be developed for all bacteria found, and the assays can be used for rapid screening of novel microbial modulators.

Rational development of novel microbial modulators requires (i) knowledge on the relevant members of the bacterial community to be screened for or against, (ii) rapid assays for quantitative analysis of such bacteria, and (iii) relevant (and practical) laboratory simulation tools, which adequately mimic microbial competition prevailing in the gastrointestinal tract. It is unrealistic to assume that any single simulation would accurately mimic all aspects of the gastrointestinal tract. Therefore, it is essential to have several targeted simulations, which collectively provide evaluation of product candidates as microbial community modulators. Relevant simulations include bacterial fermentations, and relative competitiveness of different bacteria in the proximal and distal intestine. The relative significance of the effects on proximal and distal intestine depends on the target of the microbial modulator to be developed. The two intestinal compartments support highly different microbial communities, which differ with regard to their requirements for nutritional and physicochemical conditions. Therefore, the simulation design for the screening of products aimed to be effective in the small intestine differs significantly from the simulation design intended for the caecum or colon. In addition to the simulation of bacterial growth, it is sometimes more rational to screen feed ingredients for their effect on bacterial adherence, an essential element for the colonisation and pathogenesis of some intestinal bacteria. An ideal feed ingredient inhibits the adherence of pathogens, but enhances the adherence of health promoting bacteria.

To serve their purpose simulation systems used for the product screening need to provide the information they are designed to provide. The data produced must be reproducible, and the resolution of the simulations and all assays used should be known. Indeed, the techniques available and under routine use differ significantly with regard to their resolution power. For example, when no significant difference can be found between the control and a test product (e.g. on bacterial numbers), it may either mean that the difference was less than 1000% (10-fold), but depending on the reproducibility of the method, it may also mean that the difference was less than 5%. With the adequate replication and the correctly chosen methods it is possible to achieve any desired degree of resolution. Small differences in the measured parameters may be of high significance due to the long usage times (weeks to months) of the products in their intended applications.

Overall, the questions related to the ban of growth promoting antibiotics, and their novel replacement strategies are of high relevance. Already, a considerable amount of money has been used to find new ways to cope without the AGPs but so far, with only moderate success. Consequently, it seems that a systematic approach and a combination of the newest techniques are required for the successful development of novel management strategies and for answering questions on the safety, mode of action and efficacy of the different strategies.

# Use of dynamic in vitro model of the gastrointestinal tract (TIM) in studying replacements for AGPs

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The development of alternatives for antimicrobial growth promoters is no simple task. Besides safety requirements and sensory properties, significant health benefits are essential. With the growing insights in physiological and molecular mechanisms linking diet to health benefits, a range of laboratory methods is becoming available for supporting health claims. In feed products, health benefits are usually not related to the presence of one or two specific compounds, but to a wide range of components and to product structure. In such complex matrices the use of validated and accurate laboratory models can contribute to a cost- and time- effective development of new products. In this paper we will give an outline of methods for assessment of the impact of health related compounds.

## Dynamic gastrointestinal systems

At TNO, a multicompartmental, dynamic, computer-controlled models (TIM) have been developed, which closely simulates the *in vivo* conditions in the stomach and small intestine (TIM-1; Figure 1) and in the large intestine (TIM-2; Figure 2). These models simulate the peristaltic movements in the gastrointestinal (GI) tract, mixing and moving the contents gradually through the stomach and intestine of humans, pigs and calves and dogs. This allows to mimic the exact gastric emptying and intestinal transit times as occur in real live situations after the intake of different types of foods, such as bread, milk with cereals and pasta products.



Figure 1. TNO gastro-Intestinal Model simulating the stomach and small intestine (TIM-1).

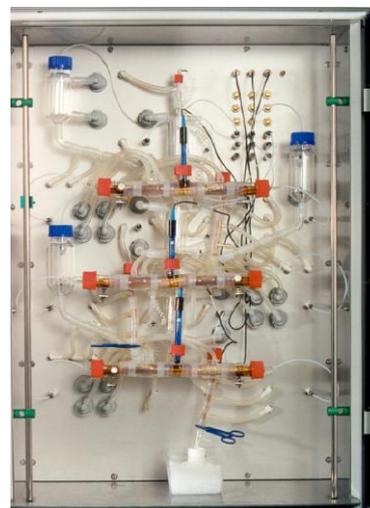


Figure 2. TNO gastro-Intestinal Model simulating the large intestine (TIM-2).

Saliva, gastric juice, bile, and pancreatic juice are 'secreted' into the corresponding compartments using computer-controlled pumps. The large intestinal model contains a complex, metabolic active microflora of human or animal origin. Fermentation and bioconversion/bioactivation of undigested compounds take place in the same rate as in

humans or animals (e.g. pigs, dogs). TIM has unique dynamic features: the successive conditions in the stomach and intestinal compartments are closely simulated. The strength is that experiments can be performed under strictly controlled and specified conditions, resulting in reproducible simulations of specific GI conditions and the opportunity to take samples in time from different sites. To ensure that the results obtained in TIM are relevant for the situation in target animals, extensive validation and application studies have been performed.

### **Digestion and availability for absorption**

Important factors in relation to the digestibility of food products and the availability for absorption of nutrients and/or bioactive compounds are the successive dynamic conditions in the gastrointestinal tract, such as pH in combination digestive enzymes and secretion of bile for the formation of (mixed) micelles of fatty acids and fat soluble nutrients. Because these digestive processes are accurately simulated in the TIM systems, it is an excellent method to study the stability, release, digestibility, absorption and/or bioconversion of nutrients and bioactive compounds. Validation experiments in comparison to human and animal studies showed the high predictive quality of the system for proteins, minerals, vitamins as well as for bioactive food compounds, the formation of toxic compounds or the binding of mycotoxins. In combination with cultured cell lines (e.g. Caco-2 cells) or intestinal segments, also the passive and active mucosal transport and cell metabolism can be studied. The combination of these laboratory methods offers the possibility to put the obtained data in computer-simulation programmes (physiological based kinetic modelling) to predict short- and long-term plasma levels of specific nutrients, such as demonstrated for folate.

### **Probiotics and organic acids**

The selection of functional microbial strains (probiotics) should be based on scientific criteria, which are associated with the specific health-claims of the products. On the other hand probiotics are only active in the host if they fulfil a number of general criteria, for example related to the survival and metabolic activity of the micro-organism(s) during passage through the gastrointestinal tract. The resistance of probiotics against the successive stress conditions in the gastrointestinal tract (enzymes, low pH, bile) is an essential selection criterion. The fate of ingested bacteria in the TIM system was compared to that in humans with the same products and bacterial strains. The data on survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in the model fit in closely (ANOVA:  $P = 0.88$ ) with those obtained with these strains in healthy volunteers using an intubation technique. The data on survival of *L. bulgaricus* in the duodenum and ileum were also consistent with literature data of other human studies. Studies in the dog GI model (FIDO) showed similar results. This means that the dynamic model has a high predictive value for the kinetics of ingested lactic acid bacteria in humans and animals.

The model can be used to assess the successive influences of for example gastric acid and bile concentrations on the fate of functional bacterial strains, the interaction of probiotics and pathogens, and the influences of specific feed ingredients (e.g. organic acids) on the survival of potential beneficial or pathogenic micro-organisms (report).

### **Prebiotics and dietary fibres**

In a recent overview of research with the TIM system on dietary fibres methods and results have been described among others in the field of (i) degree of digestibility in the small intestine, (ii) binding of cholesterol and fat, (iii) degree of fermentability in the large intestine, and (iv) energy value.

Digestibility of dietary fibres is determined by the amount monosaccharides available for

uptake by the body. Analysis of the dialysate fractions from the intestinal compartments allows calculations of the kinetics and amounts of bioaccessible monosaccharides. Collection and analysis of ileum effluent allow determination of the indigestible fraction that arrives in the large intestine.

Fermentation of carbohydrates by the intestinal microbiota in TIM-2 leads to the production of metabolites, e.g. short chain fatty acids (SCFAs), lactic acid, CO<sub>2</sub>, H<sub>2</sub> and CH<sub>4</sub>. The organic acids are taken up by the epithelial cells of the colon and metabolised. Butyric acid (butyrate) is metabolised as energy source by the colonocytes. Taking the data from the digestibility in TIM-1 and the fermentability in TIM-2, the energy value of carbohydrates can be calculated.

### **Impact on health of the activity of the large-intestinal microbiota**

Diet is one of the factors that influences the composition and activity of the microbiota. Carbohydrate fermentation and its production of SCFAs are considered to be beneficial for health. Protein fermentation on the other hand leads to potentially toxic fermentative metabolites such as ammonia, phenolic compounds, and sulphur containing compounds. Over the years several dietary components have been tested in TIM-2 for their potential to produce butyrate and/or to increase the balance between health-promoting and toxic microorganisms and/or metabolites. For instance, high amylase resistant starches and D-tagatose have been shown to lead to ratios of 50-60% butyrate (manuscripts in preparation). In addition, inulin has been shown in TIM-2 to shift the balance of health promoting over toxic metabolites towards a healthier colon. The model shows to be a better tool to mechanistically study the production of metabolites than experiments with animals or humans. The reason for this is that in TIM-2 all metabolites are being collected, whereas in the body the metabolites are being used (e.g. butyrate by the epithelial cells) and therefore lost for analysis. So, data from faecal samples do not at all reflect the metabolic activity in the proximal colon, whereas TIM-2 allows sampling at the site of fermentation.

### **Conclusion**

With the growing insights in physiological and molecular mechanisms, linking diet and ingredients to health benefits and the rapid development of analytical tools in combination with validated laboratory models of the GI tract, a range of methods has become available for the development and optimisation of feed compounds as alternative to AGPs.

### **References**

A list of references in relation to the presented items is available from the authors: havenaar@voeding.tno.nl

# Nutritional solutions beyond AGPs

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The restrictions on the use of prophylactic antibiotic growth promoters (AGPs) in animal feed were introduced in the European Union in 1999. By 2006 all growth promoters will be banned within the EU. In the global market environment, the ban does not affect only EU poultry producers but also countries exporting poultry products to the EU. Furthermore, the growing focus on the production of 'natural' as well as 'organic' poultry in many markets outside the EU provide the poultry producer with opportunities to exploit these higher value segments. The removal of AGPs tends to increase the costs of poultry production, as AGP removal from feed typically reduces weight gain and feed conversion and is responsible for increasing mortality. The incidence of poor litter quality may also increase, increasing the risk of carcass downgrades and microbial contamination with food poisoning bacteria such as *Salmonella* and *Campylobacter*. The procedures published in 2003 regarding the use of some ionophorous anticoccidials in the EU suggest that the legislative changes affecting poultry feed and production will not stop with the AGP ban and all within the poultry sector will need to adapt in order to at least maintain profitability of poultry production. Different strategies, combining nutrition and management, have been implemented to cope with the reported negative side effects of in-feed AGP withdrawal. The situation also fuelled an interest in research to evaluate novel approaches of poultry feeding and production. Commercial and scientific evidence suggests that some commonly used feed additives such as exogenous enzymes and betaine combined with changes in management may offer considerable improvements in production economics. However other ways such as vaccination, the use of microbial cultures, prebiotics, organic acids, plant products, feed ingredients, fat sources and feed technology are also being considered. The ultimate goal for the poultry producer is to adopt cost-effective consumer friendly strategies which suppress the proliferation of the pathogenic bacteria previously controlled by AGPs. One option to achieve this goal is to minimise the nutrients available for the growth of disease-causing bacteria in the gut of the bird, whilst maximising the nutrients available to the bird to achieve profitable growth.

## **The benefits of AGPs**

The mode of action of AGPs is not fully understood considering their long history of use in animal feed. It is believed that the main effects are mediated via the bacterial gut flora [1] by selectively targeting and destroying undesirable intestinal bacteria. The long-term benefit of AGPs has been seen in terms of improved and more uniform bird growth and reduced incidence of bacterially induced gastrointestinal tract diseases. However, the magnitude of the response to AGPs depends on a number of parameters including farm management, exposure to pathogens, environmental stresses and diet type. Rosen [2], and Thomke and Elwinger [3] reviewed a large number of studies to summarise the effect of these factors on the efficacy of AGPs. They suggested that, on average, AGPs improve FCR by approximately 3% (4-6 points). Clearly an average 3% reduction in feed conversion as a consequence of AGP removal negatively impacts the profitability of poultry production and additional financial losses will be incurred as a result of increased mortality, performance variability, increased contamination and condemnation of carcasses in the processing plant.

## **Nutrition and the intestinal microflora**

Ultimately, both the transient and resident bacteria in the intestine rely on nutrients that are

ingested by the bird for their metabolism and growth. Wagner and Thomas [4] found that the type of ingredients included in a diet can affect microbial populations present in the gastrointestinal tract. Other factors such as gastrointestinal tract pH, the rate of feed passage, feed particle size, gizzard grinding action, presence of exogenous and endogenous enzymes, high oxygen tension, and endogenous antimicrobial compounds such as bile salts help limit microbial proliferation in the small intestine.

When digestion and absorption are optimal, there is a limited amount of easily fermentable substrate (e.g. starch, protein) available for rapid digestion by the microflora in the distal GI tract. Conversely, reduced nutrient digestibility by the bird typically occurs when feeding less digestible feed ingredients [5] or in a situation of high endogenous losses, for example in the presence of gut lesions caused by coccidiosis or other enteric disease challenges. The unabsorbed nutrients provide easily fermentable substrate for microbes and lead to their proliferation in different parts of the gastrointestinal tract.

*Campylobacter jejuni* and most types of *Salmonella* infections are of specific importance, as they constitute a health risk to consumers of poultry products. Other bacteria cause concern because they induce intestinal disease in the birds, reducing profitability by causing sub-clinical infections, which reduce bird growth and feed conversion. Among the most gut-specific pathogens *Clostridium perfringens* is assumed to represent the main health problem associated with the ban on the use of in-feed antibiotics as growth promoters.

### **Nutritional solutions beyond AGPs – feed the bird not the bug**

In general, it has been found that a combination of different approaches is required in order to compensate for the performance and economic losses associated with AGP withdrawal. Use of high cost, highly digestible feed ingredients limit the 'by-pass' of nutrients from the bird to the microflora. However, this expensive approach may not be the most economic option. Another nutritional approach is to use feed additives that are effective in improving nutrient digestibility of the diet, increasing nutrient absorption by the bird, thereby reducing nutrients for the growth of the microflora.

Feed enzymes have been used extensively for approximately 15 years to improve nutrient digestibility, bird performance and body weight uniformity in poultry fed wheat/barley-based diets. More recently, feed enzymes have also shown value in improving performance in birds fed corn/sorghum/soy-based diets. A number of authors [6-8] have reported that feed enzymes could play a role in influencing gut microbial populations in broilers fed wheat-based diets. Choct et al. [9] noted that the effects of certain anti-nutritional factors, such as soluble non-starch polysaccharides, could contribute to both the levels and types of in gut microflora. The relationship between the use of enzymes in corn-based diets and their indirect effect on microbial proliferation has been also found [10,11].

Other feed additives can impact bird performance and nutrient digestibility through indirect mechanisms. The removal of AGPs often increases the incidence of enteric diseases such as necrotic enteritis (NE). Elwinger et al. [12] suggested that NE often follows coccidia challenge. Feed additives such as betaine that can help reduce lesion damage associated with small intestinal coccidia also result in improvements in bird performance and nutrient digestibility [13]. It can be speculated therefore that improvements in intestinal integrity result in better nutrient digestibility. Betaine's ability to improve intestinal integrity and nutrient absorption during coccidia challenge, indirectly reduces nutrient supply to the gastrointestinal microflora. In feed formulation, betaine can be used to replace some added methionine and replace all added choline to reduce feed costs. Other approaches such as feeding undefined and defined (probiotics) microbial cultures or products that supply nutrients for beneficial microflora (prebiotics) or products

such as organic acids, phytochemicals, feed ingredients, fat sources, feed technology and management are being considered by commercial industry.

## Conclusions

The feed and animal production industry is facing a number of challenges, not the least of which are the pressures to produce high quality products to satisfy customer needs in a cost effective manner. The industry is facing a future without the benefit of some biologically and economically effective feed additives such as AGPs (and potentially ionophorous anticoccidials). Feeding poultry AGP-free feeds requires further research and commercial evaluation. Nevertheless, it is clear that the causes of microbial proliferation, especially of pathogenic bacteria, must be reduced either via improved management practices or via currently available nutritional means. As examples, feed enzymes and betaine can contribute significantly to the replacement strategy of AGPs, by altering the nutrient supply to intestinal microflora and by improving intestinal integrity of the bird. Furthermore, these two ingredients can be used cost-effectively as they both reduce feed costs and improve broiler growth and carcass quality (e.g. breast yield) in AGP free diets.

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# The likely benefits of feed enzymes in AGP free diets

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## AGP usage and benefits

Antibiotic growth promoters (AGP) were used routinely in Europe in the past and are still employed in the majority of the rest of the world as a means to improve the performance of animals whilst at the same time to reduce the incidence and severity of disease. Such effects are likely mediated through changes in the enteric flora which subsequently influence a multitude of factors including digestive enzyme output, gut motility, the quantity and quality of mucin secretion, rate of loss of enterocytes and alterations in the enteric and systemic immune system status and capability to name a few. With so many biological activities influenced directly or indirectly through the use of AGP, it is not surprising that the responses noted in the literature are varied (greatest effects when reactive variables are all at their most responsive, and least when they are all optimal, e.g. germ free). The net result on use of an AGP as far as commercial animal production is concerned is an improvement in the nutritive value of the ration, the extent of improvement observed being very much greater when performance in the absence of AGP is poor. As a result, the term “pronutrient” has been coined to describe their mode of action, since they clearly work to improve the availability and/or utilisation of the nutrient(s) limiting growth, whether it be via improved diet digestibility, reduced endogenous losses, or diminished energy losses via heat or gas. It therefore appears rational to consider that the benefits delivered on use of an AGP may also be derived through alternative mechanisms, and provided all the activities of the AGP are duplicated, then it should be possible to replace them without loss in performance.

However, despite such detailed understanding of the effects of antibiotics on the animal, it is still unclear which micro-organism(s) are the key growth depressing agents, and moreover which of these are controlled on the use of a specific AGP. Such limitations in knowledge are a consequence of the problems involved in accurately mapping the entire microbial community, problems which have only recently been addressed. As a result, until now, it has not been possible to identify the structure of the resident microbial population associated with poor performance and susceptibility to disease states, and conversely, those associated with high rates of growth. Recent publications suggest that classical culture techniques were effective in identifying less than 10% of the inhabitants of the intestinal tract, so it is not surprising that our understanding of exactly how AGPs functioned through changing microbial population structures is limited. Consequently, the implications of their removal were also not clear. It follows, therefore, that it is very difficult to determine whether an “alternative” is in fact capable of replacing an antibiotic and if so, how consistent such a product will be. There clearly are many conditions which influence the response obtained on use of an AGP [1], conditions which must be investigated in multiple studies if there is to be a real test as to whether a robust, “all weather” alternative to AGP has indeed been presented. The literature on feed enzymes is probably the most comprehensive of all additives purported to (partially) replace AGP, and have recently been reviewed empirically, allowing some test of this theory.

## Feed enzyme usage and benefits

Feed enzymes have been developed over the past 15 years to improve the nutritive value of diets based, principally through targeting the cell walls of cereal grains or phytate phosphorus. Much has been published relating to their modes of action, with particular reference to that of the cell wall degrading enzymes, xylanases and cellulases respectively.

Whilst much of the research on the cell wall degrading enzymes has focused on whether they target insoluble or soluble fractions (the cell wall and viscosity hypothesis respectively), it is becoming increasingly apparent that the response obtained is dependent to some extent (perhaps more than is given credit) upon the participation of the resident intestinal microflora. The focal point for much of the research to date has been on the effects such enzymes have on improving diet digestibility, with little regard for the effect such an event would have on restriction of supply of nutrients to the lower small and large intestinal microfloral populations. In contrast to this effect of microfloral “nutrient restriction”, degradation of cereal cell walls produces oligomeric carbohydrates which clearly provide substrate for select bacterial populations, and as a result there is a considerable net change in fermentable substrates presented in the intestinal lumen. These effects clearly influence the structure and size of the intestinal microfloral population. Thus it has been postulated that there is some overlap in the mechanisms by which AGP and enzymes operate and hence their interaction will not necessarily be synergistic. Multifactorial analysis of the data present in the literature in which both AGP and enzymes were used in a full factorial manner suggests that this may be the case [2]. Whilst both enzymes and AGP gave largely similar responses, their interaction was subadditive, suggesting that there may be some overlap in the mechanisms through which these products elicit their responses.

Whereas the effects of cell wall degrading enzymes on the intestinal microfloral status of the monogastric are documented, little such data exists for phytase. As a result it may be concluded that the benefits of phytase in AGP free diets are limited to supply of additional nutrients for growth in a “stressed” animal. However, recent evidence suggests that phytate may exert antinutritional effects at the level of the intestinal tract, manifest by increased endogenous losses, which are reduced on supplementation with phytase. Such an effect is unlikely to take place without influencing the microbial populations in the intestines, hence it remains to be seen whether phytase has a larger role to play than previously identified.

### **Commercial experience**

Notwithstanding the fact that the scale of animal performance response on use of enzymes and AGP is similar, suggesting performance can be restored on the removal of AGP by the use of an enzyme, it is clear from commercial experience that the removal of AGP did result in reduced performance. In practice, when AGPs were removed, those feed compounders that could use an enzyme (i.e., were feeding responsive diets) were mostly already doing so. Thus the performance loss could not be mitigated by their introduction. Moreover, despite the fact that both product types influence the intestinal microflora, it is clear that the response to enzymes is largely diet dependant (i.e., the target substrate needs to be present) and thus there will not be a response if the diet does not contain the target grain or substrate. AGPs were not necessarily so diet dependent. Additionally, the removal of AGP has precipitated novel conditions such as dysbacteriosis, which are not addressed though administration of an enzyme. As a result, and as suggested above, it is unlikely that enzymes, or any other non-antimicrobial additive for that matter, will be able to replace all the activities of an AGP, rather they may be able to substitute for some. Whilst enzymes clearly are of great value in AGP free diets, it is not realistic to expect that they can replace AGPs, and as a result further alternatives need to be found.

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# Interfacing gut health and nutrition: the use of pre- and probiotics to maximise growth performance

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The reduction in the use of antibiotic growth promoters (AGPs) has led to increased focus on natural growth promoters. Prebiotics and probiotics are familiar supplements in a wide range of diets for farm animals and pets. It has been widely recognised that these additives could have a significant impact on animal growth performance and health. Following the definition of prebiotics by Gibson and Roberfroid [1], prebiotics are short chain carbohydrates that neither are hydrolysed nor absorbed by the host, and therefore are available to the beneficial microflora in the intestine. In contrast, the concept of probiotics is based on direct supplementation of live cultures of these beneficial bacteria. Following the Rosen's argument [2], both terms are unsatisfactory, in that they fail to capture the true nature and effects on the intestinal microbiota. Fundamentally both supplements have the same mode of action: the manipulation of the indigenous microflora in order to reduce the risk of proliferation of potential pathogens and increases resistance to infection. Characteristics and postulated beneficial effects of ideal prebiotics and probiotics have been summarised by Simmering and Blaut [3]. A number of known microorganisms (predominantly the bacterial species *Enterococcus*, *Bacillus*, *Bifidobacterium* and *Lactobacillus*) as well as a smaller number of unidentified microbial cultures (CE) are currently used as probiotics. The dominant prebiotics in use are mannanoligosaccharides (MOS) and fructo-oligosaccharides (FOS, including inulin). All these products focus on improving intestinal health. However, despite the fact that these supplements are widely used in animal feed, the exact mode of action that leads to their beneficial effects in the intestine of farm animals is only partly known.

## Interfacing gut health, immunity and nutrition

The impact of live microbial cultures on intestinal health for human nutrition has been widely discussed. Mechanisms which prevent the colonisation and growth of enteric pathogens including *Salmonella*, *Escherichia coli*, *Campylobacter* or *Clostridium* species using microbial cells include enforcement of the physical barrier (modulation of paracellular permeability, mucosal trophic action) and the functional barrier (mucosal immunity) (summarised in Fioramonti et al. [4]). Although these mechanisms also apply to farm animals the key for optimal growth performance lies in the interaction between supplemented cultures and the indigenous microflora of the host animal such as competitive adhesion to epithelial receptors, reduction of intestinal pH or modification of bile salts [5]. Colonisation resistance and competitive exclusion is often associated with the number of beneficial bacteria in the intestine. A recent study by Smirnov et al. [6] showed that the adding of a commercial microbial culture significantly increased the proportion of lactobacillus species in the ileum of broiler chickens. Koenen et al. [7] showed that feeding fermented liquid feed fermented with *Lactobacillus plantarum* and *L. paracasei* can increase lactobacilli in the GI tract. On the other hand Jin et al. [8] reported that adding a mixture of 12 different strains of Lactobacilli (species *L. acidophilus*, *L. fermentum*, *L. crispatus*, and *L. brevis*), had no effect on lactobacilli in the small intestine of broiler chickens. Gardiner et al. [9] showed that oral administration of porcine derived *Lactobacillus* strain to pigs resulted only in a small effect increase in caecal lactobacillus counts. However, despite the apparent lack of increase of beneficial bacteria the same study also found a significant reduction in pathogenic indicator species (*Enterobacteriaceae*) in both faecal and caecal samples. Attempts to evaluate the potential changes in the intestinal microflora through the addition of microbial cultures depends on the actual survival rate of the culture in the intestinal tract as well as identifying

actual changes in the intestine. Using genetic fingerprinting technologies, it has been identified that some *Lactobacillus* strains administered to pigs to some extent become established in the intestine [9].

Carbohydrates such as fructo-oligosaccharides (FOS), oligo-fructose (OF), transgalacto-oligosaccharides (TOS) or inulin can selectively stimulate the growth of beneficial microorganisms in the intestine of lab animals [10,11]. Since pathogens like *E. coli* or *CP* are unable to use these carbohydrates as an energy source, the number of FOS, OF or inulin fermenters will increase. However, it has now been reported that bacterial strains belonging to *Enterobacteria* are also able to use inulin as an energy source [12]. However using 16s RNA techniques the same study also showed an increase in bifidobacteria rRNA in the small intestine. In contrast to these types of carbohydrates, mannan oligosaccharides derived from the outer cell wall of a specific strain of *Saccharomyces cerevisiae* have a direct impact on unwanted enteric bacteria by blocking the type-1 fimbriae which enables pathogens such as *Salmonella* ssp. or *E. coli* to attach to the intestinal lining [13].

Live microbial cultures or oligo- and polysaccharides also play a key role in the development of the gut-associated immune system (GALT). For example, feeding *L. paracasei* to meat-type birds increased phagocytic and bacterial activity of cells in the ileum and caecum, however no such response was found when feeding the same strain to layer-type birds. Differences in the innate immune system of meat and layer-type birds could explain these results [7]. Adding MOS to diets for turkeys can increase plasma IgG and bile IgA [14] or can alternate the leukocyte populations in piglets [15] or they can increase IgA titers in sow milk [16]. Distinct carbohydrate structures can have very specific biological activities. For example, sugars (monosaccharides) combine to form giant molecules such as cellulose; they are already known to regulate hormones, organise embryonic development, direct the movement of cells and proteins throughout the body, and regulate the immune system [17]. Understanding the structure and sequence of individual monosaccharides that form oligo-or polysaccharides is the basis for developing new carbohydrate based immunomodulators. Research suggests that we can influence some of the control mechanisms of the immune system through selected dietary carbohydrates as the digestive tract offers a large surface for carbohydrates to interact with intestinal cells and the immune system as well as with bacterial cells.

## Effectiveness

Regardless of the actual mode of action and the specific impact on the gastrointestinal microflora and GALT, the effectiveness of most feed supplements is measured in terms of improvement in growth performance. The response of these types of feed additives can vary widely because effects will depend on the interactions between microbial communities in the gastrointestinal tract as well as interactions between the microbial community of the animal and the environment. The effectiveness of a limited number of trials (22 feeding trials from 7 publications) with cultures of *E. faecium*, *Bacillus cereus* and *S. cerevisiae* on fattening broilers and turkeys have been summarised by Simon and Jadamus [18]. Trends towards improved weight gain and feed conversion ratio are in the range of 1-2%. Examples of possible benefits in growth performance in pigs fed with *Bacillus licheniformis* are listed in [19]. To the best knowledge of the author, comprehensive evaluation on the effectiveness of commercially available additives in the category pre-or probiotics is limited to one product only. Effectiveness of the addition of mannan oligosaccharides (Bio-Mos™, Alltech) to broiler, turkey, pig and rabbit diets have been evaluated in five individual meta-analyses and have recently been published in scientific journals and trade magazines. The individual data sets are based on published and unpublished data and include a total of 55 comparisons in nursery pigs [20], 44 comparisons in broilers (pen studies only) [21], 15 comparison in broilers (field studies) [22], 27 comparisons in turkeys [23] and 20 comparisons in rabbits [24]. These reports clearly show a significant improvement in life weight gain (depending on

species between 2-4%) and a reduction in FCR (between 2-5%) when Bio-Mos is added to the diet.

Future research might add to existing knowledge on the effectiveness of these supplements as well as improving the understanding of their mode of action. The challenge for producers however is to find suitable, reliable and most importantly cost effective feed supplements for a sustainable and successful animal production in the future.

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# Acidification of diets as an alternative

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In view of growing evidence of bacterial resistance to antibiotics, the European Union (EU) has decided to ban the use of antibiotics as animal feed additives, effective 2006. As a first step, in December 1998, four products – virginiamycin, spiramycin, tylosin phosphate, and bacitracin-zinc – were banned. Therefore animal producers are looking for alternatives to antibiotic growth promoters that offer comparable effects while not creating bacterial resistances. Organic acids offer the best potential as alternative for pigs and may also improve the production of poultry. Growth promoters optimise digestive processes; they decrease the number of undesirable microorganisms in the digestive tract and reduce nutrient losses during digestion. Increased digestibility means more nutrients are available for absorption and retention. The mode of action of organic acids is comparable, because they are also active against microorganisms in the feed and intestine and they are able to reduce nutrient losses during digestion. Organic acids have been used for decades in feed preservation, protecting the feed from microbial and fungal destruction. Propionic acid, in particular, plays an important role in the inhibition of mould growth during storage and, consequently, prevents the formation of mycotoxins [1].

## Mode of action of organic acids

The mode of action of organic acids takes place in three different areas: in the feed, in the intestinal tract and in the metabolism. Feed, even under favourable conditions always contains microorganisms. The application of organic acid depresses the metabolic activity of susceptible germs and therefore reduces the microbial load of feed. The antibacterial activity of organic acids is related to the reduction of pH, as well as their ability to dissociate. Furthermore, the antibacterial activity increases with decreasing pH-value. Organic acids are lipid soluble in the non-dissociated form, in which they are able to enter the microbial cell. Once in the cell, the acid releases the proton in the more alkaline environment, resulting in a decrease of intracellular pH. This influences microbial metabolism, inhibiting the action of important microbial enzymes, and forces the bacterial cell to use energy to release protons, leading to an intracellular accumulation of acid anions. While lactic acid bacteria are able to grow at relatively low pH levels, the growth of other bacterial species, like *Escherichia coli* and *Salmonella* is impaired. In the animal, dietary acidification also increases gastric proteolysis and protein digestibility. The acid anion has been shown to complex with Ca, P, Mg and Zn, which results in an improved digestibility of these minerals. The acid anion plays an important role regarding the antibacterial effect of organic acids and their salts. Several investigations have shown a strong bactericidal effect of organic acid without significantly decreasing the pH-value in the gastro-intestinal tract. In addition, organic acids serve as substrates in the intermediary metabolism [2]. Most organic acids contain a significant energy content. This quantity of energy is completely metabolised and can thus also be taken into account in the calculation of the energy content of the complete diet. As an example, propionic acid contains 1.5 times more energy than wheat.

## Acidification of diets for pigs

Thirty years ago, first results were published which proved that organic acids are able to positively influence animal performance when added to diets for pigs [3,4]. During the past fifteen years, intensive research has been conducted on the nutritive effectiveness of organic acids in pigs confirming their positive influence on growth performance and feed conversion

[5]. In this aspect, formic acid has gained special attention [6,7]. As reported by Gabert and Sauer [8], the nutritive effect of organic acids is most pronounced in weaning pigs. Weaning of piglets is often associated with growth depression and diarrhoea following low feed and water intake [9]. Moreover, the gastrointestinal tract is not fully developed and thus the digestive capacity of piglets is limited. These limitations also include the incapability of maintaining a low pH in the stomach, which decreases the breakdown of dietary proteins and supports the proliferation of enterotoxic bacteria. As a consequence, the incidence of diarrhoea increases. A more rapid reduction in the pH of the stomach stimulates the secretion of pepsin and pepsinogen, which may enhance dietary protein digestion. Lower pH conditions have a direct inhibitory effect on the bacteria population in the lumen [10].

### **Effect of the acid anion**

Kirchgessner and Roth [11] have studied the effect of acid anions using formic acid (1.2%) and sodium formate (1.8%) in feed for weaner piglets. Improvements in weight gain and feed conversion upon sodium formate supplementation were about half of the improvements of the formic acid treatment. This difference can be attributed to the effect of formate, while formic acid provides a stronger effect due to the additional acidification. Results obtained by Kirchgessner et al. [12] indicate, that calcium formate reduces microorganisms of the accompanying flora (*E. coli*, *Enterococci*) in the duodenum of piglets, almost as effectively with formic acid when applied in a formate-equivalent dosage. Further possible actions of organic acids and their salts in the small intestine can be concluded from in vitro studies conducted by [13]. In these experiments, increasing amounts of a mixture of 1/1 propionic and formic acid were added to a grower feed and the adherence potential of *E. coli* to the gut wall was investigated. With increasing dosage of the acid mixture, the ability of *E. coli* to adhere decreased. The risk of diarrhoea caused by *E. coli* is closely related to their adherence to the gut wall. Only after binding to the gut wall these microorganisms produce and liberate their enterotoxins. Based on the relatively high pH conditions in the small intestine it can be concluded, that this effect of organic acids could be related to the bactericidal and bacteriostatic properties of the anions of the acids. Performance improvements in by organic acids are also possible in fattening pigs, however, the effects are smaller than in piglets. Nevertheless, Baustad [14], using 0.6% formic acid in fattening pigs, reported improvements in daily weight gain of 8.2 and feed conversion of 8.2%.

### **Combinations of acid and salt**

The nutritive efficacy of organic acids is stronger compared to their salts. When salts are applied, only the anion of the acid can be effective in the small intestine, the acidification or the decrease of the acid binding capacity in the feed or stomach contents is not included. Potassium-diformate (trade name: Formi<sup>®</sup>), a specifically produced salt of formic acid (HCOOH·HCOOK), combines the chemical properties of acid and salt. In contrast to formic acid, Formi is non-odorous and non-corrosive. Formi is the first approved alternative to feed antibiotics in the EU\*. A large number of trials have confirmed the positive effect of Formi on growth performance in pigs. Statistically significant effects are already obtained upon the addition of 0.6% Formi [15]. Increasing amounts of Formi in the diet result in continuous positive effects on weight gain and feed conversion [16]. On average, the addition of 1.2% Formi to the feed, improves weight gain by 12 and feed conversion ratio by 5.4%. There is, however, considerable variation between trials, which might be explained by differences in feeding and housing regime, as well as different hygiene conditions. Overland et al. [15] and Roth et al. [17] demonstrated a dose related response of Formi on growth performance and feed conversion also for growing-finishing pigs. In comparison to piglets the effects are smaller in growing-finishing pigs, because older animals have a more mature digestive

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\* Commission Regulation (EC) No. 1334/2001 of 2 July 2001 and Commission Regulation (EC) No. 676/2003 of 14 April 2003.

system. As shown by Danielsen [18] and Daza et al. [19], Formi gives a comparable growth performance as feed antibiotics. The study of Daza et al. [19] was carried out under practical conditions with 252 weaned piglets. Diets were supplemented with either 1.2% Formi or 40 ppm avilamycin and compared to the negative control without growth promoting additives. Formi improved growth rate of piglets by 8.6% and feed efficiency by 7.3%. No significant differences between avilamycin and Formi were found. Formi also had a positive influence on the health status of piglets similar to the antibiotic. In a kinetic study with pigs, Mroz et al. [20] showed that 85% of the formate in Formi appears in the duodenum. Thus, significant amounts are present to exert antimicrobial effects in the small intestine. Formi also reduced pH in the stomach and duodenum. Supplementation of 0.9% Formi significantly reduced the pH of duodenal digesta by on average 0.4 pH units from 5 to 65 minutes after feeding. The antimicrobial effect of Formi is stronger against potential pathogens like coliform bacteria than towards the desirable bacteria such as lactic acid bacteria. This leads to a shift in the composition of microbes to a more balanced microflora in the gut, which also improves the general health status of the pigs.

### **Organic acids and phytase act synergistically**

The combined use of organic acids and microbial phytase has shown synergistic effects on animal performance [21,22]. Addition of phytase allows to reduce P- and Ca-levels in the diet, which lowers the acid binding capacity in the feed and therefore supports the acidifying potential of acids.

### **Organic acids in poultry**

Although organic acids have not gained as much attention in poultry as in pigs, positive results against *Campylobacter*, *Salmonella* and *E. coli* were also reported for poultry [23,24]. The effects on animal performance following dietary supplementation of organic acids in poultry are less consistent than in pigs [25]. However, positive influences on either feed conversion ratio or growth performance have been reported by Vogt et al. [26]. Similar observations were made by Selle et al. [27] using Formi in broiler diets.

### **Conclusions**

Organic acids can accomplish much more in pig nutrition than just optimising the feed quality. They lower the risk of infections with pathogenic bacteria like *E. coli* and *Salmonella* and add to better digestion and utilisation of nutrients. As the first approved alternative to feed antibiotics, Formi is an efficient and safe-to-use product for pig production. In poultry, organic acids help to control undesired microorganisms to prevent infections with *Salmonella* or *Campylobacter*. However, performance-enhancing effects in poultry are not consistent and require further research. In pig production, there is evidence, that a combined use of organic acids and microbial phytase has synergistic effects on animal performance.

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# **Are herbs, botanicals and other related substances adequate replacers of AGPs?**

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Twenty years from now there will be almost 9 billion inhabitants on earth who expect to get enough food to meet their nutritional needs. This goal can only be archived in future if the world food production increases by about two percent per year. It is expected that world animal production will follow this trend. World food production must grow without increasing the environmental waste load. This precondition demands the efficient use of all available resources both traditional and modern technologies also of feed additives in a responsible way.

Especially in highly developed countries concerns about food safety and quality get predominant. The ban of antibiotics as feed additives in animal nutrition is therefore in discussion or already implemented. The search for alternatives opens a new field for new strategies to improve the health situation of the animals. The use of herbs, botanicals or other related substances is therefore of central interest.

By definition herbs are non-wooden flowering plants that have special properties or taste. A compound that is produced by parts of plants is called a "botanical". Botanicals and within the botanicals essential oils as extracts after hydro-distillation have special properties in relation to taste or pharmaceuticals are of central interest in animal nutrition. In the foreground are the activities on feed intake, antimicrobial and antioxidative properties. In many Asian and South American countries such drugs were used over centuries. More and more such experiences are utilised also in modern animal production. The main reason for that development is that consumers trust more herbs or botanicals as feed additives compared to products coming from industry.

In addition to their effect on feed intake herbs and botanicals often increase the secretion of digestive fluids and improve the immune system of animals. Besides improved health aspects, a better nutrient digestibility, reduced frequency of digestive disorders and also increased performance of animals are the consequences. The antimicrobial, coccidiostatic as well as antiviral effect of herbs and their preparations have been studied by many authors. The variation of these results is wide, which can be explained by the differences in the composition of such products. Furthermore, results from in vitro studies must be carefully compared with those from practical experiments. The antioxidative effect can improve the health status of the animals but is more important in the sense of the improved product quality.

## **Bacteriophage: a safe and natural alternative to antimicrobial growth promoters**

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Bacteriophage are viruses that infect and kill bacteria. Bacteriophage do not infect either animal or plant cells making them a potentially safe alternative to antibiotics to both prevent and treat bacterial diseases in animals and humans. Bacteriophage lytic to a sero-type O2 strain of *Escherichia coli* were isolated from municipal wastewater treatment plants and poultry processing plants. This *E. coli* isolate is pathogenic to poultry, causing a severe respiratory and systemic infection. Two bacteriophage isolates were selected to use in studies designed to determine the efficacy of these bacteriophage to prevent and treat severe colibacillosis in poultry. Colibacillosis is induced by injecting  $6 \times 10^4$  cfu of *E. coli* into the thoracic airsac when the birds are 1 week of age. Initial studies demonstrated that mortality was significantly reduced from 85% to 35% when the challenge culture was mixed with equal titers of bacteriophage, and the birds were completely protected when the challenge culture was mixed with  $10^8$  pfu of bacteriophage. In subsequent studies, we have shown that an aerosol spray of bacteriophage given to the birds prior to this *E. coli* challenge could significantly reduce mortality even when given 3 days prior to the *E. coli* challenge. Our research on treating colibacillosis in poultry has demonstrated that an intramuscular injection of bacteriophage given 24 or 48 h after the birds were challenged rescued the birds from this severe *E. coli* infection. In studies where bacteriophage and antibiotic therapy are combined we have demonstrated a significant synergistic interaction improving the therapeutic efficacy of both bacteriophage and the antibiotic. This research suggests that the levels of antibiotics used can be decreased if combined with bacteriophage therapy, which should increase the effective life of antibiotics. Our research has demonstrated that bacteriophage can be used to both prevent and treat colibacillosis in poultry and may provide an effective alternative to antibiotic use in animal production. The real challenge is how to make bacteriophage a practical alternative to antibiotics given the complexity of modern animal production systems.

# Intestinal genomics for the evaluation of alternatives to AGPs: current situation and perspectives

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AGPs as feed additives have proven to be effective in improving growth and feed efficiency. Increasing restrictions on the use of AGPs have caused an urgent need for the development of alternatives to AGPs. A range of (preferably) natural replacers for AGPs have been proposed and tested: pre-, pro-, and synbiotics, enzymes, (organic) acids, herbs and herbal extracts etc. None of the alternatives tested yet are as efficient as AGPs. The search for effective alternatives to AGPs is hampered by lack of knowledge about the mechanisms of AGPs mediated growth enhancement. The general assumption is that AGPs act as either a prophylacticum to endemic subclinical infection thus saving the animals energy, or that they somehow affect the efficiency of digestion by modulating the composition of the intestinal microflora. These two mechanisms are not mutually exclusive. In fact, most mechanisms proposed have both in common, the intestinal microbiota provides both nutritional and defensive functions for the host. The following mechanisms have been suggested for AGP mediated growth enhancement: enhanced uptake of nutrients, reduction of growth depressing microbial metabolites (such as ammonia, and bile degradation products), reduction of microbial use of nutrients, and reduction of the metabolic costs of the (innate) immune system by inhibiting pathogens. The reason for our lack of understanding lies in the complex inter-relationship of feed with microbiota and host.

The intestinal wall interacts with nutrients, exogenous compounds and microflora, and its condition is influenced by the complex interaction between these factors and genetic elements. The intestine is best described as a complex and dynamic ecosystem [1]. Concerning the microflora, as with most ecosystems, the true extent of biodiversity in the intestinal tract remains to be determined. Intestinal microbial metabolism constitutes an important biochemical activity in the body, with important consequences for health and disease [2]. In their turn, the epithelial cells lining the intestines are influenced by the intestinal content (food and microflora) in terms of differentiation and functionality. Furthermore, the interactions of the immune cell populations with the other components of the intestinal mucosa are essential in the maintenance of equilibrium with commensals and in the defense against pathogens [3,4]. How little we understand as yet finds a perfect illustration in the remark by Gaskins et al. [5]: '*it is curious that a class of organisms that appears to depress growth (Lactobacillus and Enterococcus), are also often used as probiotic organisms for promoting growth in livestock*'.

It is concluded that because of the complexity of intestinal interactions, analysis is usually refractory to the reductionist approach. The recent development of genomic techniques offers a solution to the latter problem, allowing for analysis of multiple responses captured in gene expression profiles [6]. Gene expression profiling holds tremendous promise for dissecting the regulatory mechanisms and transcriptional networks that underlie complex biological processes.

Given the above, it is also clear that functional data have to be obtained from functional whole mucosa since *in vitro* tests cannot be assumed to predict functionality in the body [2]. In addition, results obtained in *in vivo* small rodents models can only be extrapolated to other species with great caution. Furthermore, species specific differences imply that in intestinal research preferably the target animal should be used.

The basic principle of microarray technology is the miniaturization of current hybridization systems. Essentially, thousands of individual genes are immobilized on a solid surface (e.g. glass slide) either as cDNA or oligonucleotides. Test RNA from two different sources are either labeled with red fluorescence or labeled with green fluorescence, and then are co-hybridized to the arrayed genes. Genes expressed equally by both sources will fluoresce with both colors, resulting in yellow. Those present only in one source fluoresce either red or green. The individual gene expression level is presented as the ratio of red versus green fluorescence. Using this method, gene expression profiles of tissues comparing various of physiological and pathological conditions can be obtained. Gene expression profiles can be linked to functional properties (such as health and growth), and the genes and pathways involved can be identified through bioinformatics. In the photolithographically synthesized array experiments, the two samples under comparison are labeled with the same dye and individually hybridized to arrays.

The two different types of microarrays (cDNA microarrays and oligonucleotide microarrays) both have notable and distinct advantages. The primary disadvantage of oligo-based arrays is that oligonucleotide sets are very expensive because of the extensive sequence data and gene-specific oligonucleotide design and synthesis. The great advantage is the reproducibility. cDNA arrays can be prepared directly from existing cDNA libraries. Construction of such cDNA arrays is dependent on the availability of collections with unique genes. Alternatively, cDNA arrays can be prepared *de novo* from specific tissues under different conditions, and apparent important genes identified by sequencing afterwards. The advantage of the latter method is that it is independent from known sequence information, and that relevant genes are likely to be present on the microarray. Furthermore, the most important point for all types of array is that one can relate the expression profile with function, e.g. optimal growth. To this end, it is not strictly necessary to know the identity of the genes involved. Ideally, the cDNA spotted should represent all relevant genes expressed. Complicating the design of intestinal microarrays is the complexity of the intestine. Intestinal epithelium consists of a diversity of cell types of different maturity [7], showing heterogeneity from crypts to villi tops, and along the duodenal to colonic axis [8]. Also, expression of intestinal genes is influenced by a wide variety of factors and conditions. The ideal microarray would require cDNA from all different cell types, conditions, ages, location, feed etc. However, this could lead to underrepresentation or absence of relevant gene expression by dilution [8]. It is clear that in constructing a microarray it is important to strike a balance between completeness and practical limitations.

Relatively limited work appears to be in progress on farm animal intestinal microarrays, despite the importance of the intestines for production animals. What can be found in the public domain, is that the Murtaugh Laboratory of the University of Minnesota works on the creation of a microarray for immune genes of the porcine intestine (<http://www.ahc.umn.edu>). In our laboratory, a pig and a chicken intestinal cDNA microarray have been developed (<http://www.ard.asg.wur.nl/>) for the broader purpose of animal health and growth. The microarrays were constructed, and validated. Currently, expression profiles are determined in a variety of controlled conditions. Comparisons are made between control animals and with different enteric infections in the presence or absence of microflora. Furthermore, experiments are underway with pre- and probiotics, and different (functional) feeds. Striking differences in mucosal expression profiles are observed. Sequencing of apparent important genes is now in progress. The first results show clear responses of immune, metabolic and differentiation associated genes which will be related with health and growth by bioinformatics. Ongoing sequencing of important genes will allow for further optimization of the arrays by elimination of redundancy and non-essential genes. Furthermore, a limited number of relevant genes would reduce the costs of the production of an oligo based variant with the associated advantage of reduction of experimental noise. Our aim is to produce an oligo based microarray containing a limited number of genes essential for intestinal functions.

Alternatively, since the mapping of the genome of several husbandry animals nears completion, it will soon be possible to use whole genome oligo based arrays. Either type of microarray can be used as a predictor of properties of feed and feed components for disease prevention, and health and growth promotion. In addition, it would also help to identify genetic biomarkers (SNPs) associated with health and growth.

In conclusion, genomic analysis offers the possibility to identify mechanisms and pathways, allowing for rational design of alternatives to AGP rather than by trial and error, which is time-consuming and costly. While genomics is relatively expensive as yet, cost reductions can be foreseen, making it economically feasible.

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## Further reading

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Rothschild, M.F., 2003. Advances in pig genomics and functional gene discovery. *Comparative Functional Genomics* 4: 266-270.

# **Sense and non-sense of innovative approaches to replace AGPs**

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Yesterday we were living in an economically mastered world, today we still are but changes might be around the corner. In a democratic system with conscious (developed) people, governments (have to) make decisions which fulfil (favour) the wishes of its people. Recently, in Western countries a bigger awareness for animal welfare and food safety has arisen and might, as a consequence, soon dictate the ways and methods of raising our animals. These concerns will have to be taken into consideration by the industry and the veterinarian and will probably result into a shift from quantity- towards quality-considerations. The demand for food quality (taste and safety) but at a cheap price should be of major concern to the industry. For the last ten years, industry invested in a higher-value food, such as health-promoting or -conserving food. However these “functional foods” are not experiencing the breakthrough that was expected some 10 years ago. In a recent survey of 2004 in Flanders by Prof. Wim Verbeke, 66% of responders rejected “functional food” when it tasted worse than its traditional variant (De Morgen, 12 January 2005). Four years before, that was less than 50%. Unfortunately, when the society speaks as a consumer, it most often does not act accordingly. How do we as scientists react to this new evolution on animal welfare concern and food safety in animal production? As a general statement, I would state that pressure for higher production rates at the expense of animal (physiological) welfare is not (longer) justifiable but that due to the intensive animal production systems, we have a duty (obligation) to protect our production animals from economical losses due to unbalanced feeds, disease or dysbacteriosis. A change from growth-promoters to animal health- or welfare-promoters should be a challenge for the industry.

Intensive animal production systems in particular for pigs and poultry are for economical reasons well represented in the industrial world. These intensive production units are characterised by a high density of animals, which propagates the dissemination of infectious organisms, including those with a preference for the intestinal tract. These intestinal infectious diseases which are of bacterial, viral, protozoan or helminth origin, have a negative influence on the health of the animal and consequently on the production and revenue of the system. They cause major losses through epidemic and endemic disease outbreaks. For these reasons, prophylactic measures such as hygiene, well-balanced feeds, drugs and vaccines are of prime importance in the management of these production systems. Today, efforts are focused on the reduction (abolishment) of prophylactic drugs in feed, and animal products are these days under tight scrutiny for the presence of any residues of these drugs. Because of this awareness, industry is investing a lot in the search for harmless prophylactics in natural food components.

In order to modulate or influence the immune response or responsiveness of an animal by nutrition it is important to understand the basic immune effector mechanisms and factors influencing these. A fundamental knowledge on the intricate communication of the immunocompetent cells, their ontogeny and their induction sites and signals is required for understanding and improving dietary modulation of immune responsiveness. Moreover, a good understanding of the intimate relationship between the resident bacterial flora/pathogens and the host intestinal tract is quite important.

In general we can distinguish two main categories in this search, a search for food components which have a direct effect on the pathogen or gut flora and secondly those

which have an indirect effect on the gut pathogen or flora by targeting the intestinal and/or immune cells of the host. Some compounds might even act in both ways. The first category of compounds includes medium-chain fatty acids killing pathogens, sugars or lectins inhibiting colonisation of the gut by competition or blocking, and competition in colonisation by changes in the resident flora. The second group of modulators targets the innate immune system of the host by interacting with host receptors. To understand this innate immune alertness of the mucosal tract, it is imperative to focus on the acute phase of the inflammatory response. A lot of food components such as vitamins A and D,  $\omega$ -6/ $\omega$ -3 fatty acid ratios, oligosaccharides and others can indeed stimulate/modulate innate immunity at the mucosal site and protect against intestinal invaders. However, their mechanisms of action are not always fully understood and are now under thorough investigation. More recently, new groups of receptors, including the Toll-like receptors, have been discovered, which specifically recognise molecules with certain patterns common to the microbial world, referred to as pathogen-associated molecular patterns (PAMPs). These patterns are characteristic for lipopolysaccharides, lipoproteins, peptidoglycans, glycolipids and other molecules of microbial/yeast/fungal origin. They induce an immune alertness in the host that could be exploited in animal feeds for mucosal protection against pathogens.

Considering this new scientific knowledge on communication between host and microbes in the intestinal world, innovative approaches are certainly making sense as long as they encompass equally well the health and welfare of the animal. But does not everything have its price? Does constant immune alertness not consume energy at the expense of production?

# Setting and meeting standards for the efficient replacement of pronutrient antibiotics in poultry and pig nutrition

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Six questions concerning the rectification of set-backs due to the precautionary proscription of the use of prescription-free antibiotics in animal production are considered herein.

- How can the adverse performance effects of a pronutrient antibiotic ban in poultry and pigs best be quantified?
- What is holo-analysis and what can it contribute?
- What independent variable factors, other than dosage, are influential?
- How are comprehensive multi-factorial response models used in praxis?
- What are the prospects for the use of admixtures?
- Which are promising candidate replacement products?

Large data banks mined from the extensive literature on negatively-controlled nutritional response tests on antibiotics are used to provide predictive models for feed intake, liveweight gain, feed conversion and mortality effects in broilers, turkeys, layers, piglets and slaughter pigs over a wide range of conditions. Holo-analysis provides mathematical models summarising all available data in terms of all available independent variables in order to provide response estimates with associated confidence limits.

Multi-factorial models contain up to 20 highly-significant independent variables, quantifying the genetic, management, environment, diet composition and nutrient content effects. In the longer term, when data suffice, similar models will be elaborated for potential replacements, as already e.g. for non-starch polysaccharidases and phytases in broilers and pigs, with analogous models for acids, oligosaccharides and nutrients in the pipeline. Pending the availability of sufficient data to model individual replacement products for comprehensive comparisons *inter se* or versus specific antibiotics, existing antibiotic models can be used for iso-cost assessments of the results of (i) individual replacement tests; (ii) replacement dose-response tests; (iii) individual replacement/antibiotic comparison tests with and without negative controls; (iv) arbitrary admixtures; and (v) the mean values of replacement data sets too small as yet for modelling.

A Seven Question Test can be used to assess developmental replacement products. Main candidates as antibiotic replacements are acids, anticoccidials, botanicals, enzymes, nutrient, microbials and oligosaccharides, of which exogenous enzymes, some anticoccidials and organic acids appear currently to be the best prospects.

## MINI-SYMPOSIUM

### **Rapid detection of many illegal antibiotics in feed, a way forward for the control in animal husbandry: the Feedstuffs-RADIUS project\***

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It has been common practise in agriculture to add antibacterial drugs as growth promoters to the feed of entire herds and flocks at subtherapeutic levels over extended period of time. These performance enhancing antibacterial growth promoters are regulated by Council Directive 70/524/EEC [1] as zootechnical feed additives and specified tolerances for their inclusion are given in the annex of that document.

Whenever drug preparations are administered to food-producing animals, residues thereof in edible tissues, milk or eggs are likely. Residues of antibacterial drugs in food could lead to allergic reactions but the greatest threat is the development of resistant strains of bacteria which could lead to the improper respond to normal drug treatment in humans. For these reasons the European Commission decided to ban some of the regulated growth promoters. With Council Regulation 2821/98 [2] zinc bacitracin, spiramycin, tylosin and virginiamycin, and with Commission Regulation 2788/98 [3] olaquinox, were banned from animal feed.

Since this ban the microbiological based "Community methods of analysis", referred to in Council Directive 95/53/EC [4], were no longer usable. While suitable to control if a label declared feed additive was present in the feedingstuff within specified tolerances, these methods were unsatisfactory to allow the identification of unknown or undeclared antimicrobial agents. Moreover, these microbiological methods were seen as being time consuming, labour intensive, laboratory based and did not lend themselves for on the spot use.

The Feedstuffs-RADIUS project had the aim of having a direct bearing on the ability of Member States to check compliance with the ban. The development of two distinct analytical approaches, namely, rapid, robust and user-friendly portable immunoassay screening test backed up by a sensitive, chemical confirmatory method, utilising mass spectrometric identification and quantification, should allow a harmonised EU-wide approach to this problem.

As the RADIUS project nears completion the outcomes have been to deliver two distinct classes of analytical tests. The first procedure is a multi-ELISA method capable of detecting all the above named compounds at or below 1 ppm inclusion in animal feed. It is hoped that this method will be commercialised by a RADIUS partner to allow wide access to the methodology.

The second major outcome is the development, validation and ring testing of a sensitive LC-MS/MS procedure, which allows the simultaneous detection of all five antibiotics in animal feed. This method is available free of charge by requesting a CD-ROM from the project co-ordinator.

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\* The Feedstuffs-RADIUS project ('Rapid antibiotic detection for illegal and unlicensed substances in animal feedingstuffs') was funded by the European Commission.

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1. Council Directive 70/524/EEC of 23 November 1970 concerning additives in feeding-stuffs. Official Journal of the European Union L 270, 14.12.1970, pp.1-17.
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## Towards a control strategy for banned antibiotics and growth promoters in feed: the SIMBAG-FEED project\*

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In order to protect human health the authorisation of several antibiotics (avoparcin, zinc bacitracin, spiramycin, tylosin and virginiamycin) and growth promoters (carbadox and olaquinox) as feed additives has been withdrawn in the European Union in 1999. The effective and efficient control of the possible illegal use of these substances requires the availability of multi-screening and confirmation methods that can be implemented in an overall control strategy.

The main technical and scientific objective of the EU-project SIMBAG-FEED is to develop, improve and validate adequate methods which can be implemented in an overall control strategy applying the following techniques: microbiological inhibition, high voltage electrophoresis (HVE), Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS/MS). The methods should be: (i) sufficiently sensitive (antibiotics 1 mg/kg; olaquinox 3 mg/kg; carbadox 4 mg/kg); (ii) multi-analyte; (iii) able to distinguish between the banned substances and registered feed additives and antibiotics used as veterinary drugs in medicated feed; (iv) robust techniques widely available in (official) control laboratories; (v) reliable (unequivocal confirmation; identification is more important than quantification).

Methods have been developed and in-house validated. Between-lab validation of the methods has been performed in two laboratories that were not involved in method development. Validation has been performed according to Commission Decision 2002/657/EC [1].

Based on the results of the validation studies, the candidate methods for the proposed control strategy were selected and collaboratively tested. The participants of the collaborative studies were selected based on the results of the training period that was organised prior to the collaborative studies. The results of the collaborative studies are available, the statistical evaluation is ongoing. In this lecture, the results of method development and validation and, as far as available, statistical evaluation of the collaborative studies will be presented.

The proposed control strategy is reflected in Table 1. For the antibiotics a three-step control strategy is proposed, existing of screening by microbiological inhibition followed by post-screening by TLC or HVE followed by confirmation by LC-MS/MS. For the growth promoters

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\* EU project "Screening and identification methods for official control of banned antibiotics and growth promoters in feedingstuffs").

the proposed control strategy exists of screening by LC-UV followed by confirmation by LC-DAD or LC-MS/MS. The methods are described in more detail further on in this abstract.

Table 1. The proposed control strategy.

Technique	Purpose	OLA	CAR	TYL	SPIR	VIRG	BAC	AVO
Micro	Screening AB	N.A.		+				
HVE	Post-screening AB	N.A.		+			+	
TLC	Post-screening AB	-		+			-	-
LC-UV	Screening GP	+					-	-
LC-DAD	Confirmation GP	+					-	-
LC-MS/MS	Confirmation GP and AB	+					+	

(N.A. = not applicable; AB = antibiotics; GP = growth promoters)

### Microbiological screening method for the 5 antibiotics

For all 5 antibiotics one extraction method combined with three test plates has been developed. Extraction is performed at low pH with a mixture of acetone, hydrochloric acid and water. No concentration or clean-up step is necessary, after centrifugation the samples are subjected to the test plates.

The two labs that performed between-lab validation confirmed the results of in-house validation. Tylosin, avoparcin and spiramycin could be detected at the target concentrations. Virginiamycin and zinc bacitracin spiked at 1 mg/kg were detected in 90% and 75% of the samples; 2 mg/kg virginiamycin and zinc bacitracin could be detected in all spiked samples. To distinguish between the five banned antibiotics and (carry over of) antibiotics used as veterinary medicine (e.g. tetracyclines, quinolones, other macrolides,  $\beta$ -lactams, aminoglycosides, sulphonamides) three additional testplates are necessary. The other antibiotics registered as feed additives (avilamycin, flavomycin, monensin, salinomycin) showed no interference.

### HVE post-screening method for the 5 antibiotics

Two extraction procedures have been developed. Avoparcin and zinc bacitracin are extracted at low pH with a mixture of acetone, hydrochloric acid and water. Tylosin, spiramycin and virginiamycin are extracted with methanol 50%. No concentration or clean-up step is necessary, after centrifugation the extracts are subjected to HVE in a buffered agar gel. The antibiotics are located by bioautography.

In-house as well as between-lab validation (n=20) showed that avoparcin, tylosin and spiramycin could be detected in all samples spiked at 1 mg/kg, except for milk replacers where the detection limit is slightly higher for tylosin and spiramycin. A concentration of 1 mg/kg virginiamycin and zinc bacitracin was detected in circa 50% of the samples; a concentration of 2 mg/kg could be detected in 95-100% of the samples. All blank feeds showed no inhibition.

In medicated feed, large zones of inhibition due to added antibiotics may cover up smaller zones of inhibition due to the target compounds. These interfering substances have been defined in the method description.

## **TLC post-screening method for tylosin, spiramycin and virginiamycin**

One method has been developed for tylosin, spiramycin and virginiamycin. After extraction with methanol/water and purification by liquid-liquid partition with chloroform, part of the concentrated chloroformic phase is subjected to two TLC silica gel plates. The compounds are separated with different eluents. Antibiotics are detected by bioautography.

In-house and between-lab validation proved that the method makes it possible to detect and identify spiramycin, tylosin and virginiamycin in feedingstuffs, excluding mineral feeds and premixtures. The limit of detection is about 1 mg/kg for spiramycin, 0.5 mg/kg for tylosin and 1 mg/kg for virginiamycin, but in some milk replacers, it can be slightly higher than 1 ppm for virginiamycin.

## **LC-UV screening or LC-DAD confirmation method for olaquinox and carbadox**

After extraction with a mixture of methanol and water, and purification by a short aluminium oxide column or by alumina Solid Phase Extraction (SPE), the sample extract is analysed on a RP-C<sub>18</sub> column with UV detection at 375 nm or diode-array detection.

In-house and between-lab validation showed that the method is robust, specific and suitable for the detection and quantification of both compounds. The limit of quantification of the method has been demonstrated to be better than 3 mg/kg and 4 mg/kg respectively for olaquinox and carbadox. The choice of the LC-column is a very important factor for the HPLC analysis of carbadox.

With the use of UV detection the method is suitable for the control strategy as a screening method. When using diode-array detection it is suitable as a confirmatory method.

## **LC-MS/MS confirmation methods for the 5 antibiotics, olaquinox and carbadox**

Two multi-analyte methods have been developed and validated with good results. Feed samples containing tylosin, spiramycin, virginiamycin, carbadox and olaquinox are extracted with a water/methanol solution. Avoparcin and zinc bacitracin are extracted from feed with a hydrochloric acid/acetone mixture. Aliquots of the sample extracts are diluted and loaded on SPE OASIS HLB® columns. Compounds of interest are eluted and diluted or re-dissolved after evaporation. The resulting extracts are analysed by LC-MS/MS.

Applying a triple quadrupole instrument, the results of in-house validation showed that identification of the compounds at the target concentration is possible. The between-lab validation as well as the training period of the collaborative study have shown that the developed methods can be applied on different types of mass spectrometers with good results for qualitative analyses. Problems can occur when using an ion-trap instrument for the analysis of avoparcin, especially for the lower concentration range. However, small adjustments in the sample preparation (for example concentration of the sample extract) can easily overcome this problem.

It has been shown in the in-house and between-lab validation as well as in the training period of the collaborative study that for quantitative purposes Multi Level Standard Addition (MLSA) is necessary. As a result of MLSA, at the target levels the Coefficients of Variation (CV's) for repeatability and reproducibility are generally higher than the maximum values according to Commission Decision 2002/657/EC [1]. However, it should be taken into account that the substances of interest are banned and any presence of these compounds, which could be demonstrated satisfactorily, is a violation of such ban.

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## **Acknowledgements**

The SIMBAG-FEED project is funded by the European Commission, DG RTD, 5th Framework Programme, Competitive and Sustainable Growth (GROWTH) Programme, Contract no. G6RD-CT-2000-00413.

# Novel approaches for the determination of probiotics in feed in the context of official control

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A variety of bacteria and microfungi are used in animal feed as probiotic additives to obtain beneficial effects for the host. Many of the microorganisms used as probiotic feed additives are closely related to those used in human food. Authorised probiotic feed additives include the bacterial genera *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Bacillus* and the yeast *Saccharomyces cerevisiae*. Probiotic feed additives are considered to have positive effects for a range of animals such as the ability to increase their body weight; enhance the efficiency of antimicrobial treatment of *Campylobacter* infections; reduce faecal shedding of enterohemorrhagic *Escherichia coli* O157:H7; improve digestive tract health and stimulate the immune response. Probiotic feed additives have been applied in animal nutrition for over 15 years. Microorganisms entering the food chain in association with animal feed were comprehensively regulated in Europe via Council Directive 93/113 EC [1] which has since been updated. For Official Control purposes in the regulatory framework of probiotic feed additives, robust, reliable and standardised methods are necessary for the determination of the concentration of viable microorganisms and for strain identification.

In an effort to arrive at standardised methodologies for selected genera of probiotic microorganisms used as feed additives a number of laboratories participated within a European Community Standards, Measurements and Testing (SMT) program in collaboration with the European Federation of Feed Additives Manufacturers (FEFANA). Within the project (SMT4 CT98-2235) existing methods for the enumeration and identification of probiotic bacteria and *S. cerevisiae* used as feed additives were examined and selected, with modifications as appropriate, for use as prospective control methods. This paper will provide an overview of the performance of the enumeration media used in the project with emphasis on good method repeatability and reproducibility and on their ability to selectively enumerate specific microorganisms when present in various concentrations i.e. as majority, equal or minority components, in mixtures within probiotic feed preparations.

A number of techniques are available for the identification of probiotic microorganisms at the strain level. These include biochemical or molecular techniques based on restriction endonuclease analysis (REA), rRNA genes, polymerase chain reaction (PCR), amplified fragment length polymorphism (AFLP), and pulse field gel electrophoresis (PFGE). Within the project (SMT4 CT98-2235), PFGE was reviewed and recommended by the project partners for the bacterial genera *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Bacillus* as a technique that was robust, reliable, user friendly and provided the required unambiguous fingerprints for authorised probiotic strains. For the yeast *S. cerevisiae*, a PCR-based method, which had a history of use in the feed industry, was tested in an inter-laboratory comparison and revealed appropriate performance data. This paper will provide an overview of available methods that may offer potential as official control methods applicable to probiotic microorganisms used as feed additives.

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1. Council Directive 93/113/EC of 14 December 1993 concerning the use and marketing of enzymes, micro-organisms and their preparations in animal nutrition. Official Journal of the European Union L 334, 31.12.1993, pp.17-23.

# Pitfalls and challenges for the official control of enzymes in feed

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For more than fifteen years, there is a significant increase of the utilisation of fungal or bacterial enzymes in the animal-feed industry, particularly in the alimentation of monogastrics such as pigs and poultry. The enzymes incorporated in the animal feed are hydrolases, which are used directly as feed additives and can be distinguished in two main categories:

- enzymes degrading vegetal cell walls (xylanases, cellulases,  $\beta$ -glucanases); and
- enzymes with specific effects on antinutritional factors (phytases).

The major goals of the incorporation of these enzymes in animal feedingstuff are essentially the improvement of the growth performances and the decrease of polluting faeces contents.

From a regulatory point of view each enzyme manufacturer must provide, in his authorisation dossier according to Council Directive 93/113/EEC [1], a method to measure the activity of the enzyme(s) in the feed additive, in the premixtures and in the feeds. As enzymes used in animal feed are produced by a great variety of microorganisms that have affinities for different substrates, optimal pH and temperatures, the producers have developed a great variety of methods (colorimetric, viscosimetric, immunological, gel diffusion) to measure the enzymatic activity in additive.

In principle any of the above assay methods can also be used in premixture and feed to determine levels of enzyme activities. But "in-premixture and in-feed methods" are not yet universally accepted. There are among others two main problems to be overcome and which need further research: the detection limits of the methods and interferences from matrix components.

At present, the lack of validated reference methods and the diversity of the protocols proposed by the different enzyme manufacturers make official controls of guarantee labelling impossible. Each guarantee of enzymatic activity is expressed as producer's method dependent unity. Official control of all marketed products supposes that as many different protocols have to be used as there are different products and different enzymatic activities.

As a consequence it is necessary to develop reference methods for controlling dosage of enzymes. A strong co-operation about such a complex subject between official, normative and industrial authorities is the essential condition to take up the challenge of the enzymes' assays harmonisation.

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1. Council Directive 93/113/EEC of 14 December 1993 concerning the use and marketing of enzymes, micro-organisms and their preparations in animal nutrition. Official Journal of the European Union L 334, 31.12.1993, pp.17-23.

# Review of the possibilities to detect herbal active principles and prebiotics in feeds

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In the farmed animal nutrition the progressive withdraw of the antimicrobial feed additives as gut flora modifiers and coccidiostats is prompting the market to explore the possibility to replace them with natural substances mainly recovered from botanical preparations. Such antimicrobial substances are present as complex mixtures in the sage (*Salvia officinalis*) for instance, and possibly concentrated by different processes, such as extraction, distillation, purification; up to 100 different components can be separated in the sage essential oil. Moreover, botanical preparations from different species could be added to the same feedingstuff, in order to potentiate the antimicrobial activity and/or with the claim to exploit at the same other beneficial effects, such as the antioxidant, antiparasitic and the anti-inflammatory ones.

The above mentioned factors determine the complexity of the overall analytical approach. Pragmatically, the attention can be focused on the characterisation of the single preparations, where the application of vibrational spectroscopic methods has been recently described to succeed to identify the extract according to the botanical species of origin. On the other side, the identification and quantification of those components responsible of the main antimicrobial activity is routinely carried out with the use of High Resolution (HR) chromatographic techniques such as gas chromatography (GC) combined to mass spectrometric (MS) detectors; GC-MS seems the most appropriate technique especially for the analysis of hydrophobic and semi-volatiles molecules mainly present in essential oils. For dried extracts and water-soluble components of garlic (*Allium sativum*), liquid chromatography coupled to MS with Atmospheric Pressure Chemical Ionisation has been proved to be effective in the characterisation and determination of the actives principles.

The analytical chemistry analysis should be integrated with the analytical microbiology approach for the dosage of the pharmacological activity of the botanical preparation. This requires the selection and use of the most appropriate bacterial strains, in the case of antimicrobial substances. From the comparison of the chemical data with the microbiological ones, it could be possible to evaluate the potency, that can vary basically according to the place where the botanical species has been cultivated, to the seasonal timing of the harvesting, to the storage conditions, and to the extraction method used.

Other complementary analytical aspects consolidated in the literature consist on the detection of possible environmental contaminations, such as pesticides, heavy metals, phycotoxins and phytotoxins as well on the detection of possible xenobiotics added for fraud or resulting from carry-over phenomena.

Prebiotics such fructo-oligo-saccharides (FOS) are currently proposed in feeds at 0.5% to modulate the gut flora versus lactic bacteria competing with *Salmonella* and other pathogens, and to improve the absorption of oligo-elements such as calcium and magnesium. Their detection and characterisation could be achieved with Matrix Assisted Laser Desorption Ionisation - Time Of Flight - Mass Spectrometry technique (MALDI-TOF-MS), whose application has been already described in vegetable samples.

This review indicates that the human competence and the analytical capabilities requested for detection of antimicrobials from botanical preparations in animal nutrition should be even more performing than the classical ones in use for veterinary drugs and feed additives.

# **New authorisation of feed additives in the EU: the role of the Community Reference Laboratory and the network of European Laboratories**

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Feed additives are used in animal feed e.g. to improve productivity or health of the animal. Before placing a feed additive on the market, the industry has to apply for an authorisation. One of the main objectives of the authorisation procedure is to evaluate whether the use of the feed additive under consideration could have an adverse effect on animal and human health. Another topic is the evaluation of the analytical method proposed by the applicant to determine the additive in feed and in some cases also in food. The latter aspect is important when the authorisation establishes maximum residue levels (MRLs) of the additive in the animal derived food products. Within the European Union the authorisation procedure has recently been modified as laid down by Regulation (EC) 1831/2003 [1], giving full responsibility regarding the risk assessment to the European food safety authority (EFSA). In addition, the legislation nominates the Joint Research Centre (JRC) as the Community Reference Laboratory (CRL), evaluating the analytical method to detect the feed additive. A very recent Commission Regulation implementing Regulation (EC) 1831/2003 details the tasks of the CRL and of the consortium of National Reference Laboratories (NRL) supporting the CRL in the evaluation of the analytical method proposed by the applicant.

## **The tasks of the CRL**

Amongst other tasks the CRL is responsible for the following tasks:

- maintaining a sample bank of the feed additive;
- evaluating the analytical method and testing the method in specific cases;
- co-ordination of validation studies of analytical methods if required;
- maintaining the consortium network of National Reference Laboratories; and
- reporting the results of the evaluation to EFSA (European Food Safety Authority).

## **The evaluation of analytical methods for feed additives - a challenging task**

The evaluation of the analytical methods suitable for the detection of feed additives is extremely challenging given the diversity of the substances involved. This becomes obvious when looking at the groups of feed additives covered by the new Regulation as shown in Table 1.

The complexity of the issue is also demonstrated by the list of currently authorised feed additives [3] that all need to be re-authorised according to the new procedure. Quite different substances are included ranging from trace elements to coccidiostats, probiotics and enzymes. The evaluation of the analytical methods for the determination of these compounds requires sound knowledge on a broad range of rather different methods indicating that such a task can only be managed when making use of available expertise of Member States' expert laboratories in the various topics. The role of the Member States' laboratories in the authorisation procedure and the relation to the CRL has been specified in the implementation regulation indicating their importance in this process.

Table 1. Groups of feed additives.

<b>Technological</b>	<b>Sensory</b>	<b>Nutritional</b>	<b>Zootechnical</b>	<b>Coccidiostats and histomonostats</b>
Preservatives Antioxidants Emulsifiers Stabilisers Thickeners Gelling agents Binders Substances for control of radionucleide contamination Anticaking agents Acidity regulators Silage additive Denaturants	Colourants Flavouring compounds	Vitamins Trace elements Amino acids Urea	Digestibility enhancers Gut flora stabilisers Substances which favourably affect the environment	

### **The evaluation of the analytical methods**

The new procedure foresees that the analytical methods are evaluated by a step wise approach assuming that in many cases a close examination of the method protocol and the results from the in-house validation study without conducting experiments is sufficient for a decision about the suitability of the method. For instance, if the analytical method is well known or has already been validated by an intercomparison study the method can be considered as fit for the purpose. In cases in which there are doubts about the method protocol and/or the in-house validation report, the regulation allows for testing the method in the CRL or in the consortium's laboratory. In very special cases the CRL can also organise an interlaboratory study to validate the method.

Evaluating the analytical methods is not an easy target considering the differences of the various methods. For instance, the determination of certain veterinary drugs such as coccidiostats requires the application of classical liquid chromatography, referring to internationally accepted guidelines [4,5] to check whether the method is fit for the purpose. However, in the case of other additives such as the determination of probiotics or phytase, the above mentioned guidelines are only partly applicable, thereby making the evaluation of the methods more difficult. For each method evaluation the CRL will nominate a laboratory from the consortium as rapporteur laboratory that is experienced enough in the specific topic to conduct the evaluation. It is also foreseen that all laboratories of the consortium can give their comment on the evaluation of the rapporteur laboratory before submitting the final report to EFSA. This procedure clearly demonstrates that the network of National Reference Laboratories plays a pivotal role in the evaluation procedure. In order to select the suitable NRL for a specific task the CRL is running a database containing the expertise profile of each laboratory. In addition the CRL is organising two workshops per year with the NRLs, DG SANCO and EFSA discussing specific aspects of the method evaluation. The workshops are supported by scientific lectures on selected topics.

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# PANEL DISCUSSION

## Panel discussion: background material

**Asko Haarasilta**

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### **What do we know about the effects of banning or not banning?**

Phasing out at European level

Regulation (EC) No 1831/2003 on additives for use in animal nutrition, Article 11, 2: "By way of derogation from Article 10 and without prejudice to Article 13, antibiotics, other than coccidiostats and histomonostats, may be marketed and used as feed additives only until 31 December 2005; as from 1 January 2006, those substances shall be deleted from the Register".

*Phasing out at Member State level*

- Legislative measures have been taken in order to ban the use of antimicrobial growth promoters in the Nordic countries (1986 in Sweden, 1999 in Denmark and Finland).
- In practise, feed industry of these Nordic countries does not use feed additive antibiotics other than coccidiostats.
- In many other Member States there are also non-antibiotic feeding concepts in use, although EU ban on feed antibiotics only comes into force in the beginning of 2006.
- The influence of the ban of antimicrobial growth promoters has varied depending on the strategy each country has used in the animal husbandry, in the administration and in the feed industry itself while transferring to a non-antimicrobial growth promoter-based production.

*Sweden*

- Formulation of feeds was significantly changed concerning energy, protein and fibre content.
- Energy levels of feeds decreased, which affected at least temporarily the daily gains.
- Temporary increase in use of medicated feeds after the national ban on antimicrobial growth promoters entered into force.

*Denmark*

- No significant effects on daily gain figures have been perceived.
- Slight increase of use of medicated feed but also due to other reasons.

*Finland*

- Ban on antimicrobial growth promoters did not decrease growth rates.
- Daily gain figures and feed conversion ratio have stayed at the same level as during the use of antimicrobial growth promoters.
- No increase in the production of medicated feeds.

In conclusion, (i) the ban of antimicrobial growth promoters has put an end to public speculation in this area in the Nordic countries, and (ii) its effect on the competitiveness of the domestic husbandry has been counterbalanced through new feeding concepts and better treatment methods

### **How has the ban on antimicrobial growth promoters influenced nutritional research of domestic animals?**

- Ban has activated and increased research and development in feed industry, aiming at supporting and developing healthy intestinal micro flora.

- Idea: good micro flora strengthens the animal husbandry.
- Through research a number of natural feed ingredients or feed additives developed, which can be used in feed production.

In conclusion, (i) the ban on antimicrobial growth promoters has decisively encouraged more profound nutritional research, (ii) in future this kind of research will further support the development of feeding and feeds, and (iii) the ban on antimicrobial growth promoters has supported good management of animal farms

### **What are the consequences of banning or not banning?**

- Ban is based on both:
  - scientific knowledge about the effect of antibiotics on the development of resistant bacterial strains, and
  - safety of animal-based production of foodstuffs and building of good image for animal husbandry.
- The feed industry accepts the scientifically proven facts as a basis for the ban on antimicrobial growth promoters.
- Crucial for feed industry to pay attention to the latest relevant scientific information and to improve image of feed industry.
- Influences the conclusions and operation plans of feed industry.
- Ban eliminates one of main public concerns regarding safety of foodstuffs and animal husbandry.
- Feed industry's point of view:
  - ban belongs to basket of good management measures,
  - possible to develop well working feeding programs without antibiotic growth promoters,
  - production efficiency may partly decrease, but possible to compensate the decrease in efficiency through new feed recipes and new feed ingredients or additives.

### **What is the next thing to do and how to accomplish it?**

- European feed industry has concentrated its research and development resources on non-antibiotic feed programs.
- Administrative authorities and producers in different countries are carrying out animal husbandry management programs.
- Aim of feed industry overall is to answer the customer needs of the market in question.
- Feed industry companies develop their own concepts taking advantage of the new feed ingredients and additives on the market.
- Crucial to create co-operation between feed industry and feed additive industry.
- Strengthened co-operation between research institutes and industry companies needed.
- Additional need for legislative measures.

### **How can we be sure about achieving anything?**

Future evidences, indicating success in non-antibiotic feeding:

- Maintaining good health status of animals = no increase in intestinal disorders → requires
- changes in feeding and farm management.
- Good animal health → ensures good productivity.
- Feed additive antibiotics no longer create resistant bacterial strains.
- Consumers trust in healthy and safe production of animal husbandry.
- Consistent principle: "healthy animals give good production results".
- Consumers have improved image of animal husbandry.
- Good news about animal husbandry in the public media.

## Panel discussion: background material

### Willem Penning

Head of Unit Animal Nutrition, Directorate Food Safety: production and distribution chain, The European Commission Health and Consumer Protection Directorate-General, Brussels, Belgium

The emergence and spread of antimicrobial resistance has become a major public health problem, within the Community and worldwide. Overuse and misuse of substances to kill or inhibit the growth of microorganisms (including bacteria, viruses and fungi), and certain parasites (for example protozoa) have favoured the growth of resistant organisms. This so-called "antimicrobial resistance" can spread to other microbial populations. Infections by resistant organisms endanger the human population, animals and plants, including those not previously in contact with antimicrobial agents.

Recent scientific opinions pointed out that prompt action is needed in the following areas: prudent use of antimicrobial agents, prevention of diseases, development of new products and methods of treatment and monitoring the situation.

Antimicrobial resistance is already addressed by the Community through various individual measures. The Commission has an overall approach to this question, based on the provisions of Article 152 of the Treaty establishing the European Community which provides that a high level of health protection shall be ensured in the definition and implementation of all Community policies and activities.

On this basis, the Commission is putting in place a Community strategy on four key areas of action:

- **Surveillance:** Monitoring the evolution and the effects of interventions through the establishment/strengthening of accurate surveillance systems on antimicrobial resistance in the human and veterinary sector and the consumption of antimicrobial agents.
- **Prevention** of communicable diseases, and infection control to reduce the needs for antimicrobial agents. This includes the prudent use of antimicrobial agents which entails the need for improved product information for authorised antibacterial medicinal products and the promotion of educational and behavioural actions towards the professionals and the general public.
- **Research and product development:** New modalities for prevention and treatment of infections and continued support of research for new drugs and alternatives.
- **International co-operation:** Antimicrobial resistance does not respect frontiers. An effective strategy requires close co-operation and consultation between the Commission, the Member States and other involved parties, especially at international level.

Measures are being taken in order to decrease the total consumption of antibiotics. For example, antibiotics used as feed additives will no longer be authorised as from 1 January 2006. Following the bans on avoparcin in January 1997, ardacin in January 1998, and in December 1998 of a further four antibiotics (bacitracin zinc, virginiamycin, tylosin phosphate and spiramycin), there are only four substances (flavophospholipol, monensin sodium, avilamycin and salinomycin sodium) still authorised as growth-promoting agents. These substances do not belong to classes used in human and/or veterinary medicine.

## **Panel discussion: background material**

**Paula J. Fedorka-Cray**

U.S. Department of Agriculture, Agricultural Research Service,  
Antimicrobial Resistance Research Unit (USDA-ARS-RRC), USA

- Is there any concern/evidence that the increased use of therapeutic antimicrobials that has occurred following the removal of subtherapeutic antimicrobials in some countries will result in a larger resistance problem over time.
- What does 'sound science-based' research really mean?
- Can we continue to ensure an adequate food supply at an affordable price if large-scale animal production is compromised?
- Although we know that subtherapeutic antimicrobials have a growth promotant effect, how do they really work?

## Panel discussion: background material

Gordon D. Rosen

Pronutrient Services, UK

- What do we know about the effects of banning antimicrobial growth promoters?
- What is the consequence of banning or not banning?
- What do we do next and how do we do it?
- And how do we know that it will actually achieve anything?

As background to these four questions a pilot survey has been carried out on problems caused by the banning in different parts of the world of antibiotic growth promoters. Answers to the following question were sought internationally from a random sample of 50 including 10 users, 10 consultants, 10 academics, 10 editors and 10 replacement suppliers. What do you consider are the two most important problems arising from the banning of veterinary prescription-free antibiotics in animal production?

The 100 problems nominated in the answers comprise 21 replicated and 16 singular topics, categorised for present purposes as health, nutrition, replacement and miscellaneous. Overall, these problems are in replacement (31), health (30), nutrition (22) and miscellaneous (17). The largest group is recovery of lost productivity (10). There are 7 each for increased necrotic enteritis, inadequate replacement tests and nutritional/managerial/replacement interactions and 6 each for increased piglet enteritis and the plethora of proffered replacements. Users have 19/20 for health or nutrition. Consultants have 15/20 for health and replacements. Editors and suppliers rate 10/20 and 9/20 respectively for replacements, whilst academics have a more even split of 6/5/5/4 for miscellaneous, health, nutrition and replacements respectively. It would appear then that users, as yet, are not so much concerned about replacements, perhaps not until complete bans take effect, e.g. that due on 01/01/2005 in the EU, though their consultants and suppliers, together with editors, are much more actively concerned thereon. It might be interesting to expand this survey to include the views of legislators, welfarists, wholesalers, retailers and consumers.

Hence, the following four motions are proposed for **Antimicrobial Growth Promoters: Worldwide Ban on the Horizon?** – the international debate conference for the feed & food chain.

- That the conference deems that the effects of banning antimicrobial growth promoters are very difficult, virtually impossible, to determine in praxis, so that we should now focus our efforts on the search for effective prescription-free antibiotic replacements, paying due attention also to the influence of the diverse management systems and dietary regimes in which they are deployed.
- That the conference considers that the relative consequences of banning or not banning are largely conjectural and unquantifiable, so that speculation thereon should be abandoned in order to concentrate on means for the control of microbial resistance problems due to the veterinary medicinal use of antibiotics in prophylaxis and therapy.
- That the conference, in the light of international and inter-regional world trade problems due to legislative bans by some but not by others, proposes a world-wide precautionary ban, simultaneously to be supported by appropriate investments aimed to minimise the incidence of any harmful side-effects of antibiotics used in human medicine.
- That the conference should adopt the aforementioned triad in order to concentrate the efforts of all interested parties on the expansion of research and development programmes for replacement products in order to contain food price inflation due to any ban.

# **Appropriate use of AGPs – oxymoron or opportunity?**

**Stephen W. Page**

Advanced Veterinary Therapeutics, Australia

When introduced in the early 1950s, antimicrobial growth promoters made an enormous contribution to the rapid expansion of livestock production. Over the next half century and until the present day the AGPs have continued to provide significant benefits to animal health, the environment and food safety. During (and because of) this long history of use, the impact on the selection of antimicrobial resistance and its dissemination has been almost continuously the subject of keen interest. There is now almost universal acceptance by national and global agencies that the most appropriate means of evaluating the importance of risks is through a structured process of formal, scientific, objective and transparent risk assessment. Such structured risk assessments of the human health impacts of antimicrobial resistance arising from the use of AGPs in food animals have recently been undertaken and are entering the public domain to invite and provoke discussion. Armed with an improved understanding of both the benefits and the risks associated with the use of AGPs it is now possible to consider situations of optimised use – where benefits can be realised with no significant adverse impact on human health. This presentation provides an overview of the key elements of appropriate use of AGPs and the range of risk management initiatives that have been applied elsewhere in a variety of fields and that could find a future role in the risk management of the AGPs.

## POSTERS

- P1 *Termination of antimicrobial growth promoters use in Sweden, Norway and Denmark: how did it affect usage of therapeutic antimicrobials in animals?*  
K. Grave<sup>1,4</sup>, V.F. Jensen<sup>2</sup>, K. Odensvik<sup>3</sup> and M. Bangen<sup>4</sup>  
<sup>1</sup>Norwegian School of Veterinary Science, Department of Food Hygiene and Infection Biology, Norway, <sup>2</sup>Danish Institute of Food and Veterinary Research, Department of Epidemiology and Risk Assessment, Denmark, <sup>3</sup>Apoteket AB, The Veterinary Pharmacy, Sweden and <sup>4</sup>Veterinary Drug Information Centre, c/o Norwegian School of Veterinary Science, Norway
- P2 *Virginiamycin and its impact on microbial community structure, streptogramin-resistance gene pool and streptogramin resistance in enterococci isolated from commercial poultry environment and consumable, processed chicken*  
G. Avellaneda<sup>1</sup>, M.D. Lee<sup>1</sup>, A.P. de Oliveira<sup>1</sup>, K. Williams<sup>2</sup>, D.G. White<sup>2</sup>, A. Debnam<sup>3</sup>, C. Jackson<sup>3</sup>, J. Lu<sup>1</sup>, T. Liu<sup>1</sup>, C.L. Hofacre<sup>1</sup> and J.J. Maurer<sup>1</sup>  
<sup>1</sup>The University of Georgia, Department of Avian Medicine, USA, <sup>2</sup>U.S. Food and Drug Administration, Center for Veterinary Medicine, USA and <sup>3</sup>USDA-ARS Russell Research Center, USA
- P3 *Reduction of antimicrobial resistance as induced by flavophospholipol*  
P.J.G. Oostenbach  
Intervet International, the Netherlands
- P4 *Effects of HMB on poultry digestive microflora*  
P.A. Geraert<sup>1</sup>, Y. Mercier<sup>1</sup>, P.M. Becker<sup>2</sup> and J.D. van der Klis<sup>2</sup>  
<sup>1</sup>Adisseo, France and <sup>2</sup>Animal Sciences Group, Wageningen University and Research Centre, the Netherlands
- P5 *Combination of medium chain fatty acid and organic acids provides a cost-effective alternative to AGP in pig nutrition*  
J.T.P. van Dam<sup>1</sup>, M.A.M. Vente-Spreuwenberg<sup>2</sup>, and H.P.T. Kleuskens<sup>1</sup>  
<sup>1</sup>Selko, the Netherlands and <sup>2</sup>Nutreco Swine Research Centre, the Netherlands
- P6 *A blend of acids for an optimised stomach function*  
H. Ghesquiere  
Impextraco, Belgium
- P7 *Organic acids and essential oils in AGP free diets*  
E. M. R. van Kol  
Franklin Products International, the Netherlands
- P8 *Brewer's yeast cell walls: a proven alternative for avilamycine in piglet feed*  
L.W. Bos and L.C.M. van Enkevort  
Denkavit Nederland, the Netherlands
- P9 *Use of zinc-bacitracin and mannan oligosaccharides in broiler chickens infected with necrotic enteritis*  
M. Choct<sup>1</sup>, M. Porter<sup>1</sup>, Z. Ao<sup>1</sup>, A. Kocher<sup>2</sup> and L. Nollet<sup>2</sup>  
<sup>1</sup>University of New England, Australia and <sup>2</sup>Alltech Biotechnology Centre, Ireland

- P10 *Mannan oligosaccharides, organic acids and probiotics in diets of piglets from 21 to 39 days of age*  
A. Corassa<sup>1</sup>, D.C. Lopes<sup>1</sup>, C. Bellaver<sup>2</sup> and S. Healy<sup>3</sup>  
<sup>1</sup>DZO/UFV, Departamento de Zootecnia, Brazil, <sup>2</sup>National Center for Swine and Poultry Research, Brazil and <sup>3</sup>Alltech, Belgium
- P11 *Effect of dietary mannan oligosaccharide on plasma IgG concentrations in piglets*  
B. Hengartner, M. Henggeler, S. Kohler and P. Spring  
Swiss College of Agriculture, Switzerland
- P12 *Effect of yeast cell wall on performance, intestinal morphology and immune response of broiler chickens fed low or high non-starch polysaccharides diets*  
R. Morales<sup>1</sup>, M. Francesch<sup>1</sup>, E. Auclair<sup>2</sup>, F. García<sup>3</sup>, R. Ducatel<sup>4</sup>, F. van Immerseel<sup>4</sup>, N. Andrea<sup>4</sup> and J. Brufau<sup>1</sup>  
<sup>1</sup>IRTA, Department of Animal Nutrition, Spain, <sup>2</sup>Lesaffre Feed Additives (LFA), France, <sup>3</sup>Saf-Agri, Mexico and <sup>4</sup>Gent University, Department of Pathology, Bacteriology and Avian Diseases, Belgium
- P13 *Effect of dietary carbohydrates on the resistance in the gut against colonisation of E. coli K88 in piglets*  
P. van Leeuwen, J.M.A.J. Verdonk, A.J.M. Jansman and J.D. van der Klis  
Animal Sciences Group of Wageningen University and Research Centre, the Netherlands
- P14 *Effect of inulin in broiler feed on the technical performance of broiler chickens*  
A. Veldman<sup>1</sup>, J. Pos<sup>1</sup>, H. Enting<sup>1</sup>, P.G. Groot Koerkamp<sup>2</sup> and M.W.M. van den Ende<sup>2</sup>  
<sup>1</sup>Schothorst Feed Research, the Netherlands and <sup>2</sup>Suiker Unie, the Netherlands
- P15 *On the influence of  $\beta$ -glucans from Saccharomyces cerevisiae on sow and litter performance during lactation*  
D. Förster<sup>1</sup>, F. Große Verspohl<sup>2</sup>, A. Berk<sup>1</sup> and H. Westendarp<sup>2</sup>  
<sup>1</sup>Federal Agricultural Research Centre, Institute of Animal Nutrition, Germany and <sup>2</sup>Fachhochschule Osnabrück, Faculty of Agricultural Science and Landscape Architecture, Germany
- P16 *Use of different essential oils in rearing piglets*  
K. Erlbacher, D. Förster and A. Berk  
Federal Agricultural Research Centre, Institute of Animal Nutrition, Germany
- P17 *Evaluation of a product based on essential oils for piglets*  
H. Perdok<sup>1</sup>, S. Tibble<sup>2</sup> and P. Langhout<sup>1</sup>  
<sup>1</sup>Provimi, the Netherlands and <sup>2</sup>SCA-Iberica, Spain
- P18 *The influence of botanical extracts on growth performance of broiler chickens*  
F. Recoquillay<sup>1</sup>, B. Medina<sup>1</sup>, V. Borja<sup>2</sup>, F. Calafat<sup>2</sup>, A. Fontgibell<sup>2</sup>  
<sup>1</sup>Phytosynthèse, France and <sup>2</sup>Estació Nutricional de Control d'Aliments i Pinsos (ENCAP), Spain
- P19 *The effect of a plant extract preparation in turkey feeds without antibiotic growth promoters*  
A. Veldman<sup>1</sup>, H. Enting<sup>1</sup>, J. Pos<sup>1</sup> and L. Mazuranok<sup>2</sup>  
<sup>1</sup>Schothorst Feed Research, the Netherlands and <sup>2</sup>Axiss, France

- P20 *'Rumen-up': new plants and plant extracts to decrease methane and nitrogenous emissions from ruminants and to alleviate nutritional stress*  
 R.J. Wallace<sup>1</sup>, R. Ningrat<sup>1</sup>, K. Becker<sup>2</sup>, E. Hoffman<sup>2</sup>, S. Muetzel<sup>2</sup>, N. Selje<sup>2</sup>, M. Fernandez<sup>3</sup>, S. López<sup>3</sup>, D.E. Beever<sup>4</sup>, K.E. Kliem<sup>4</sup>, R. Morgan<sup>4</sup>, F.L. Mould<sup>4</sup>, C. Duffy<sup>5</sup>, R. Losa<sup>6</sup>, and M. Frehner<sup>6</sup>  
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- P21 *'REPLACE': plants and their extracts and other natural alternatives to antimicrobials in feeds*  
 R.J. Wallace<sup>1</sup>, T. Wood<sup>1</sup>, K. Becker<sup>2</sup>, E. Hoffman<sup>2</sup>, N. Selje<sup>2</sup>, S. López<sup>3</sup>, K.E. Kliem<sup>4</sup>, R. Morgan<sup>4</sup>, F.L. Mould<sup>4</sup>, C. Duffy<sup>5</sup>, R. Losa<sup>6</sup>, M. Frehner<sup>6</sup>, F. Jackson<sup>7</sup>, O. Højberg<sup>8</sup>, B. Jensen<sup>8</sup>, A. Bomba<sup>9</sup>, M. Kaldhusdal<sup>10</sup> and D. Jozefiak<sup>11</sup>  
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- P22 *The effect of exogenous enzymes on weight gain, bacterial cell numbers and IgA concentration in the distal gastrointestinal tract of broiler chickens*  
 A.J. Cowieson<sup>1</sup>, M. Hruby<sup>1</sup>, T. D'Alfonso<sup>2</sup> and D. Korver<sup>3</sup>  
<sup>1</sup>Danisco Animal Nutrition, UK, <sup>2</sup>Danisco Animal Nutrition, USA and <sup>3</sup>University of Alberta, Canada
- P23 *Improved intestinal health and immunity in broiler chicks when fed a combination of Betafin® and Avizyme 1500*  
 H. Kettunen<sup>1</sup>, H. Simmins<sup>2</sup> and N. Rautonen<sup>1</sup>  
<sup>1</sup>Danisco Innovation, Enteromix Research, Finland and <sup>2</sup>Danisco Animal Nutrition, UK
- P24 *Enhanced IFN- $\gamma$  expression by Progut<sup>TM</sup> in the intestinal mucosa in Eimeria-challenged broiler chicken*  
 P. Nurminen<sup>1</sup>, J. Nurmi<sup>2</sup>, J. Vuorenmaa<sup>3</sup> and N. Rautonen<sup>1</sup>  
<sup>1</sup>Danisco Innovation, Enteromix Research, Finland, <sup>2</sup>University of Turku, Finland and <sup>3</sup>Suomen Rehu, Finland
- P25 *Antimicrobial agents, probiotics, prebiotics and herbal extracts as growth promoters of weanling pigs*  
 C.E. Utiyama<sup>1</sup>, V.S. Miyada<sup>1</sup>, L.L. Oetting<sup>1</sup>, P.A. Giani<sup>1</sup>, A.R. Ruiz<sup>1</sup> and W. Beeks<sup>2</sup>  
<sup>1</sup>Universidade de São Paulo, Escola Superior de Agricultura Luiz de Queiroz, Brazil and <sup>2</sup>Alltech Netherlands, the Netherlands
- P26 *A novel approach to improve performance in poultry production by using a combination of natural substances*  
 G. Schatzmayr<sup>1</sup>, A. Klimitsch<sup>1</sup> and E.M. Binder<sup>2</sup>  
<sup>1</sup>Biomim GmbH, Austria and <sup>2</sup>Erber AG, Austria
- P27 *Mode of action of a phytogenic product in weaning piglets*  
 A. Kroismayr<sup>1</sup> and W.M. Windisch<sup>2</sup>  
<sup>1</sup>Biomim GmbH, Austria and <sup>2</sup>University of Natural Resources and Applied Life Sciences, Department of Food sciences and Technology, Austria

- P28 *Development of a competitive exclusion product for poultry meeting the regulatory requirements for registration in the European Union*  
R. Plail  
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- P29 *Fermented liquid feed and fermented grain to piglets – effect on gastrointestinal health and growth performance*  
N. Canibe and B.B. Jensen  
Danish Institute of Agricultural Sciences, Department of Animal Health, Welfare and Nutrition, Denmark
- P30 *Feed supplementation strategies for reducing reliance on antibiotics in poultry production*  
G.R. Huff, W.E. Huff and N.C. Rath  
U.S. Department of Agriculture, Agricultural Research Service, USA
- P31 *A new surgical approach for post-gastric, intraluminal challenges with specific pathogens, viruses and(or) enterotoxins in conscious piglets*  
Z. Mroz<sup>1</sup>, S.J. Koopmans<sup>1</sup> and W. Krasucki<sup>2</sup>  
<sup>1</sup>Animal Sciences Group of Wageningen University and Research Centre, Division of Nutrition and Food, the Netherlands and <sup>2</sup>Agricultural University of Lublin, Poland
- P32 *The use of in vitro methods to evaluate the micro-encapsulation of capsicum oleoresin for its application in growing pig diets*  
J.-P. Meunier, B. Tournant, M. Alric and J.-M. Cardot  
Université d'Auvergne, Centre de Recherche en Nutrition Humaine, Equipe de Recherche Technologique 'Conception, Ingénierie et Développement de l'Aliment et du Médicament', France
- P33 *Screening method for banned feed additives: avoparcin, zinc-bacitracin, virginiamycin, tylosin, spiramycin*  
H.J. van Egmond<sup>1</sup>, W. Driessen-van Lankveld<sup>1</sup>, C. von Holst<sup>2</sup>, U. Vincent<sup>2</sup> and J. de Jong<sup>1</sup>  
<sup>1</sup>RIKILT-Institute of Food Safety, the Netherlands and <sup>2</sup>EC-DG JRC Institute for Reference Materials and Measurements (IRMM), Belgium
- P34 *Post-screening method for banned feed additives: avoparcin, zinc-bacitracin, virginiamycin, tylosin, spiramycin*  
H.J. van Egmond<sup>1</sup>, W. Driessen-van Lankveld<sup>1</sup>, H. Vastenburger<sup>1</sup>, L. Kortenoeven<sup>1</sup>, W. Wagner<sup>2</sup>, G. Rath<sup>3</sup> and J. de Jong<sup>1</sup>  
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- P35 *Confirmatory analysis of banned feed additives: avoparcin, zinc-bacitracin, virginiamycin, tylosin, spiramycin, carbadox and olaquinox*  
T. Zuidema, B.J.A. Berendsen, Y.J.C. Bolck, J.J.P. Lasaroms, J.A. van Rhijn and J. de Jong  
RIKILT-Institute of Food Safety, the Netherlands

- P36 *Assessment of performance of a prototype test kit for five banned antimicrobial growth promoters*  
C. Situ<sup>1</sup>, C.T. Elliott<sup>1,2</sup> and P. van Wichen<sup>3</sup>  
Queen's University Belfast, Department of Veterinary Science, UK, <sup>2</sup>Department of Agriculture and Rural Development, Veterinary Sciences Division, UK and <sup>3</sup>Euro-Diagnostica, the Netherlands
- P37 *Quantification of five natural growth promoters in feed*  
D. Bellenot<sup>1</sup> and V. Hocde<sup>2</sup>  
<sup>1</sup>Institut technique interprofessionnel des plantes à parfum, médicinales et aromatiques (Iteipma), France and <sup>2</sup>CCPA-Deltavit, France

## Termination of antimicrobial growth promoters use in Sweden, Norway and Denmark: how did it affect usage of therapeutic antimicrobials in animals?

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Ever since the introduction of antimicrobial drugs as growth promoters (AGPs) in the 1950's, controversy has existed about the nature and magnitude of human health risks caused by such use of antimicrobials. As a consequence of this discussion, the Parliament in Sweden decided to impose a ban on use of antimicrobial growth promoters (AGPs) in 1986, primarily to maintain the consumers' confidence but also for precautionary reasons. Almost a decade later avoparcin was banned in Norway and Denmark, and in the mid 1995, the Norwegian food animal industry voluntarily terminated all use of AGPs. Shortly after, the Danish food animal industry decided to voluntarily discontinue the use of all AGPs and, during the period 1998-1999, all AGPs were gradually phased out in Denmark.

An additional outcome of use of AGPs is a preventive effect against some bacterial diseases e.g., diarrhoea in pigs and necrotic enteritis in broilers. Consequently, it was expected that the termination of use of AGPs would lead to an increased incidence of such bacterial diseases and thus that an increased usage of therapeutic antimicrobial drugs in terrestrial animals would occur. To evaluate the effects of the AGP termination, monitoring programs to estimate usage of veterinary antimicrobial drugs before and after the termination were implemented in both Sweden, Norway and Denmark [1-4], respectively. The aim of the present paper is to present and discuss usage data of AGPs and therapeutic veterinary antimicrobials in terrestrial animals in Sweden, Norway and Denmark before and after the termination of AGP use.

In 2003, the overall usage, in weight of active substance, of antimicrobials in animals in Sweden had been reduced to approximately 1/3 of the amounts used in 1984 (AGP ban in 1986) [1,2]. In Norway, the corresponding figure declined by 53% from 1993 (two years before the AGP termination) to 2003 [3]. In Sweden and Norway, the consumption of therapeutic antimicrobials has declined substantially since termination of AGPs [1-3]. The overall reduction of antimicrobials in terrestrial animals in Denmark declined by 52% from 1994 (the year before the avoparcin ban) to 2003 [4]. The utilisation of therapeutic antimicrobials in animals in Denmark increased by 42% from 1998, the last year with considerable use of AGPs, to 2003. The increased consumption of veterinary therapeutic antimicrobials in Denmark in this period is confined to the pig production and is partly explained by a 7% increase in the pig production. Major regional differences in antimicrobial consumption imply that other factors than the AGP ban is responsible for the increase.

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P2

**Virginiamycin and its impact on microbial community structure, streptogramin-resistance gene pool and streptogramin resistance in enterococci isolated from commercial poultry environment and consumable, processed chicken**

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With FDA approval of the streptogramin, Synercid for treating vancomycin-resistant enterococci (VRE), there have been concerns that veterinary use of another streptogramin, may compromise the effectiveness of Synercid to treat VRE infections. However, few studies have shown how virginiamycin usage in food animals impacts the bacterial community, resident resistance gene pool and development of streptogramin resistance.

For three commercial, broiler chicken farms participating in this study, poultry houses were paired into two groups, houses that did not receive antibiotic growth promoters (AGP) in feed and those that received virginiamycin. Prior to this study the participating poultry company did not use virginiamycin, but instead used flavomycin as the AGP. Litter was collected from the broiler houses and carcasses were obtained from processed flocks. Enterococci were isolated from the poultry litter and carcass rinses using selective agar, species identified by PCR and antibiotic susceptibility determined by microbroth dilution for a panel of seventeen antibiotics. Community DNA was extracted from poultry litter and carcass rinses and the composition of the microbial community was determined using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rDNA. Streptogramin, macrolide, and tetracycline resistance genes, as well as class 1 integrons were identified from microbial community DNA by PCR.

There was no significant difference in streptogramin MICs ( $p=0.16$ ) or in prevalence of resistance among enterococci from control or virginiamycin treatment groups. There were no statistically significant changes ( $p = 0.29$ ) in microbial community structure demonstrated by Shannon-Weaver diversity indices as a response to virginiamycin usage. However, there were inter-farm differences in the presence or absence of major bacterial phylotypes identified by T-RFLP. Streptogramin resistance genes *vatA*, *vatB*, and *vatE*, macrolide-lincosamide-streptogramin B (MLS) resistance genes *ermA* and *ermB*, and tetracycline resistance gene *tetM* were present in the microbial community of poultry litter. Only *ermB* and class 1 integrase *intI1* was present among microbial communities present in litter and broiler carcass. There was inter-farm ( $X^2$  test:  $p<0.05$ ) variability in the distribution of *vatA* and *vatE* among litter microbiota. However, there were no differences in their distribution with regards to virginiamycin usage.

This study did not detect changes in microbial community structure or differences in the carriage of streptogramin and streptogramin-related resistance genes among enterococci cultured from different treatment groups.

## Reduction of antimicrobial resistance as induced by flavophospholipol

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The demands for pork and poultry products are strongly influenced by the consumers concern for healthy and safe food. The topic of food safety mainly concentrates on *Salmonella* contamination and is recently joined by the topic of antimicrobial resistance due to the use of antimicrobial growth promoters (AGPs) in animal feed. Latest findings on flavophospholipol, registered as AGP for pigs, poultry and several other species, describe a reducing effect on the development of antibiotic resistance and on the frequency of plasmid transfer.

For the first investigation, conducted at the University of Maastricht (the Netherlands) by van den Boogard (2001), 168 piglets were infected per oral administration with non-pathogenic *E. coli* strains, with a known multi-resistance pattern. Four weeks later the animal's faeces were analysed for the occurrence of *E. coli* and vancomycin resistant enterococci (VRE). In addition the isolated *E. coli* strains were tested for their sensitivity to various antibiotics (ampicilline, oxytetracycline, trimethoprim, sulphamethoxazole, ampicilline + oxytetracycline). For the trial the piglets were divided into three groups. The feed for group I was supplemented with 9 ppm flavophospholipol, for group II with 15 ppm avoparcin and group III served as control. Just prior to transport to slaughter, animal's faeces were analysed again in the same manner as described above.

The second study was conducted by Riedl et al. (2001). Three vancomycin A resistant field strains of *Enterococcus faecium* were co-cultivated with some *E. faecium* strains which were still sensible to vancomycin A. The transfer frequency of vancomycin A resistance encoding plasmids was examined after giving various concentrations of flavophospholipol to the nutrient solutions. For control vancomycin A itself was mixed into the cultivated solution in different concentrations.

The first study clearly shows an increase of antibiotic resistance of the isolated *E. coli* strains to all tested antibiotics in the control and in the avoparcin group. In the flavophospholipol group the occurrence of antibiotic resistances was unchanged or slightly reduced. The prevalence of VRE in the vancomycin-group increased significantly during the trial as compared to the control and flavophospholipol group. The other experiment resulted in a decrease of the transfer of vancomycin A resistance encoding plasmids in a dose dependent manner in the presence of flavophospholipol. In the presence of vancomycin A this effect lacked.

Flavophospholipol does not induce an antibiotic resistance to antibiotics used for veterinary or human therapy as it is not related to any antibiotic used for these purposes. Furthermore it is capable of reducing antibiotic resistance to other antibiotics. This effect seems to be yielded by interfering in the biosynthesis of the plasmid bridge (pylus) which is a prerequisite for the genetic transfer from one bacterium to another.

## Effects of HMB on poultry digestive microflora

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The bactericidal effect of HMB (2-Hydroxy-4-Methyl ThioButanoic Acid) on bacteria of the digestive tract was tested by comparison of digestive fermentations from growing broilers (42 days) fed a corn-soybean based-diet supplemented with DL-methionine or DL-HMB (0.09 %) vs. unsupplemented animals. Digestive tract contents (ileal and caecal) were sampled and microbial activities were estimated by quantification of gas volume production with an in vitro fermentation test for 24 hours.

Gas volumes were significantly lower with digestive contents incubated for 12 hours: -19 % in ileum and -10 % in caecum, ( $P < 0.05$ ) from broilers receiving a diet supplemented with HMB by comparison with control or DLM supplemented broilers. This would suggest that HMB has a bacteriostatic or antimicrobial effect on gut microflora of growing broilers.

In another study, the bactericidal effect of HMB was tested on several bacteria species to assess bacteriostatic and antimicrobial effect using the minimal inhibiting concentrations (MIC) and the minimal bactericidal concentrations (MBC) tests, respectively. The following results were observed:

	MIC		MBC	
	DL-MET	DL-HMB	DL-MET	DL-HMB
<i>Campylobacter coli</i>	1.25	2.5	> 5	2.5
<i>Campylobacter jejuni</i>	2.5	0.039	2.5	0.039
<i>Escherichia coli</i>	5	> 5	> 5	nd
<i>Salmonella enteritidis</i>	5	> 5	5	nd
<i>Salmonella typhimurium</i>	2.5	> 5	2.5	nd
<i>Enterococcus faecalis</i>	> 5	> 5	nd	nd
<i>Lactobacillus lactis</i>	> 5	> 5	nd	nd
<i>Bifidobacterium pullorum</i>	5	> 5	> 5	nd
<i>Clostridium perfringens</i>	> 5	5	nd	nd

The results were highly demonstrating for a strong bactericidal effect of DL-HMB on *Campylobacter jejuni*. This specific effect of HMB could be very beneficial for food safety since *C. jejuni* is one of the most cause of human infections due to chicken meat eating after *Salmonella*. These different studies clearly illustrate a specific effect of dietary HMB on digestive microflora of broiler.

## Combination of medium chain fatty acid and organic acids provides a cost-effective alternative to AGP in pig nutrition

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The future ban of antimicrobial growth promoters (AGPs) in the European Union (EU) will probably affect performance and health of a large part of the young farm animal population. Therefore, the use of natural alternatives for AGP has received much research attention. Based on broad experience in preservation of animal feed and drinking water, Selko R&D has studied innovative combinations of medium chain fatty acids (MCFA) and organic acids (OA) in collaboration with Nutreco Swine Research Centre. MCFA have a distinct antibacterial mode of action from organic acids (Stratford and Anslow, 1996; Van Immerseel et al., 2004). The effects on antibacterial profile, as well as piglet health and performance were investigated.

In the *in vitro* trials, it was shown that a combination of OA and MCFA (Selacid<sup>®</sup> Green Growth) inhibited both gram-negative and gram-positive bacteria; however, the inhibitory effect on *Lactobacillus* spp. was small. The minimal inhibitory concentration of a combination of OA and MCFA against *Clostridium* was as low as 1.5 g/kg (Nutreco PRRC, 2004).

Weaned piglet trial # 1 showed superior effect on ADG and FCR of combined MCFA and OA, as compared with separate treatments (ADG, -5.4, +8.5 and +15.4% difference with control for OA, MCFA and combination product, respectively; FCR, -5.4, -7.0 and -10.1% difference with negative control feed, respectively). The returns on investment (ROI) of the combination product were estimated at 4.0. Weaned piglet trial #2 showed that the combination of MCFA and OA improves daily gain by 9% and had a ROI of 2.5. Piglets showed fewer incidences of faeces with abnormal colour (e.g. *E.coli* diarrhoea; Nutreco SRC, 2003). Ongoing field studies in fattening pigs indicate that from 20-40 kg BW, ADG and FCR also show favourable tendencies (respectively, +6.8% and -3.7% as compared to negative control feed, TN Belgium, 2004).

It is concluded from *in vitro* and *in vivo* research that a combination of MCFA and OA (Selacid<sup>®</sup> Green Growth) offers a cost-effective alternative to AGP in pig nutrition.

## A blend of acids for an optimised stomach function

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Stressed animals, certainly recently weaned piglets, get in trouble due to an impaired acid secretion in the stomach. The barrier mechanism of the stomach against pathogenic bacteria is reduced; optimal growth of e.g. *E. coli* occurs at a neutral pH, but for an effective elimination of *E. coli* a pH as low as 3.6 is required in case only inorganic acids such as HCl are present. The digestion of proteins is also impaired, since the activity of the proteolytic enzymes in the stomach requires a pH lower than 5 [1] and is optimal only at pH = 2.3 [2]; poor digestion of protein also results in a misbalance of the flora in the small intestine.

Acid production and stomach pH are dynamic. Within each animal, the HCl secretion starts at the moment of feed intake only and several minutes are required before the lowest pH is reached. In part of the animals in the flock, the pH may reach a value below 2, while the average over the flock may be situated between 3 and 5; the most problematic part of the flock may not shed sufficient HCl to reach a value of 5. Also the flora is variable: it quantitatively ranges from  $10^3$  to  $10^7$  cfu/gram at the exit of the pylorus, while the nature of the organisms present is also highly variable [3].

Determination of the acids present in the stomach of healthy animals is an indication of what a good mixture of acids should represent. Figures are found in the literature of Ehlert et al. [4] who quantified different types of acids in the stomach content of piglets at the age of 7 weeks; the piglets were weaned 2 weeks earlier, thus at the age of 5 weeks: 30 to 80 mmol of lactic acid (= 2.72 – 7.26 g/kg DM), 60 to 180 mmol of HCl (= 2.19 – 6.56 g/kg DM) and 25 to 90 mmol of VFAs (some 1.85 – 6.67 g/kg DM) or a total of 115 to 350 mmol of acid pro kg of dry matter. In the laboratory, one can also determine the quantity of acid required to reduce the pH of compound feed to e.g. pH = 3; for an average piglet starter diet this is some 300 mmol HCl pro kg of feed [5]. This 300 mmol would correspond with e.g. 10.94 kg of HCl or 18.02 kg of acetic acid pro MT of feed.

Adding such quantities to commercial feed is perhaps not feasible from a financial viewpoint. Neither are those quantities required for all animals, since most of them already secrete fair quantities of gastric acids. The nutritionist has to define what “safety level” he can afford.

Commercial products should provide the best possible mix to cope with the wide variation:

- Inorganic acids must immediately release their  $H^+$ , thus reducing the pH in the stomach. Orthophosphoric acid is a good choice, since it releases as much as 3 protons and thanks to the low  $pK_a$ -value ( $pK_1 = 2.15$ ) continues to do so till the optimal pH of 2 is reached.
- Organic acids may also release their  $H^+$ . Depending on the  $pK_a$  values of the concerned acids in relation to the stomach pH, they may also remain undissociated. Even though dissociation is required for pH reduction, their intrinsic bactericidal activity is only valid in the undissociated form. Undissociated acids pass the bacterial membrane, reduce the pH in the cytoplasm and thus kill the bacteria.
- By providing a several organic acids, the variability in the flock and the variation over time is countered. The following variation in stomach pH is considered:
  - pH > 5,0, all acids release their  $H^+$  for pH reduction. At this pH level, this is the most efficient mode of action: reducing the bacterial growth rate and activating pepsin from pepsinogen.
  - $5,0 > \text{pH} > 3,0$ , at pH =  $pK_a$ , each acid is 50 % dissociated (= pH reducing) and 50 % undissociated (= bactericidal). Thus, with reducing stomach pH the mixture passes from the dissociated form to the undissociated form; the higher the gastric HCl

secretion by the animal, the higher the bactericidal effect of the organic acids. At pH = 4.88 the 50/50 ratio is reached for propionic acid, while this is the case for formic acid at pH = 3.74. So, providing a mixture of organic acids is providing bactericidal activity in a larger percentage of the flock of animals.

- pH < 3.0, nearly the entire quantity of organic acids is present in its undissociated form, thus providing optimal bactericidal activity. And also an optimal pepsin activity is reached.
- Emulsifiers in the acid mix, prevent the organic acids from forming globules, thus ensuring an even spread over the feed bras.
- Natural extracts, such as Oregano extract, further enhance the antibacterial activity of the mixtures. Thymol and carvacrol degrade the bacterial cell membrane and inhibit albumin synthesis in the pathogens.

The efficiency of adding acids to animal feed is improved by using an optimised blend of inorganic and organic acids, emulsifiers and eventually natural extracts. Economic efficiency is maximised in all animals, even at suboptimal dosing level rates.

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## Organic acids and essential oils in AGP free diets

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AGP free diets have been part of the range of feeds for feed milling organisations for several years now. In the beginning feed mills looked for one product that could replace the function of the AGP. During the development of AGP free diets and during the first experiences of such diets in practice, it has become clear that it will be difficult to replace AGPs just by one feed additive, and no further additives or changes to feed compositions. Especially in poultry, nutritionists have realised that more changes are necessary.

Organic acids have been considered as first alternative for AGPs. Their function is also antibacterial, one of the important functions carried out by the AGPs. However acids also have a pH lowering effect. Both effects, on pH and antibacterial, are responsible for improved growth and feed conversion. Those effects are described for a wide range of organic acids. The antibacterial mode of action of organic acids is that they are capable of passing the cell wall of pathogens. This is only possible for the complete acid molecule and not for the dissociated acid anion. The result of this is that organic acids show their maximum effect in lower pH environments.

AGPs are effective both in the stomach and in throughout the intestines. Organic acids can effectively reduce the bacterial contamination in low pH environments like the stomach, and keep numbers relatively low afterwards, however organic acids are somewhat less efficient under the conditions of the intestinal tract. Antibacterial effects, like reported for organic acids, are also reported for several essential oils. Although their efficiency related to dosage rate is high, their economic efficiency is generally low caused by the high cost / kg of essential oil. Some of the oils however show synergy with organic acids. Theoretically this can be explained by an effect of the essential oils on the cell wall, making them more permeable. This increases the migration of organic acids in the cell wall, improving the efficiency of the organic acids also at higher pH levels.

Results, *in vitro*, in poultry and in swine, show good effects against pathogens like *E.coli* and on animal performance. So far also practical results are very promising. Further research needs to determine the limitations and economics of scale of such synergistic formulas in practice.

## **Brewer's yeast cell walls: a proven alternative for avilamycine in piglet feed**

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Since the use of antimicrobial growth promoters in animal feed will be prohibited in the European Union as from 2006, feed producers are now searching for feed ingredients that can correspond to these antibiotics. It is often recognised that mannanoligosaccharides (MOS) and  $\beta$ -glucans are beneficial to animal health. The cell wall of brewer's yeast cells contains both MOS and  $\beta$ -(1,3)-(1,6)-D-glucans. Biolex®-MB 40 is a brewer's yeast product that consists primarily of extracted, intact, yeast cell walls. Mannanoligosaccharides are known to be able to bind to receptors of the intestinal cell wall, specific pathogenic bacteria and mycotoxins. It is assumed that  $\beta$ -glucans have a stimulating effect on the  $\alpha$ -specific immune system. In order to find a reliable alternative for antibiotics in piglet feed, Denkavit Nederland has compared a traditional antimicrobial growth promoter with the brewer's yeast product Biolex®-MB 40.

Two trials were conducted at the experimental farm "De Grutto" of Denkavit Nederland. A total of 606 piglets (crossbred F1(NLxGYZ)xTempo) were weaned on average at 26 days after birth. The piglets were weighed and in both trials, the animals were equally divided in three groups with a total of 202 piglets per treatment. The piglets were housed in pens of eight animals. Piglets of the control group (CO) were fed a standard weaner diet (NE 10.0 MJ, Lys 1.2%) including organic acids (Denkacid Dry) but without additional antimicrobial growth promoters or other additives. The other piglets received the same weaner diets which were either completed with 40 ppm avilamycin (AV) or with 0.2% Biolex®-MB 40 (MB). The diets were fed over a period of 13 days. Feed intake was measured per pen. After the trial period, the piglets were weighed again. Growth and feed conversion rate were determined. Compared to CO, growth of AV increased with 6.4% whereas growth of MB increased 8.0%. Feed intake of AV and MB were respectively 1.9% and 3.4% higher compared with CO. Feed conversion rate of AV and MB improved with 4.4% and 4.2% respectively.

These trials show a clear improvement of the technical results of piglets fed weaner diets either completed with a traditional growth promoter or with brewer's yeast cell walls, compared to piglets receiving a standard diet without these supplements. Furthermore, the supply of brewer's yeast cell walls to piglets on an experimental farm tended to give a higher growth, an increased feed intake and a comparable feed conversion rate compared to piglets that received a diet including an antimicrobial growth promoter. Therefore, Biolex®-MB 40 seems to be a proven alternative for avilamycine in piglet feed.

## Use of zinc-bacitracin and mannan oligosaccharides in broiler chickens infected with necrotic enteritis

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The occurrence of necrotic enteritis (NE) caused by  $\alpha$ -toxin of *Clostridium perfringens* is a common problem in modern broiler production. *C. perfringens* is a natural inhabitant of the intestine of broiler chickens and when present at low numbers the bacteria is considered to be part of a healthy and stable intestinal microflora. However, damage to the intestinal mucosa through coccidial infection or a change in the normal intestinal microflora as a result of a change in diet can predisposes birds to the rapid proliferation of *C. perfringens* leading to reduced growth performance or death. Antibiotic growth promoters (AGPs) are commonly used to control the occurrence of NE. Increased occurrence of bacterial resistance to AGPs and the worldwide trend to reduce inclusion of AGPs in broiler diets has forced the poultry industry to search for new methods to prevent NE.

In this study the efficacy of a mannan oligosaccharide derived from the outer cell wall of a specific strain of yeast (Bio-Mos™, Alltech, Inc.) was compared to commonly used AGP (zinc-bacitracin) using a NE challenge model. The challenge model consisted of a dual infection with *Eimeria* spp. on day 9 followed by the challenge with *Clostridium perfringens* (day 13, 14 and 15). The effects of Bio-MOS on the resistance and resilience of broiler chickens to *C. perfringens* challenge were investigated by offering six dietary treatments. These were: (i) control- no challenge; (ii) negative control – no additives + challenge; (iii) positive control with monensin (1000 ppm) and zinc-bacitracin (50 ppm) + challenge; (iv) negative control with Bio-Mos 2 kg/t starter, 1kg/t finisher) + challenge; (v) negative control with monensin and Bio-Mos + challenge; (6) positive control with monensin, Bio-Mos and ½ zinc-bacitracin (25 ppm).

Zinc-bacitracin was completely effective against NE as no bird given the antibiotic had *C. perfringens* in the intestine or NE lesions. Bio-Mos alone failed to prevent the outbreak of NE but numerically reduced NE lesion scores and Cp counts in the intestine. Adding Bio-Mos in combination with a coccidiostat successfully prevented the outbreak of NE. Addition of Bio-Mos to broiler diets enhanced absorptive capacity of the small intestine under Cp challenge. Although Bio-MOS could not give the birds the same degree of protection against *C. perfringens* infection as the antibiotic did, it numerically reduced the *C. perfringens* count and NE lesion scores in the gut, and also numerically improved the body weight.

Treatment	21-d		42-d		Mortality
	BW (g)	FCR	BW (g)	FCR	%
Unchallenged control	716 <sup>bc</sup>	1.403 <sup>b</sup>	2529 <sup>bc</sup>	1.858 <sup>ab</sup>	0.7
Challenged control	607 <sup>d</sup>	1.540 <sup>a</sup>	2425 <sup>c</sup>	1.869 <sup>ab</sup>	13.3
Monensin+Zn-bacitracin	774 <sup>ab</sup>	1.338 <sup>b</sup>	2703 <sup>ab</sup>	1.738 <sup>b</sup>	0
MOS	621 <sup>cd</sup>	1.609 <sup>a</sup>	2527 <sup>bc</sup>	1.914 <sup>a</sup>	14.7
MOS+Monensin	721 <sup>bc</sup>	1.375 <sup>b</sup>	2660 <sup>ab</sup>	1.732 <sup>b</sup>	1.6
MOS+Monensin+Zn-bacitracin	837 <sup>a</sup>	1.343 <sup>b</sup>	2775 <sup>a</sup>	1.750 <sup>b</sup>	0
P-Value	0.001	0.001	0.003	0.030	---

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## **Mannanooligosaccharides, organic acids and probiotics in diets of piglets from 21 to 39 days of age**

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An experiment was conducted with 200 newly-weaned pigs, maintained from 21 to 49 days of age under sanitary challenge in order to investigate the effects of use of mannanooligosaccharides, organic acids and probiotics on performance, pH of the gastrointestinal tract, diarrhoea score and intestinal morphology. The experimental design consisted of blocks randomised in five treatments, ten replicates and four animals by experimental unit. The treatments were defined by diets: T1 – no additives (control), T2 - mannanooligosaccharides (MOS, Bio-Mos<sup>TM</sup> Alltech, Inc.), T3 - acidifier + probiotic (A+P), T4 - a combination of MOS and acidifier + probiotic (COMB), and T5 - with antibiotic (AGP). Pigs from 21 to 35 days in the control group had higher feed intake than the animals in the other groups, while those fed A+P had lower average daily weight gain when compared with the other treatments. From 36 to 49, and 21 to 49 days, average daily weight gain was higher for ANT when compared with the control diet and A+P. The treatments with additives produced better feed conversion rates than the control diet from 21 to 49 days. Diarrhoea scores and pH values were not influenced by the treatments. At 28 days, duodenal villus height was higher in animals fed antibiotics, MOS and the control diet; however, no treatment effects were found for jejunum and ileum. Diets supplemented with MOS and COMB provided a similar performance to that achieved with AGP for pigs from 21 to 49 days of age.

## **Effect of dietary mannanoligosaccharide on plasma IgG concentrations in piglets**

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Strong immune protection is essential for good piglet health and performance. The first days of life the piglet depends on the passive immune protection, which it receives through the sow's colostrum. As this protection diminishes over time and establishment of the active immune defense takes time, a gap in protection results around the 2nd and 3rd week of life. In order to minimise this immune gap, it is very important that the piglets can take up a maximum concentration of Ig from the colostrum. Means that maximise Ig uptake can help in reducing the risk of disease.

The objective of the present study was to evaluate if mannanoligosaccharide (MOS) has the ability to affect plasma IgG concentrations, thus enhancing colostral Ig uptake in piglets. The trial was conducted in a commercial pig farm in Switzerland. The trial was set up as a complete randomised block design with 9 sows and their litters. Four piglets per sow served as control and four piglets received 0.75 g of MOS (Bio-Mos<sup>TM</sup>, Alltech, Inc.) right after birth and 0.75 g of MOS 24 h later. The MOS was suspended in 10 ml of water and administered orally with a syringe. At the age of 3 days and 17 days blood was taken by jugular venipuncture from all piglets and plasma IgG concentrations were determined by a radial diffusion assay. Data were analysed by ANOVA.

The application of MOS tended to enhance plasma IgG concentrations 3 days after birth (control: 13.23 mg/ml; MOS: 15.90 mg/ml; SE: 1.18;  $P < 0.15$ ). Data for day 17 could not be evaluated as most of the samples were below detectable limit of the analytical assay ( $< 6.25$  mg/ml). The data suggest that early application of MOS could have the potential to enhance IgG uptake from colostrum in neonates. This novel approach of using MOS deserves further investigation.

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## **Effect of yeast cell wall on performance, intestinal morphology and immune response of broilers chickens fed low or high non-starch polysaccharides diets**

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Yeast cell walls (YCW) is used in poultry nutrition as an alternative to in-feed antibiotics, to improve intestinal health and to reduce productivity losses. The two principal components of the YCW are mannose-based carbohydrates, which may play an important role blocking or inhibiting pathogen bacterial attachment, and 1-3, 1-6  $\beta$ -glucans, which can act as immunological modifiers by stimulation of innate immune system. One experiment was performed to assess the efficacy of YCW on performance, intestinal morphology and immune parameters of broiler chicken fed maize or wheat-barley-rye diets. Four hundred and sixty one day old Ross 308 broilers chicks were used and distributed in a completely randomised design with a 2 x 2 factorial model, with four dietary treatments replicated five times each. Dietary treatments were: i) corn diet; ii) corn diet + 500 ppm of YCW; iii) wheat-barley-rye diet and iv) wheat-barley-rye + 500 ppm of YCW. Feeds did not contain enzymes, antibiotic growth promoter or anticoccidial drugs. Performance was measured from 0 to 43 days. At day 23, samples of jejunum from ten birds per treatment were obtained to evaluate intestinal morphology. Two vaccines vs. Newcastle disease were applied to the birds, at 9 days of age (virus attenuated live vaccine "La Sota" by drinking water) and at 14 days age (emulsified inactivated vaccine by subcutaneous injection in the neck) to stimulate humoral immunity. The antibody titers were measured at 14, 21 and 27 days after first vaccine application. At day 37, individual weights of lymphoid organs (bursa of Fabricius, thymus and spleen) were taken to calculate % relative weight of these organs respect to bird body weight. In overall experiment, YCW increased ( $P < 0.05$ ) body weight gain of birds fed both types of diets (2516 g control vs. 2574g YCW diets) and feed to gain ratio of birds fed maize diets was better ( $P < 0.01$ ) than those fed wheat-barley-rye diets. YCW increased ( $P < 0.01$ ) villous length (from 1025 to 1297 $\mu$ m), mucus thickness (from 39.1 to 70.7 $\mu$ m) and number of mucous cells (from 407 to 1214) with both types of diets. Although no differences between treatments were observed in antibodies titer vs. Newcastle disease vaccine at different days (14, 21 and 27 days post-vaccine), percentage of spleen relative to body weight was reduced ( $P < 0.01$ ) by YCW (0.131 % for control group vs. 0.106 % for YCW group). Weight of Fabricius bursa relative to body weight was numerically higher ( $P > 0.05$ ) in birds fed YCW than those fed control diets (0.213% vs. 0.187%) and YCW increased ( $P < 0.05$ ) the ratio % of relative of Fabricius bursa / % relative of spleen from 1.43 to 2.02.

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## Effect of dietary carbohydrates on the resistance in the gut against colonisation of *E. coli* K88 in piglets

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The gastro-intestinal tract (GIT) comprises many locations differing in ecological circumstances, inhabiting a wide variety of commensally living microbes each contributing to the resistance against colonisation of pathogens. The presence of adequate substrates for the microbes on the different locations is a prerequisite. *In vitro* studies demonstrated that the non-digestible carbohydrate (NDC) fraction of diets, which are potential substrates for microbes, may differ in rate of fermentation and are depending on diet formulation. Therefore, we studied the relationship between fermentation properties of the NDC fraction of diets fed to piglets on the resistance against *Escherichia coli* K88 colonisation. After an oral inoculation of *E. coli* K88 the excretion patterns of *E. coli* K88 in rectal faeces were determined.

The experiment was conducted with three groups of 16 cross-bred piglets at eight weeks of age. The animals were fed one of three diets, differing in fermentation kinetics as determined by a gas production test after pre-digestion [1]. Diets 1 and 2 comprised mainly fast and slow fermenting NDC, respectively, and the NDC fraction of diet 3 was intermediate. The diets were iso-energetic and standardised for ileal digestible protein. No anti-microbial compounds were added to the diets. The piglets were challenged by an oral dose with  $10^8$  cfu K88 positive enterotoxigenic *E. coli* (O149K91 K88), according to Van Leeuwen [2]. Criteria for allocation of piglets to the respective treatments were litter and body weight. Piglets were individually kept and faeces were collected daily by rectal stimulation. Moreover, at day 4 and 6, dissections on five and eleven piglets, respectively, were conducted to determine the occurrence of *E. coli* K88 in segments of the jejunum. Determined parameters were: dry matter (DM) contents in rectal faeces over a period of three days pre- and six days post-challenge, percentages of piglets with *E. coli* K88 positive faeces over a period of six days post-challenge, and the percentages of piglets which were positive on the occurrence the *E. coli* K88 in jejunal digesta collected at dissection.

At days 2 and 3 the DM contents of the faeces were on average significantly ( $P < 0.05$ ) lower compared to the pre-challenge period and decreased compared to the 4-6 days post-inoculation period. At days 3 and 4, the numbers of *E. coli* K88 positive piglets was maximum. Results show differences in faecal excretion patterns of the inoculated bacteria between dietary groups. Feeding the fast fermentable NDC the numbers of piglets excreting *E. coli* K88 in the faeces was lower and the period of excretion was shortened compared to the piglets fed the slow fermentable NDC diet. Also the percentages of piglets with *E. coli* K88 positive digesta in the small intestine were higher in the piglets fed the mainly slow fermentable NDC diet. The results of the piglets fed the mixture of fast and slow fermentable NDC, were intermediate. These observations indicate that fast fermentable dietary NDC improve gut resistance against non-indigenous microbes like *E. coli* K88 in piglets.

The results of the present study support the importance of the inclusion of fermentation characteristics in diet formulation as a tool for intestinal microflora management.

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## Effect of inulin in broiler feed on the technical performance of broiler chickens

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Inulin is a blend of fructan chains found widely distributed in nature as plant storage carbohydrates. Most of the inulin commercially available today is extracted from chicory roots. Chemically, inulin is a polydisperse  $\beta$ -(2,1) fructan. The chain lengths of chicory fructans range from 2 to 60 fructose units, with an average degree of polymerisation of 10. The unique aspect of the structure of inulin is its  $\beta$ -(2,1) bonds, which prevent inulin from being hydrolytically digested in the upper tract of monogastric animals, making them available for fermentation to short chain fatty acids by the intestinal bacteria. This property makes inulin a so called prebiotic [1]. Normal intestinal microflora such as *Lactobacillus* or *Bifidobacterium* use inulin for fermentation more efficiently than other groups of bacteria and produce short chain fatty acids and lactate on inulin. This process results in an acidic environment which suppresses the growth of putrefactive proteolytic bacteria [2]. These bacteria produce biogenic amines, ammonia, toxic phenols and volatile S-containing components in the distal part of the intestine, and will compromise animal health and growth performance [3]. In this way, inulin may enhance performance responses in monogastric animals and may be seen as an alternative for antimicrobial growth promoters. Therefore, the objective of this experiment was to study the effectiveness of adding inulin (Frutanimal<sup>®</sup>) in broiler feed on the technical performance of broiler chickens.

The experiment included 5 treatments with 6 replicates of 170 Ross 308 broiler chickens each. The experimental treatments consisted of 0.25%, 0.50% and 1.00% Frutanimal<sup>®</sup> in starter feed. Furthermore, a positive control treatment with 10 mg/kg avilamycin and a negative control treatment without additions were included. In each floor pen 85 females and 85 males were placed. Feed intake, birth weight and mortality were determined at day 15 and 37. The litter quality was visually scored from 1 (bad) to 10 (excellent) by 4 experienced persons at day 21. All experimental feeds were formulated according to Dutch practical standards to meet nutrient requirements of broilers. All experimental feeds were pelleted with steam and provided as pellets.

Inulin in starter feeds had a positive effect on bird performance in the experimental period of 0-37 days. An inclusion rate of 1.00% in the starter diet gave the best results, which was nearly as good as avilamycin in starter and grower feed. It is likely that this effect is a prebiotic effect but this has to be proven by experimental microbiological data. Inulin did not improve litter score compared to the control feed without additions. This experiment demonstrated that broiler feed without antimicrobial growth promoter resulted in significantly lower broiler performance if no alternatives were used.

Inulin (Frutanimal<sup>®</sup>) in starter feed enhances bird performance, likely according to the prebiotic concept.

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## On the influence of $\beta$ -glucans from *Saccharomyces cerevisiae* on sow and litter performance during lactation

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In search of alternatives for antimicrobial growth promoters various feed additives are used in farm animal feeding to improve animal health and performance. Among these are 1,3/1,6- $\beta$ -D-glucans extracted from yeast cell walls. They are supposed to stimulate the immune system thus enhancing animal performance as it was shown in previous studies with various types of animals [1].

To prove this, a trial was conducted feeding cell wall extracts from the yeast *Saccharomyces cerevisiae* to fifteen sows from a commercial herd between day 112 of gestation and weaning (day 21). The animals were divided into three groups of five sows each in the experiment and housed into individual farrowing crates. Diets consisted of a standard barley-soybean meal (13.0 MJ ME/kg, 17.5% XP) and were supplemented with 0 g (control), 4 g (group 1) or 2.2 g (group 2)  $\beta$ -glucan per sow and day, respectively. From day 112 of gestation until farrowing sows were fed restrictively with 1.5 kg of the lactation diet which was then increased daily by 700 g until *ad libitum* intake from day 6 of lactation on. Creep feed was offered to suckling piglets starting on day 10 after birth. Sows and piglets had free access to tap water.

Significant treatment effects on litter performance were not observed. The number of piglets born alive varied insignificantly between 10.2 (control group) and 12.2 (group 2). However, these differences were not due to  $\beta$ -glucan-supplementation so that cross-fostering occurred within 24 h *post partum*, irrespective of treatment. Nonetheless, there was a positive tendency towards higher litter weight at weaning for group 1 amounting to 11.9 kg compared to the control group ( $p = 5,6\%$ ). Moreover, litter weight gain was 8.5 kg higher than in the control group ( $P > 0.05$ ).

The results show that under practical farming conditions improved animal performance may be possible by use of  $\beta$ -glucans derived from yeast cell walls. However, further research is necessary to understand the immunological mechanisms forming the basis of this amelioration.

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## Use of different essential oils in rearing piglets

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Until the nineties antimicrobial growth promoters were used successfully to increase performance and feed conversion ratio as well as to decrease environmental burden. With the upcoming ban of these feed additives the search for alternatives is forced. More and more herbal substances and essential oils are used in animal feeding to increase feed intake by enhancing appetite or other more or less unknown properties. In *in vitro* studies it was shown that many herbal extracts or components have a partly wide antimicrobial effect. But until now, little is really known about the mode of action of these herbal substances in live stock production. The question is now if such substances can *in vivo* influence the digestive tract of pigs like antimicrobial growth promoters by increasing nutrients conversion and life weight gain.

In a trial with 240 rearing piglets (common use hybrids) the effect of 3 different essential oils on performance data should be evaluated. The animals (120 castrated males and 120 females) had a mean start life weight (LW) of  $6.7 \pm 1.0$  kg. The basal diet containing wheat (40%), barley (30%), soy bean meal (24%), fish meal (1%), soy oil (0.5%) and supplements of vitamins, minerals, trace elements and amino acids (4.5%) in mash form was offered *ad libitum* during the whole trial of 35 days. The animals were divided into 8 groups by individual LW. Group 1 got the basal diet without additives (negative control group) and group 2 got this diet supplemented with 40 mg/kg of the antimicrobial growth promoter avilamycine (positive control group). The feed of the groups 3 to 5 was supplemented each with 100 mg/kg of oregano (*Oregano vulgaris*), clove (*Syzygium aromaticum*) or cassia (*Cinnamomum cassia*). The feed of the groups 6 to 8 was supplemented each with a combination of two of these oils fifty-fifty 100 mg/kg also. The interpretation was made over the whole period (35 days) while measuring feed intake (FI), mean life weight gain (LWG) and feed conversion ratio (FCR).

The results show that none of the additives led to a significant ( $p < 0.05$ ) effect of the measured data. 3 animals dropped out of the trial independent of treatment (without indigestion). A possible effect of essential oils can be influenced by i) resorption, ii) inactivation, iii) digestion or iv) binding conditions *in vitro* or inactivated by other feed compounds.

There is a further research demand concerning the active substances in the oils and the metabolic pathways.

## Evaluation of a product based on essential oils for piglets

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With a total ban on in-feed growth promoters in the European Union from January 2006 and dwindling public acceptance elsewhere, there is a need for safe feed additives that stimulate appetite and maintain animal productivity and health. A combination of essential oils and organic acids is doing just that. A possible mode of action of these products is that they modify the intestinal flora via their bacteriostatic and bactericidal properties.

Based on many years research by the Provimi Group, supported by TNO, a commercial product based on essential oils has been developed (Cinergy). Another commonly used approach in antibiotic free piglet diets is the use of probiotics. Probiotics have been thought to stimulate the development of beneficial bacteria in the intestinal tract over detrimental bacteria via the supply of viable beneficial bacteria.

The objective of the present study was to evaluate the effect of essential oils (Cinergy) or a probiotic (Toyocerin) on animal performance in antibiotic-free piglet diets. This trial was conducted at the Swine Research Centre of SCA-Iberica in Northern Spain. The test contained four treatments groups, a negative control (without a growth promoting agent), a positive control (40 ppm avilamycin), a diet containing a probiotic (100 ppm Toyocerin) and a diet containing Cinergy (400 ppm). The experimental diets were fed for a period of 33 days from weaning at 21 days of age. All four diets contained 4 kg organic acids /Mt in the weaner phase from 1 to 11 days post weaning and 3 kg organic acids /Mt in the starter phase from 12 to 33 days post weaning. Feed and water was available *ad libitum* during the entire experiment.

Table 1. The effect of a probiotic or a mixture of essential oils (Cinergy) on technical performance of piglets.

Additive	None	Avilamycin (40 ppm)	Probiotic (100 ppm)	Cinergy (400 ppm)	P-value
Number of pigs	195	195	184	197	
Initial wt (kg)	6.46	6.44	6.41	6.45	0.998
Final wt (kg)	14.86 <sup>bc</sup>	15.05 <sup>b</sup>	14.06 <sup>c</sup>	16.58 <sup>a</sup>	0.001
Feed intake (g/d)	332 <sup>bc</sup>	344 <sup>b</sup>	314 <sup>c</sup>	367 <sup>a</sup>	0.001
Gain (g/d)	255 <sup>bc</sup>	261 <sup>b</sup>	232 <sup>c</sup>	307 <sup>a</sup>	0.001
FCR (g/g)	1,33 <sup>b</sup>	1,34 <sup>b</sup>	1,38 <sup>b</sup>	1,19 <sup>a</sup>	0.032

Overall, from weaning till 33 days post weaning, the mixture of essential oils significantly improved feed intake, live weight gain and feed conversion ratio in comparison with all three other treatments. On the contrary, the inclusion of the probiotic to the diet showed a numerical decrease in gain and feed intake.

This study supports the conviction that carefully chosen combinations of essential oils provide a natural solution to the challenge of maintaining animal performance and welfare in the post-AGP era.

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## The influence of botanical extracts on growth performance of broiler chickens

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As a consequence of the increasing concern about the potential for antibiotic resistant strains of bacteria, the European Commission decided in 1999 to ban 4 commonly used feed antibiotics. Today, the European Union has also signalised its intention to remove the other so-called antibiotics growth-promoter (AGP) by 2006 (flavomycin and avilamycin / monensin and salinomycin). Among non-therapeutic alternatives, botanical components (herbs and essential oils) showed efficient results on growth performance of broilers.

Therefore, a production trial was carried out to evaluate the efficacy of the preparation of natural plant extracts (AEN 700) on growth performance of broilers in stress conditions (bad cleaning conditions and summer time temperatures). In order to increase the stress conditions, no cleaning of the room was performed from the previous trial. Used litter was removed but neither water cleaning nor disinfection was performed before adding the clean litter. A total of 512 1-day (Ross 308) male broiler chickens were randomly distributed in 16 floor pens (32 animals per pen) during 5 weeks. Live weight (LW) of animals was weighed per pen (replicate) at arrival, at d 21 and d 35. Average Daily Gain (ADG) was calculated thereafter. Feed supplies and refusals were weighed to calculate Average Daily Feed Intake (ADFI). Both the Feed to Gain Ratio (FGR) and the European Productivity Index (EPI) were calculated as technical parameters. Animals were fed *ad libitum* with a starter feed, from d 0 to d 21, then with a grower one till 35 days of age. Both feeds were based on wheat-barley-soy and formulated according to normal broiler requirements (Starter: ME 3000 kcal/kg, CP 19.50%, Lysine 1.15%; Grower: EM 3100 kcal/kg, CP 18.50%, lysine 1.10%). Data were analysed according a factorial design by mean of analysis of variance.

No special incidences were recorded during the trial, and no outliers were detected. Animals suffered thermal stress as the trial was performed in Spanish early summer time. Temperatures averaged 28.3°C during the last four weeks of trial. Natural plant extracts significantly improved the FGR in both periods of the trial (1.5% and 1.1% for the first and second period respectively) as well as in the whole trial period (1.1%). Natural plant extracts also significantly improved the ADG in the second period (4.2%) and tended ( $P = 0.062$ ) to improved the final BW (3.0%) and the ADG (3.0%) in the whole trial period. The EPI also was numerically improved (2.3%).

This production trial confirmed that natural plant extracts could be used as non-therapeutic substitutes to AGP.

## The effect of a plant extract preparation in turkey feeds without antibiotic growth promoters

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Besides probiotics, prebiotics and organic acids, plant extracts can be used as feed additives of natural origin [1]. Plant extracts are standardised products and are characterised by a number of activities like stimulation of appetite, secretion of endogenous enzymes, immunostimulation and antimicrobial activities. The basic ingredients of plant extract preparations are so-called secondary plant metabolites like allylisothiocyanates, thymol, carvacrol, cinnamaldehyde, capsaicin, piperin and numerous other active agents. Improvements of plant extracts may be comparable with the results of broiler chickens fed a diet supplemented with in-feed antibiotic [2].

Organic acids appear to be potential alternatives for antimicrobial growth promoters. Fumaric acid has the advantage to be crystalline and odourless. Inclusion of fumaric acid in broiler diets at levels from 0.125% till 2.00% resulted in improvements in growth rate, feed utilisation or both [3,4]. Fumaric acid seems to be also attractive in turkey feeds for its properties and effects while it can be easily combined with other additives. In the present study the effect on turkey performance and litter quality was evaluated by supplementing a wheat- and soybean meal-based turkey diet with a plant extract preparation which contained *Capsicum* oleoresin, cinnamaldehyde and carvacrol (Xtract 6930™) and a combination of the latter with fumaric acid.

Commercial male BUT Big 6 turkeys were used in the period 0-84 days (12 weeks) of age. Treatments consisted of a control diet (diet 1) containing no AGP, a diet containing diet 1 plus Xtract 6930™ at 150 g/t (diet 2) and a diet consisted of diet 2 plus different levels of fumaric acid: 0-13 days – 5 kg/t, 14-27 d – 3.75 kg/t, 28-84 d – 2.50 kg/t. Feed and water were provided *ad libitum*. Each treatment consisted of six replicates of 45 turkeys in a floor pen, which was the experimental unit. Experimental observations were body weight, feed intake and mortality at day 84. Litter quality was visually scored by 5 experienced persons in week 7, 8, 10 and 12.

In the experimental period from 0-84 days, Xtract 6930™ in turkey feeds tended to an increased performance of the birds by a slightly higher feed intake and a lower feed conversion ratio (FCR). FCR corrected for body weight was significantly lower with Xtract 6930™ (- 1.8%). Xtract 6930™ combined with fumaric acid in the turkey feeds fortified these effects (- 2.5%). There were no differences in mortality, and overall mortality was rather low. Xtract 6930™ alone had no effect on litter quality but in combination with fumaric acid a better litter quality was observed in week 8.

Xtract 6930™ alone or in combination with fumaric acid in turkey feed without antibiotic growth promoters significantly improves turkey performance during the first 12 weeks of live.

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## **'Rumen-up': new plants and plant extracts to decrease methane and nitrogenous emissions from ruminants and to alleviate nutritional stress**

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The aim of this EC FP5 project (QLK5-CT-2001-00992) was to develop new plants or plant extracts as dietary additives for ruminants. Five hundred plant materials were collected from botanical and industrial collections, based on criteria such as traditional uses and likely phytochemical content. They were evaluated for their ability to prevent lactic acidosis and bloat, to decrease formation of the greenhouse gas, methane, and to decrease nitrogen excretions by inhibiting ruminal proteolysis and protozoal activity. The aim of the project was to deliver plant-based, sustainable solutions to these problems in ruminant livestock production. The samples were also investigated to ensure that potentially useful samples had no detrimental effect on the other basic functions of the fermentation, such as fibre digestion and volatile fatty acid production. A total of 23 samples was identified to have potential for development as feed additives which could manipulate fermentation in one or more of the target areas without having detrimental effects on overall fermentation. The application of the samples for these purposes is the subject of a pending patent application.

A smaller number of samples was then taken forward for more detailed experimentation on persistence and dose response. These included *Bellis perennis* (daisy, antiprotozoal), *Carduus pycnocephalus* (Italian thistle, antimethane), *Gentiana asclepiadea* (gentian, antiprotozoal), *Knautia arvensis* (field scabius, antiproteolytic), *Lactuca sativa* (lettuce, antiacidosis), *Peltiphyllum peltatum* (Indian rhubarb, antiproteolytic) and *Urtica dioica* (stinging nettles, antiacidosis). In *in vivo* tests of acceptability and toxicity carried out in sheep, none of the short-listed samples gave any indication of toxicity or problems with feed intake. In terms of dose response, the materials were generally not potent at low concentrations. Most would most likely have to be included in the diet at 3-5%, unless more potent cultivars could be found.

Differential solvent extraction and HPLC are being used to identify the likely active phytochemical components. Antimicrobial effects of samples are being assessed using both cultural and molecular profiling. Production-type trials are being carried out with three of the most promising samples. The project will terminate in 2005. Its website is at [http://www.rowett.ac.uk/rumen\\_up/](http://www.rowett.ac.uk/rumen_up/).

## 'REPLACE': plants and their extracts and other natural alternatives to antimicrobials in feeds

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Growth-promoting antibiotics will be banned in the European Union by 2006. Livestock producers need alternative means of obtaining similar production benefits to maintain profitability and competitiveness against overseas producers, including the US, where such restrictions do not exist. Ways must also be found to improve the healthiness and safety of animal products reaching the consumer, including those from organic farming. This EC-supported FP6 project (FOOD-CT-2004-506487), will examine plants, plant extracts and other natural materials as safe alternatives to feed antimicrobials. The materials will be derived from 500 samples of plant materials collected as possible feed additives for ruminants [FP5 project, Rumen-up, QLK5-CT-2001-00992], plus some additional natural materials likely to be useful in non-ruminants. Rumen-up samples, for which a large data set of background information and screening success now exists, will be tested for properties not screened in FP5: their possible impact on human and animal health (*E. coli*, parasites), food quality (fatty acids in ruminant products) and efficient use of natural resources (increased forage use by ruminants). Researchers on pigs, poultry and fish, where the impact of antibiotic withdrawal is greatest, have joined the consortium. The priorities in these species overlap with ruminants, although the precise aims and pathogen species are different. After identifying the most promising candidates for each target, a small number of samples will be taken to animal trials. The project will link fragmented research carried out with different animal species across Europe and provide a platform, via consultation with industry, farmers' and consumers' organisations, veterinarians, botanists, agronomists and economists, for the rational production of a new generation of natural feed additives. The main benefits will be a healthier, safer food chain, increased sustainability of animal agriculture and reduction in its detrimental effects on the environment. The project began in March 2004 and will terminate in 2008. Its website is at [www.replace-eu.com](http://www.replace-eu.com).

## The effect of exogenous enzymes on weight gain, bacterial cell numbers and IgA concentration in the distal gastrointestinal tract of broiler chickens

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In countries where corn is available at a competitive price, it is the first choice for many nutritionists for use in the diets of poultry. This is primarily due to the perception that corn has a relatively high and consistent nutritional value for livestock. However, it has been demonstrated that corn is variable in its chemical composition and nutritional value for poultry and that the use of exogenous feed enzymes can reduce the variability in the feeding value of corn and improve the performance of growing broiler chickens. The mechanisms by which exogenous enzymes mediate their effects are not well elucidated but are known to include improvements in nitrogen and energy retention, reduction in endogenous losses, improvements in feed intake and beneficial effects on the microbial community in the distal gastrointestinal tract (GIT).

In order to further explore the effects of exogenous enzymes on the microbial populations in the ileum and caecum of growing broiler chickens a study was conducted. A total of 585 male broiler chicks were randomly assigned to 9 experimental treatments, with 5 replicate pens of 13 chicks per treatment. The treatments comprised three experimental rations consisting of distinct batches of corn supplemented with 250 and 500g/tonne of Avizyme<sup>®</sup> 1502 (to supply a guaranteed minimum of 300, 400 and 4000 U kg<sup>-1</sup> feed of xylanase, amylase and protease respectively at 500 g/tonne). Diets were formulated to be nutritionally adequate (NRC, 1994) and were fed *ad libitum* throughout the experiment. Body weight gain and feed intake were monitored from day 1 to day 41 and on day 42 3 birds per replicate were killed and the GIT was excised. Ileal and caecal contents were removed and assayed for total bacterial cell numbers and IgA concentration by flow cytometry and ELISA methods respectively. On average, the addition of enzyme to the corn/soy rations numerically improved ( $P < 0.35$ ) body weight gain and significantly improved ( $P < 0.01$ ) feed conversion ratio by 1% (21g) and 3.5% (6 points) respectively, although there were corn/enzyme interactions. Furthermore, birds fed on diets containing exogenous enzymes had, on average, lower bacterial cell numbers in the ileum ( $2.3 \times 10^9$  vs.  $3.1 \times 10^9$ ) and a higher IgA concentration in the caecum than birds fed on the control ration (11,166  $\mu\text{g/g}$  digesta vs. 9,833  $\mu\text{g/g}$  digesta). These results suggest that the improvements in performance associated with enzyme addition may be partially explained by a reduction in bacterial cell numbers in the distal GIT, presumably caused by an improvement in nutrient absorption in the proximal GIT and so a quantitative and qualitative reduction in substrates that are available for microbial metabolism. Furthermore, it can be speculated that the reduction in bacterial load in the lower gut in the present study was partially responsible for the increase in IgA concentration in the caecum, which suggests an improvement in localised immune competence.

It can be concluded that the use of exogenous xylanase, amylase and protease in a corn/soy-based diet for poultry is effective in improving performance of growing broiler chickens. This may be partially mediated through an improvement in nutrient absorption in the proximal gut with a consequential reduction in bacterial numbers in the distal GIT, reducing the pressure on immune responses. The precise mode of action of exogenous enzymes on immune status and bacterial communities are interesting, largely unresolved and require further research.

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## Improved intestinal health and immunity in broiler chicks when fed a combination of Betafin® and Avizyme 1500

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*Eimeria maxima* (*E. max*) infection damages intestinal mucosa and decreases the rate of immunoglobulin A (IgA) secretion into the intestine. Therefore, feed ingredients that can protect the epithelium or reduce the immune suppression caused by coccidiosis could improve the disease resistance. Betafin® (Bet) has been shown to accumulate in intestinal epithelium and support its histological structure during *E. maxima* infection. Avizyme® 1500 (Avi) improves the digestibility of feeds and could ensure improved nutrition uptake also for the intestinal immune cells.

96 Broiler hatchlings were divided into four feeding treatments. The corn-soy feed, containing no AGPs or coccidiostats, was supplemented with or without 1 kg/t of Bet or 0.5 kg/t of Avi, or their combination. On day 14, half of the chicks were challenged with *E. max*, and were sampled for tissue and digesta on day 21. In jejunal digesta, the mean±SE concentration of IgA was 177±21 for healthy controls, 407±162 for healthy Bet+Avi group, 53±11 for *E. max* controls, and 116±41 for *E. max*+Bet+Avi group (NS, N=3 pools/tr.). The mean±SE jejunal crypt-villus ratio was 0.23±0.01 for healthy controls, 1.15±0.10 for *E. max* controls, 0.74±0.05 for *E. max*+Bet, and 0.75±0.06 for *E. max*+Bet+Avi, suggesting that betaine decreased the mucosal damage caused by coccidial infection ( $p < 0.01$ ). Bet+Avi also improved the weight gain (NS), and raised the ileal dry matter content of challenged chicks to the level of healthy controls.

The study suggests that the concentration of IgA in the digesta may serve as a biomarker for improved weight gain and intestinal health.

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## Enhanced IFN- $\gamma$ expression by Progut<sup>TM</sup> in the intestinal mucosa in *Eimeria*-challenged broiler chicken

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The effect of the yeast-based Progut<sup>TM</sup> on intestinal immune responses was studied in broiler chicken. Feed supplemented with 0.3% Progut<sup>TM</sup> was compared with a control starter/grower feed. At two weeks of age half of the birds in each feeding group were challenged with *Eimeria maxima* oocysts. Tissue samples were obtained from the small intestine for measurement of IFN- $\gamma$  RNA by real-time quantitative RT-PCR one week post infection at the age of three weeks. The *Eimeria* infection reduced growth in the birds by approximately 6% and stimulated IFN- $\gamma$  expression at the infection site by four-fold. A further twofold enhancement in the expression of IFN- $\gamma$  was observed in *Eimeria*-challenged chicken with Progut<sup>TM</sup>-supplemented feeds when compared with the control challenge group. Effective production of IFN- $\gamma$  is decisive for the clearance of the infection. Importantly, no elevated expression of IFN- $\gamma$  was observed in healthy non-challenged birds fed with Progut<sup>TM</sup>. Thus, Progut<sup>TM</sup> improved the ability of host defences to respond effectively in a challenge situation but did not cause inflammatory responses by itself. No significant difference in weight at three weeks of age was observed between *Eimeria*-challenged control and 0.3% Progut<sup>TM</sup> group.

In conclusion, feeding supplemented by the yeast-based Progut<sup>TM</sup> stimulated development of appropriate mucosal immune responses to fight *Eimeria* infection. The effective IFN- $\gamma$  response is likely to shorten the time needed for clearance of the infection and therefore result in improved growth rates during the growing period.

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## Antimicrobial agents, probiotics, prebiotics and herbal extracts as growth promoters of weanling pigs

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The purpose of this work was to evaluate the probiotics, prebiotics and herbal extracts as alternatives to antimicrobial agents as growth promoter of weanling pigs, based on the performance.

Five treatments were tested: control (basal diet); antimicrobial - basal diet plus antimicrobials (50 ppm of zinc-bacitracin + 50 ppm of olaquinox); probiotic - basal diet plus probiotic (1300 ppm of probiotic - *Bacillus subtilis* and *Bacillus licheniformis*); prebiotic - basal diet plus prebiotic (3000 ppm of Bio-Mos<sup>TM</sup>, MOS, Alltech, Inc.); herbal extract - basal diet plus herbal extracts (500 ppm of herbal extract - garlic, clove, cinnamon, pepper and thyme). One hundred twenty 21 days weaned pigs were used in a completed block design experiment, with 12 replications per treatment and two animals per experimental unit (a barrow and a gilt). The average daily feed intake (ADFI), the average daily gain (ADG) and the feed conversion (FC) were analysed on days 1-14, 15-35 and 1-35 post weaning. The antimicrobial agents improved the ADG during 15-35 (+22%) and 1-35 (+21.4%) days post weaning ( $P < 0.05$ ), compared to pigs fed the control diet. MOS presented similar performance ( $P > 0.05$ ) to the animals of the antimicrobial treatment during 1-14 days post weaning period, improving the ADG (+25.7%) as compared to control. During 15-35 and 1-35 days post-weaning periods, the performance of animals fed MOS was intermediate between the control and antimicrobials. The herbal extracts did not improve the performance of the pigs ( $P > 0.10$ ). The palatability may have restricted the ADFI. The probiotic did not improve the growth performance ( $P > 0.10$ ) on weanling pigs.

Bio-Mos was the most efficient alternative to antimicrobial agents. However, MOS and the other alternatives, must be better studied, in relation to its mechanisms of action, the added amounts and supply strategy in the diet. Thus, it will be possible to improve the growth performance of weanling pigs without the antimicrobial use agent.

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## A novel approach to improve performance in poultry production by using a combination of natural substances

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For an economic production of meat and eggs poultry producers need a healthy livestock that gives optimal performance. In the past antibiotic growth promoters (AGPs) were used to achieve an economic production of livestock animals. Due to the fact that especially low dosages of antibiotics increase the resistance of microbes against antibiotics, especially those which are used as therapeutics, the European Commission decided to ban the preventive use of such chemical compounds. But the economic production of poultry and also the consumers' access to "safe" meat (free of pathogens and residues of antibiotics) have to be guaranteed. Besides improving the management at production sites natural alternatives to AGPs can significantly contribute to control problems in animal production.

It is known that in the first days of life young animals are very susceptible to infections with enteric pathogens because the gut of newly hatched animals is devoid of bacteria. The establishment of a natural microflora in the digestive tract, which is an important barrier against colonisation of pathogenic microorganisms, is a very slow process that can take several weeks. Additionally, the immune system of young birds is working slowly. Therefore intensive research was carried out to find an alternative to antibiotics as AGPs. Several probiotic strains were tested for excluding and inhibiting pathogenic bacteria. Further research was carried out to enhance the proliferation of *Bifidobacteria* in the intestinal environment. Different nutrients, which cannot be converted by the animal and pathogenic bacteria but by the beneficial microflora, were tested for their stimulating effect on *Bifidobacteria*. Further research activities were the development of a Macrophage Activation Test (MAT) to evaluate various substances for their immune-modulating properties.

The outcome of these research activities was a new generation of products (Biomin<sup>®</sup>C-EX and Biomin<sup>®</sup>IMBO) that combine the positive actions of an *Enterococcus faecium* strain (probiotic), inulin (prebiotic), bacterial cell wall fragments and extracts of algae (immune stimulating substances). The probiotic and prebiotic ingredients help to establish and maintain a beneficial and protective gut microflora by competitive exclusion, production of lactic acid and by facilitating the development of beneficial *Bifidobacteria*. Cell wall fragments and phycophytic substances (algal extracts) further improve the positive effect of these components by stimulating the weak immune system of the young animals (positive effect on macrophage activity and phagocytosis). The synergistic effect of these ingredients guarantees a reliable and competitive animal production without the use of antibiotic growth promoters. In several feeding trials it was possible to prove the benefits of this product generation. Body weight was increased, feed conversion was improved, mortality was reduced, faecal pH-values were lowered and finally the organ invasion of pathogens was diminished.

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## Mode of action of a phytogetic product in weaning piglets

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As a result of the ban of feed antibiotics and the search for alternative products, botanicals, herbs and essential oils have recently found increased attention in animal nutrition. But less is known on the mode of action of these products. To get new information, the effects of a phytogetic product and an antibiotic growth promoter on performance, gut microflora, gut immunology and meat safety of weaning piglets are compared.

Three trials with 21 day old weaning pigs were carried out. In each trial 45 pigs were split into 3 groups: a negative control group, the control group (Biomin P.E.P) and a positive control group (avilamycin). On the 22nd day of each trial 12 representative animals (4 per group) were slaughtered to get tissue and chymus samples. The animals got 3 different diets. Starter feed (1-7days), weaner feed (8-21 days) and grower feed (22-50 days). Feed analysis (Weender analysis, contents of antibiotics and essential oils) of every used feed have been made. The zootechnical data (feed conversion rate, daily weight gain) was investigated for each group per trial. From the slaughtered animals chymus of 3 different gut areas (Ileum, caecum, colon) was investigated on following parameters: content of ammonia and lactic acid, content of biogenic amines and fatty acids, pH factor. Investigations of metabolites of essential oils in blood, liver, kidney, spleen and eatable meat (filet and karree) were made. Additionally, 10 different tissues of every slaughtered animal were taken to make the following investigations: (i) molecular biological analyses (quantity and quality of total RNA as an immune marker); (ii) blood count (marker of immune status); and (iii) histological analyses (morphology of different cell types (lymph nodes, crypts in epithelium of gut)).

This project is supported by 3 universities (University of Natural Resources and Applied Life Sciences, Austria; University of Veterinary Medicine, Austria; Technische Universität München, Germany) and Biomin GmbH, Austria

## **Development of a competitive exclusion product for poultry meeting the regulatory requirements for registration in the European Union**

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Competitive exclusion (CE) is a promising method to protect chicks from infections with pathogenic bacteria. In particular the CE concept is able to increase the colonisation resistance of day old chicks against Salmonella, one of the main important bacterial contaminant transmitted by poultry. A project has been conducted successfully with support of EU-funding to develop a feed additive meeting the regulatory requirements for registration. The project leads to a total quality concept for animal production – responding to changed regulations as well as changes in consumer perception.

The animal production in the European Union faces a very difficult situation, due to the foreseeable total ban of antibiotic growth promoters and the understandable consumer objections to their intensive use. Production of feed additives affords the opportunity to face occurring demands and changed regulations as prior relevance to implement new strategies and innovative concepts. There is a demand to react for both economic and social reasons. Poultry industry must be provided with alternatives to antibiotics to ensure competition for European producers. Consumer health has to be considered providing safe food of reliable and high quality.

Main impact of the project:

- substitution of antibiotics as growth promoters in animal production
- improvement of health in animal farming
- decreased occurrence of antibiotic resistant micro-organisms
- sound production processes based on natural resources
- safe and reliable food for consumers

The outstanding structure of this CRAFT project allows to follow the technological steps from the naturally derived source to the refined product. It covers at the same time all relevant scientific fields according to the partner's scope:

- microbiological aspects and design of the competitive exclusion product
- formulation and application of the product
- registration and regulatory aspects (investigate risk of antibiotic resistances)
- development of molecular biological test systems for
  - quality control
  - feeding trials
  - control of hygienic parameters
- application aspects in feeding trials and large field trials
- general improvement of
  - animal well-being
  - consumer confidence
  - market developments
- improvement of hygienic concepts regarding “farm to fork” principles
- information- and training concepts

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## Fermented liquid feed and fermented grain to piglets – effect on gastrointestinal health and growth performance

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Fermented liquid feed (FLF) has gained interest in Europe due to the need for finding alternatives to antibiotic growth promoters. Feeding swine with FLF has positive effects on the gastrointestinal microflora (e.g. reduction of coliform bacteria, *Lawsonia*, *Salmonella*). Moreover, piglets fed with FLF do not have to learn separate patterns of feeding and drinking behaviour. The latter can help preventing the gastrointestinal disorders associated with weaning due to dehydration and a drastic drop of feed intake. However, studies have pointed to reduced lysine content in FLF compared to non-FLF due to microbial fermentation, which may impair performance of the animals. Improved growth performance has been measured in heavy pigs by fermenting only the grain and adding the remaining ingredients of the diet, including the synthetic amino acids, immediately before feeding compared to animals fed non-FLF.

Three dietary treatments were designed using a weaner diet: (i) dry feed (DRY), (ii) fermented liquid feed (FLF), (iii) fermented grain liquid feed (GRAIN). The FLF was prepared by storing the feed containing all the ingredients and water in the ratio 1:2.5 in a closed tank under agitation at 20°C for five days before being offered to the pigs. The GRAIN was prepared similarly but storing only the grain (barley and wheat). At feeding, 50% of the content of the tanks containing FLF and GRAIN was taken out and then replaced with an equal amount of fresh feed and water. Additionally, for the GRAIN treatment, the cereals were mixed with the remaining ingredients of the diet in a bucket immediately before being offered to the animals. The effect of feeding the three dietary treatments to weaners on aspects of gastrointestinal ecology and on performance was investigated. Fermenting only the cereals promoted the growth of yeasts to a higher level than fermenting the whole diet (FLF), whereas the density of lactic acid bacteria was similar in both treatments. Both, fermenting the cereals alone (< 3 log cfu/g) and fermenting the whole diet (< 3.5 log cfu/g) reduced the number of enterobacteria compared to the DRY diet (5.4 log cfu/g). The FLF diet had higher concentration of acetic acid and lactic acid than the GRAIN diet, whereas the latter contained more ethanol. The counts of lactic acid bacteria grown at 25°C were higher in the FLF and GRAIN groups compared to the DRY group ( $P \leq 0.04$ ) in the stomach and distal small intestine, whereas no differences were detected in the hindgut. Although the enterobacteria counts in the small intestine and hindgut were numerically lowest in the GRAIN group, the values of the three diets were not different. The number of yeasts grown at 25°C and 37°C along the GIT was highest in the GRAIN group compared to the other two dietary groups (4.7-7.2 log cfu/g for the GRAIN and 3.3-6.9 log cfu/g for the DRY and FLF groups). The concentration of lactic acid in the proximal GIT was highest in piglets fed the FLF diet, whereas the piglets fed the GRAIN diet had the highest concentration of ethanol. The effect of diet on growth performance during six weeks post-weaning showed a higher daily body weight gain and daily feed intake in the DRY fed piglets compared to the other dietary treatments. However, although the numerical values were highest for the DRY group, no effect of diet on gain to feed ratio was detected.

The results from the present study indicate that fermented grain contains higher counts of yeasts and ethanol than FLF, which contains higher concentrations of lactic acid. These characteristics are reflected along the GIT of piglets fed these diets. Growth performance is not significantly different in piglets fed fermented grain liquid feed and fermented liquid feed.

## Feed supplementation strategies for reducing reliance on antibiotics in poultry production

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One of the mechanisms by which growth promoting antibiotics improve animal production may be through their ability to decrease sub acute bacterial disease resulting from the stress inherent in intensive production systems. Our research has demonstrated that subacute respiratory infection with *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* results in significant decreases in body weight and feed conversion. The stress of intensive poultry production can lead to changes in the immune response of birds that result in decreased resistance to respiratory infection with opportunistic pathogens.

We have conducted a series of experiments in both broiler chickens and turkeys to evaluate the efficacy of various feed additives for their ability to protect birds from low levels of bacterial challenge both with and without glucocorticoid immunosuppression. Dietary supplementation with 20 g/ton of  $\beta$ -1,3/1,6-glucan, a helical polysaccharide derived from the cell wall of *Saccharomyces cerevisiae*, has shown efficacy in reducing the effects of respiratory challenge of both turkeys and broilers. Body weights and feed conversion of both unchallenged turkeys as well as those challenged with air sac injection of 50-100 cfu of *E. coli* were improved with supplementation. Broiler chickens fed the same product at 20 g/ton for 7 days prior to challenge with 800 cfu of *E. coli* were protected from the adverse effects of challenge on both body weight and feed conversion. However, supplemented birds that were not challenged had lower body weights than non-supplemented, non-challenged birds. Water supplementation of turkeys with vitamin D<sub>3</sub> prevented disease incidence, mortality, and body weight loss due to dexamethasone treatment and *E. coli* challenge. Water supplementation of turkeys with Vitamin E or sodium salicylate prevented mortality and body weight loss in birds challenged with approximately 50 cfu of *E. coli* however again, body weights of supplemented non-challenged controls were lower than those of non-supplemented controls.

These studies illustrate the adverse effects that stress and sub acute respiratory disease can have on poultry health and production and suggest that nutritional supplementation with immunomodulators may be protective in challenging commercial environments. However, such immune modulation may be costly in decreased production values in birds raised in an environment with minimal disease challenge.

## A new surgical approach for post-gastric, intraluminal challenges with specific pathogens, viruses and(or) enterotoxins in conscious piglets

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To replace in-feed antibiotics for pigs, numerous “natural” antimicrobial growth promoters are offered worldwide. In order to evaluate their factual effectiveness against specific pathogens, viruses and(or) enterotoxins, various oral challenge models are applied. However, these models are not consistently reproducible due a relatively large variation in diarrhoea incidences and other response parameters within and between experiments. This lack of consistency in responses of piglets to oral infections is partly ascribed for a variable rate of pathogenic survival in the stomach. Intra-gastric HCl is recognised as a permeabiliser being able to disrupt the outer membranes of Gram-negative bacteria, which leads to sub-lethal or lethal injuries of their LPS layers [1]. In consequence, the growth and colonisation of these bacteria in the post-gastric region of the digestive tract (as manifested by diarrhoea) may be limited, but not necessarily as a result of adding any specific antimicrobial growth promoter. This implies that the claims for some antimicrobials growth promoters may be inappropriate and give rise to false expectations. Therefore, a new surgical approach for post-gastric intraluminal challenges with specific pathogens, viruses and(or) enterotoxins in conscious pigs is proposed in the aim to obtain a better reproducibility of the infection effects in vivo and a more objective evaluation of tested alternatives to antibiotics.

Twenty suckling piglets at 21 days of age were operated under general anaesthesia to insert polyethylene catheters (i.d. 2.00 mm, o.d. 2.24 mm) into various parts of the small intestine as follows: group 1, intraduodenal; group 2, intrajejunal; group 3, intra-ileal; group 4, intraduodenal + intrajejunal + intra-ileal). A week later, they were weaned and on day 4 post-weaning, each group was infected via the catheters with *E.coli* O157:K88 (2 x 2 ml of broth culture grown for 15 h at 37°C, which was infused at 07:00 and 17:00). In the pre- and post-infective periods, intestinal digesta were sampled via the catheters for bacterial enumerations. Also, rectal swabs were taken to check for culture of haemolytic *E.coli*. All piglets accomplished this study successfully and their growth rate was comparable to non-operated piglets (which also underwent an effective oral infection). Irrespective of the intestinal site of *E.coli* infection, consistent symptoms of a mild diarrhoea and greater haemolytic *E.coli* counts were recorded in each animal. The rate of *E.coli* shedding in faeces was the greatest when the piglets were infected at three locations (intraduodenal + intrajejunal + intraileal).

In general, these results indicate that our new surgical approach is suitable for testing antimicrobial potency of antibiotic-alternatives in diets for weaned piglets and this model can be regarded as advantageous to oral infection models

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## The use of *in vitro* methods to evaluate the micro-encapsulation of capsicum oleoresin for its application in growing pig diets

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In response to the European ban of in-feed antibiotics in January 2006 (Directive 1831/2003), the animal feed industry is actively searching for alternatives to improve livestock performance. One such possibility is additives based on plant extracts and their bio-active substances. Among these is capsaicin (trans 8-methyl-N-vanillyl-6-nonenamide), the major pungent lipophilic alkaloid of capsicum fruit (i.e. chili pepper and paprika). Previous studies have characterised it to possess (i) antibacterial activity, (ii) stimulate digestive enzyme and bile secretions, (iii) increase the feed intake, and (iv) induce the vasodilation of smooth muscle. Capsaicin in its natural form is available in liquid form as oleoresin of capsicum. However, conversion of this liquid into powder form by spray dry technology yields a highly irritant product which limits its direct usage as a micro-additive for livestock diets.

In this study a spray cooling technology (Aeromatic Fielder MP11) was used to micro-encapsulate 20% of capsicum oleoresin in a rapeseed hydrogenated vegetable oil matrix to reduce its irritating effect and also to control its release kinetics upon consumption by the animal. As one of the main factors affecting the release of bio-active compounds is particle size, two formulations differing only in particle size distribution were developed (F1 with a mean size of 125  $\mu\text{m}$  and F2 with a mean size of 500  $\mu\text{m}$ ). These were compared in a flow through cell dissolution apparatus to determine the release kinetics of capsaicin at different pH. The release kinetics of capsaicin were significantly ( $p < 0.05$ ) different between the two preparations (100 % capsaicin released in first order kinetics from F1 after 40 minutes and 60% capsaicin release in zero-order kinetics from F2).

As flow through cell apparatus systems are unequipped to measure the release of bio-active substances in the presence of the feed matrix, follow-up studies were performed on F2 using an *in vitro* multi-compartmental, dynamic, computer-controlled system (TIM-1) simulating the digestive environment of the growing pig. Results in the TIM-1 showed a difference in the release of capsaicin from F2 after 8 hours of digestion under fasting conditions alone, or in the presence of the feed matrix (64% for F2 alone versus 50% for F2 with feed). This suggested a possible interaction between F2 and the feed sample.

This preliminary study demonstrates the potential of each individual *in vitro* system in the evaluation of the release of encapsulated feed additives in livestock feeds. Their use in complement, however, provides a more accurate determination before their evaluation in costly live animal trials.

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## Screening method for banned feed additives: avoparcin, zinc-bacitracin, virginiamycin, tylosin, spiramycin

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Recently, the European Union has banned the antibiotics avoparcin, zinc-bacitracin, spiramycin, tylosin and virginiamycin as feed additives. Within the EU-funded research project SIMBAG FEED [1], a qualitative microbiological screening method is developed for the official control of banned antibiotics in animal feeds.

The method consists of an extraction step, followed by a detection procedure. One simple extraction procedure for all targeted compounds is developed, the resulting supernatant is used for the following detection step. For detection of the banned antibiotics three testplates are developed. They make use of *Micrococcus luteus* ATCC 9341 (tylosin, spiramycin, virginiamycin), *M. luteus* ATCC 10240 (zinc-bacitracin, tylosin, virginiamycin) and *Bacillus megatherium* ATCC 10778 (avoparcin). Synergistic antibiotics are added to the test-plates, to increase the sensitivity of the microorganisms. After pipetting the feed extract into wells in the test agar plates and incubation at 25/30°C, formation of zones of inhibition (> 14 mm) indicates the presence of the antibiotics.

In order to distinguish between the five banned antibiotics and (carry over of) antibiotics used as veterinary medicine (e.g. tetracyclines, quinolones, other macrolides,  $\beta$ -lactams, aminoglycosides, sulphonamides) three additional testplates are necessary. The remaining antibiotics (avilamycin, flavomycin, monensin, salinomycin) in category A showed no interference.

In November and December 2004 a collaborative study for the developed method was organised with participation of several European laboratories. This study contained over 3000 samples, around 15 laboratories and four different types of feed. The results of this study will be presented.

With the developed method animal feed can be adequately screened for the banned feed additives. Furthermore it gives the ability to incorporate the detection of the carry over of antibiotics used in feed as medication (e.g. tetracyclines, sulphonamides, quinolones) in the same procedure. The use of microbiological tests is well known in the official laboratories for the control of animal feed in the EU and implementation of a microbiological screening test will be relatively easy.

### References

1. SIMBAG FEED. Project "Screening and identification methods for official control of banned antibiotics and growth promoters in feedingstuffs", funded by EC, DG RTD, Competitive and Sustainable Growth (GROWTH) Programme, Contract no. G6RD-CT-2000-00413.

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## Post-screening method for banned feed additives: avoparcin, zinc-bacitracin, virginiamycin, tylosin, spiramycin

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The recent ban of antimicrobial growth promoters (avoparcin, zinc-bacitracin, spiramycin, tylosin and virginiamycin) by the European Union has led to the necessity to develop screening, post-screening and confirmation methods for the official control of these substances in animal feed stuffs. Within the EU-funded research project SIMBAG FEED [1], a post screening method is developed based on High Voltage Electrophoresis followed by bioautography. This method enables a preliminary identification of positive screening samples.

Two simple extraction procedures for the five target compounds are developed:

- avoparcin and zinc-bacitracin are extracted at low pH with a mixture of acetone, hydrochloric acid (HCl) and water;
- tylosin, spiramycin and virginiamycin are extracted with a mixture of water-methanol (1:1).

After centrifugation, the supernatants are subjected to high voltage electrophoresis in a buffered agar gel. The antibiotics are located by bioautography using *Bacillus megatherium* ATCC 10778 (pH 6) (avoparcin), *Micrococcus luteus* ATCC 10240 (pH 6.5) (zinc-bacitracin) and *M. luteus* ATCC 934 at pH 8 (tylosin, spiramycin) and pH 6.5 (virginiamycin). The identification is carried out by observing with which microorganisms zones of inhibition are produced and comparison of distance ( $R_f$  values) and direction of migration and detailed appearances of inhibition zones with known spike samples.

Within laboratory validation using twenty blank feeds (spiked at 1 and 2 ppm and including one milk replacer) showed good detection of 1 ppm tylosin (95% positive), 1 ppm avoparcin (100% positive) and 1 ppm spiramycin (95% positive). One ppm of virginiamycin (50% positive) and zinc bacitracin (45% positive) were detected less sensitive. A concentration of 2 ppm virginiamycin and zinc-bacitracin was in 95-100% of the cases positive. All blank feeds showed no inhibition. The negative results at 1 ppm level for spiramycin and tylosin occurred in milk replacer. For this matrix 2 ppm spiramycin and tylosin proved to be well detectable (100% positive, n=3).

The developed HVE method is not applicable for mineral feeds and the detection of zinc-bacitracin in the presence of virginiamycin is not possible. Furthermore some antibiotics uses as veterinary medicines showed interference:

- other macrolides with spiramycin and tylosin;
- tetracyclines with zinc-bacitracin and avoparcin;
- aminoglycosides/quinolones with avoparcin.

The remaining antibiotics (avilamycin, flavomycin, monensin, salinomycin) in category A showed no interference.

### References

1. SIMBAG FEED. Project "Screening and identification methods for official control of banned antibiotics and growth promoters in feedingstuffs", funded by EC, DG RTD, Competitive and Sustainable Growth (GROWTH) Programme, Contract no. G6RD-CT-2000-00413.

## **Confirmatory analysis of banned feed additives: avoparcin, zinc-bacitracin, virginiamycin, tylosin, spiramycin, carbadox and olaquinox**

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In the intensive production of animal products, feed additives are frequently used to prevent diseases and to promote animal growth. Within the European Union (EU) there is a tendency to eliminate the unnecessary use of antibiotic feed additives in view of their possible adverse effect on induction of microbial resistance to antibiotics. Several feed additives have been banned in the past years as a result. The aims for regulatory control, as a result of this ban, have changed from verification of the additive concentration, to the detection of unwanted substances. This includes quantitative analysis as well as the confirmation of the identity of detected compounds in view of legal prosecution that may result from positive findings. The methodologies suitable for this purpose were developed in the framework of a Research project financially supported by the European Commission [1]. In addition to screening methods suitable for the detection of sub-additive levels of the banned compounds, quantitative, confirmatory methods are developed, aiming for one or two multi-analyte methods covering the entire range of banned compounds.

In this paper two methods are presented for the confirmatory analysis of five macrocyclic (polypeptide) antibiotics and two growth promoters: (i) avoparcin and zinc-bacitracin, and (ii) virginiamycin, tylosin, spiramycin, carbadox and olaquinox. The few methods for instrumental analysis that have been reported in literature for the compounds mentioned are all aimed at single compounds (virginiamycin, carbadox and olaquinox) or members of one class of compounds (macrolides) and are primarily aimed at additive concentrations. Multi-analyte methods at sub-additive levels have not been available so far. The first method is suitable for the accurate quantitative analysis of  $\alpha$ - and  $\beta$ -avoparcin, zinc bacitracin A, B1, B2 and B3 at sub-additive concentration in animal feed. The second method is suitable for the accurate quantitative analysis of virginiamycin, tylosin, spiramycin, carbadox and olaquinox at sub-additive concentration in animal feed. The extraction and extract purification can be carried out using the same conditions for all compounds.

Validation data for both methods will be presented. The initial validation as well as the between-lab validation has shown that:

- Method performance is dependent to a considerable extent, on the composition of the feed. Different feeds may result in different yields of the antibiotics in the extraction step. Yields during extraction in general are reproducibility within one feed sample.
- In LC-MS detection, different feeds may yield different degree of ionisation suppression.

These two phenomena together necessitate the application of multi-level standard addition for quantification, thereby causing a five-fold (!) increase in the workload for validation. In addition multi-level standard addition will yield a higher variability in the quantification step. Furthermore, in addition to the quantitative results obtained, the identity of the analytes can be confirmed according to EU-criteria. This method can therefore be used as a follow-up method for suspect screening results and enables the enforcement of the ban on feed additives in an efficient and cost-effective manner.

### **References**

1. SIMBAG FEED. Project "Screening and identification methods for official control of banned antibiotics and growth promoters in feedingstuffs", funded by EC, DG RTD, Competitive and Sustainable Growth (GROWTH) Programme, Contract no. G6RD-CT-2000-00413.

## Assessment of performance of a prototype test kit for five banned antimicrobial growth promoters

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The uses of five antimicrobial growth promoters (AGPs) zinc-bacitracin, spiramycin, tylosin, virginiamycin and olaquinox in animal feedingstuffs have been prohibited in the European Union (EU) since 1999. EU Member States are required to ensure compliance with the prohibition on their use. However, the existing community methods of analysis are non-specific microbiological assay, incapable of effectively monitoring compliance with the ban. The development of an immunoassay-based multi-antibiotic screening test for detection of the five banned AGPs was recently achieved as part of an EC funded project (RADIUS). The resultant test developed has been adapted to an ELISA (enzyme-linked immunosorbent assay) kit format.

Microtiter plate was coated with four different polyclonal antibodies. Antibodies of bacitracin, olaquinox and virginiamycin were produced to detect the corresponding antimicrobials while antibody of spiramycin was used for the detection of both spiramycin and/or tylosin. The mixture of drug standards and drug-HRP (horse radish peroxides) were used in this system with an unofficial minimum required performance limit of 4 mg kg<sup>-1</sup> for olaquinox and 1 mg kg<sup>-1</sup> for the remaining banned substances. As a qualitative screening method, this assay was developed to target the banned substances at 20% of their previously authorised minimum inclusion rates in animal feedingstuffs.

The presentation will outline the capability of simultaneously screening for the five banned AGPs in feeds using the developed ELISA with a simple sample preparation procedure.

## Quantification of five natural growth promoters in feed

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A GC-FID (Gas Chromatography-Flame Ionisation Detector) method was developed for the quantification of five essential oils added in feed (1,8-cineol, cinnamaldehyde, thymol, carvacrol and eugenol) in the 5 ppm range. These substances were chosen because they are the main components of some essential oils, which are used as alternatives for antimicrobial growth promoters in animal feed.

Among the various extraction processes tested, the classic Soxhlet is selected for its low cost, good results and possibilities for automation. The solvent used is n-pentane, chosen for its good extraction yield, low toxicity and cleanliness of the extracts.

Ground sample (20 g) is extracted with pentane in a Soxhlet extractor for 4 h. After cooling and addition of 2 ml azulene solution (50 µg/ml) the volume is adjusted to 200 ml with pentane. Each extract is injected three times under the following conditions:

- Carrier gas, helium at a flow rate of 1.4 ml/min
- Injector, split mode with split ratio 1:5/1:9, temperature 250°C
- Detector, FID, temperature 250°C
- Injection volume, 4 µl
- Oven temperature, from 70°C to 130°C at a flow rate of 4°C/min, stabilisation for 5 min, then up to 150°C at 4°C/min and clean up by heating at 330°C
- Column, J&W DB5, 25 m x 0.25 mm ID, 0.25 µm thickness

In an interlaboratory test including eight laboratories, the best results were obtained for the three phenols (thymol, carvacrol and eugenol) with an interlaboratory repeatability of approx. 10%. The worst results were obtained with cinnamaldehyde

### Acknowledgements

The programme was supported by a grant of the French "Bretagne" and "Pays de la Loire" regions.