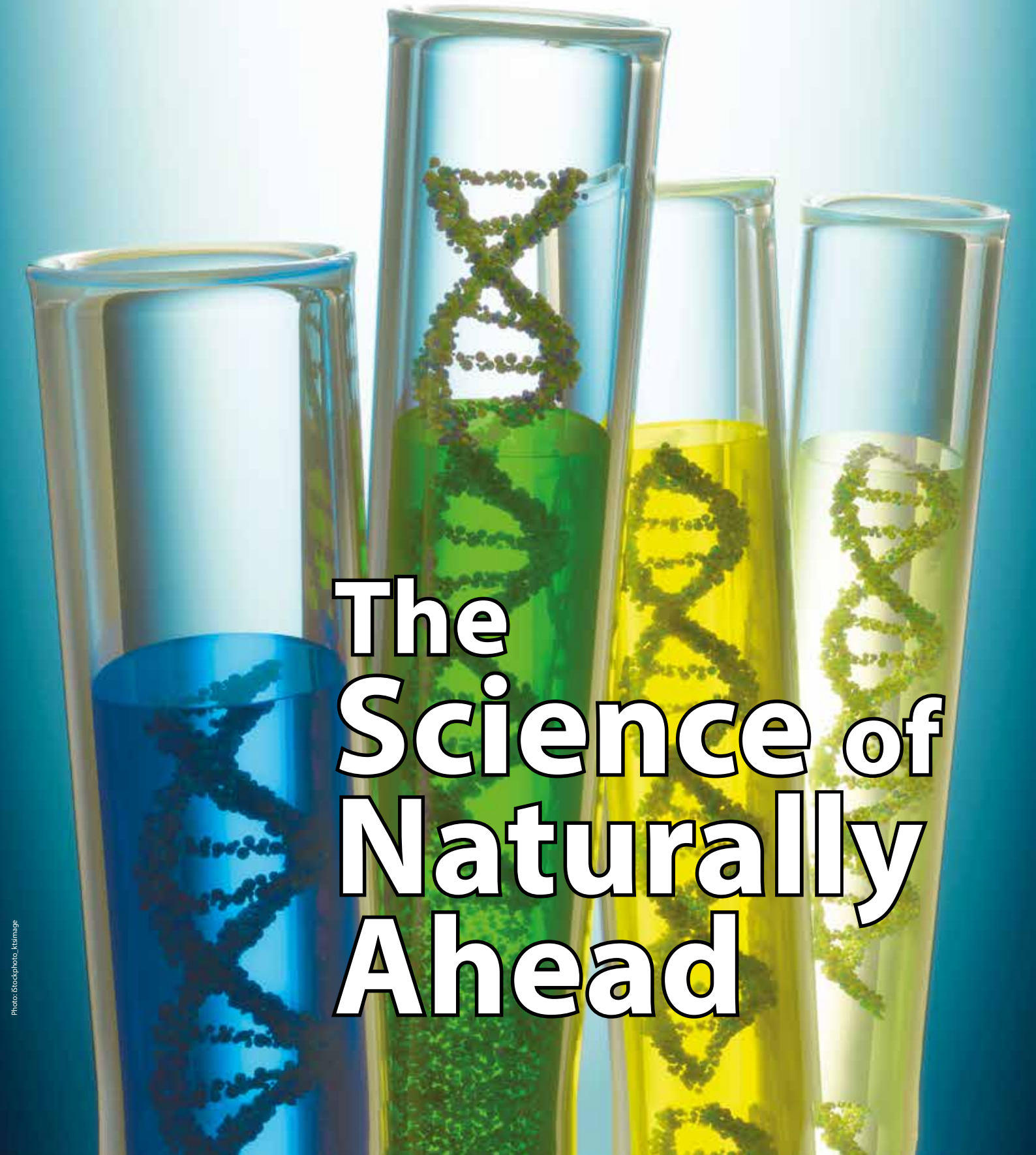
Issue R&D
A magazine of Biomin

Science & Solutions



The Science of Naturally Ahead

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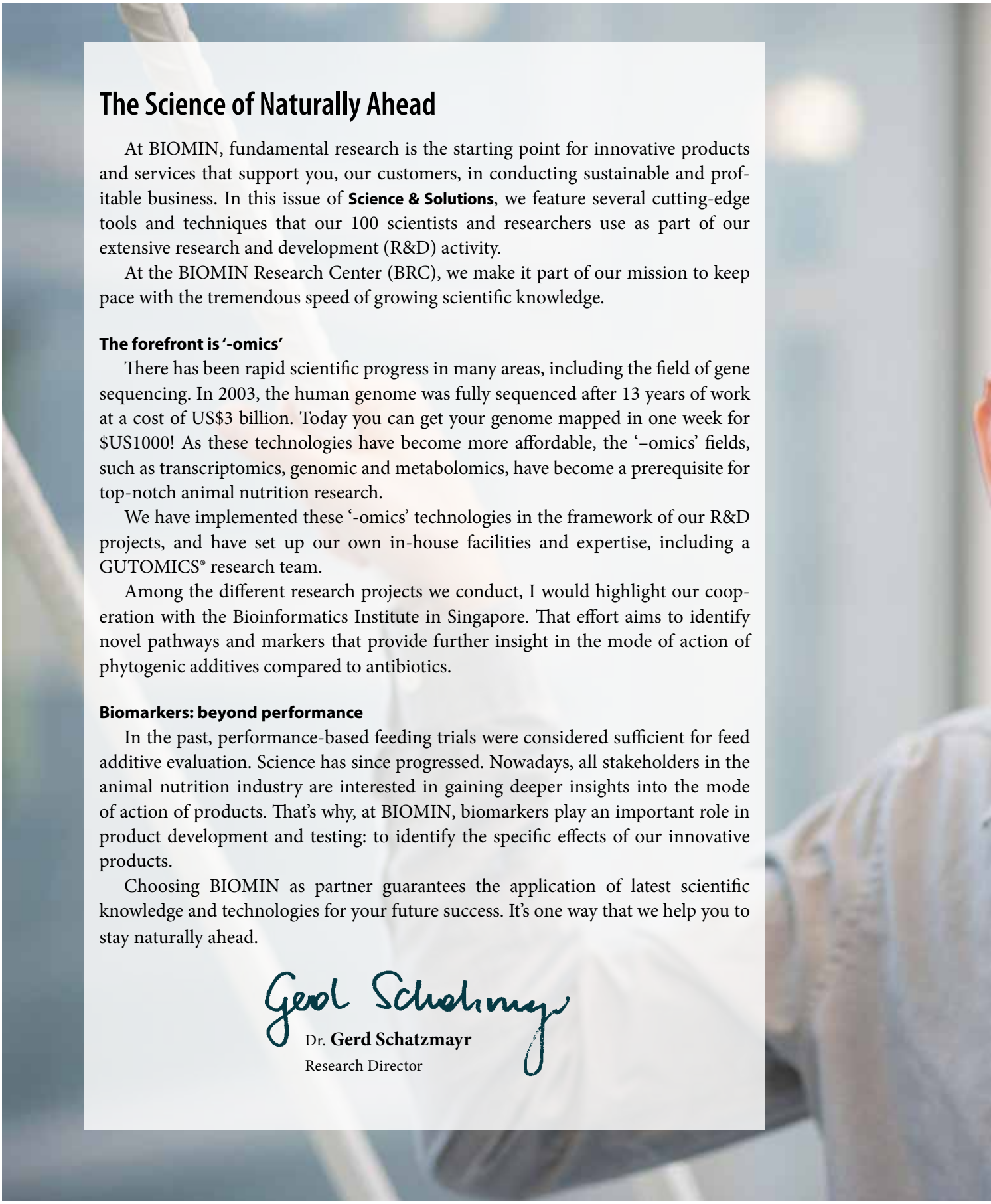
Editorial



The Science of Naturally Ahead

At BIOMIN, fundamental research is the starting point for innovative products and services that support you, our customers, in conducting sustainable and profitable business. In this issue of **Science & Solutions**, we feature several cutting-edge tools and techniques that our 100 scientists and researchers use as part of our extensive research and development (R&D) activity.

At the BIOMIN Research Center (BRC), we make it part of our mission to keep pace with the tremendous speed of growing scientific knowledge.

The forefront is '-omics'

There has been rapid scientific progress in many areas, including the field of gene sequencing. In 2003, the human genome was fully sequenced after 13 years of work at a cost of US$3 billion. Today you can get your genome mapped in one week for $US1000! As these technologies have become more affordable, the '–omics' fields, such as transcriptomics, genomic and metabolomics, have become a prerequisite for top-notch animal nutrition research.

We have implemented these '-omics' technologies in the framework of our R&D projects, and have set up our own in-house facilities and expertise, including a GUTOMICS® research team.

Among the different research projects we conduct, I would highlight our cooperation with the Bioinformatics Institute in Singapore. That effort aims to identify novel pathways and markers that provide further insight in the mode of action of phytogenic additives compared to antibiotics.

Biomarkers: beyond performance

In the past, performance-based feeding trials were considered sufficient for feed additive evaluation. Science has since progressed. Nowadays, all stakeholders in the animal nutrition industry are interested in gaining deeper insights into the mode of action of products. That's why, at BIOMIN, biomarkers play an important role in product development and testing: to identify the specific effects of our innovative products.

Choosing BIOMIN as partner guarantees the application of latest scientific knowledge and technologies for your future success. It's one way that we help you to stay naturally ahead.

Gerd Schatzmayr

Dr. **Gerd Schatzmayr**
Research Director

Contents

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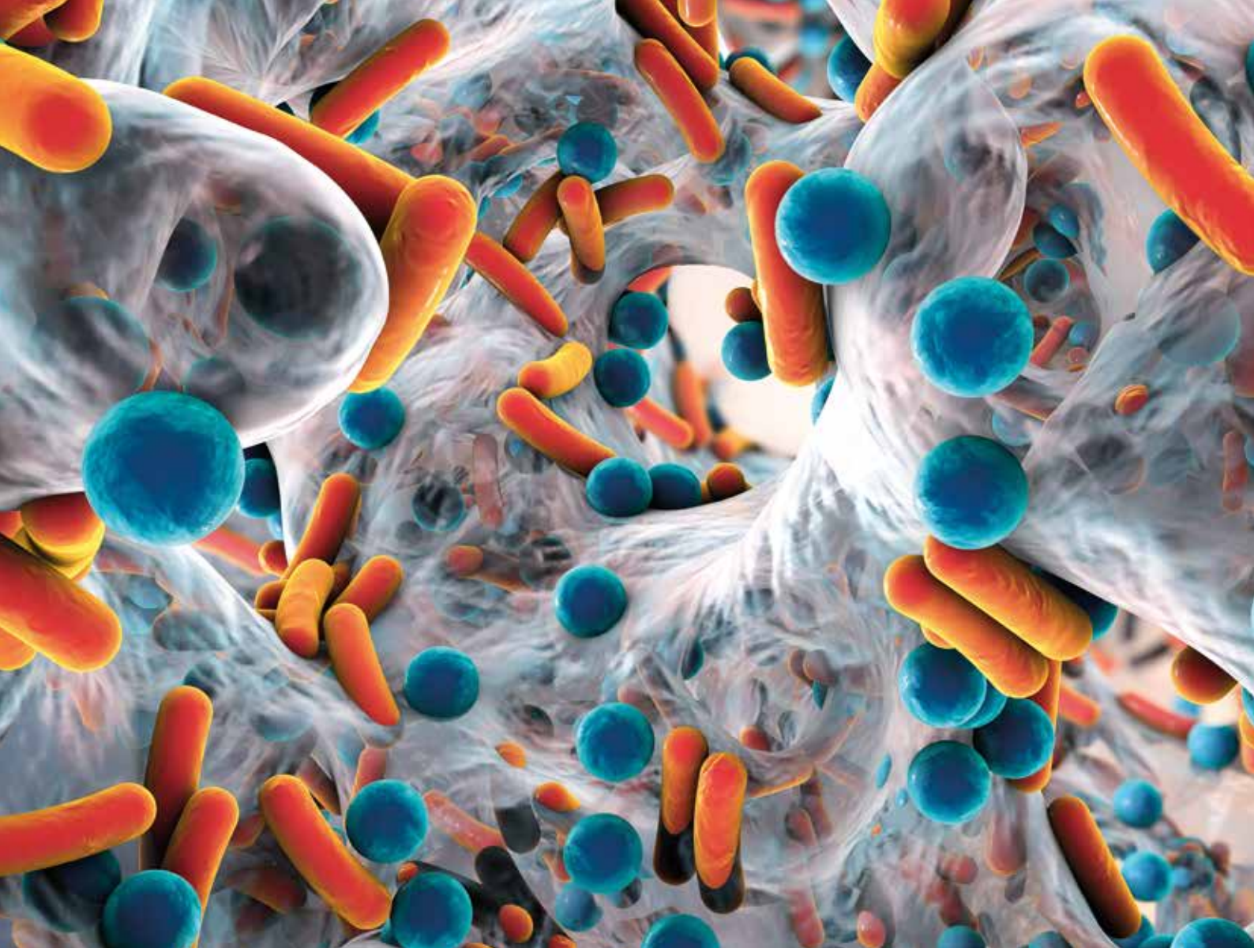

Two Advanced Tools for Understanding Antimicrobial Resistance

EPI assays and qPCR are two of several tools that BIOMIN scientists and researchers use to better understand and potentially combat the mechanisms of antibiotic resistance.

By **Andreas Köstelbauer**, Research Associate, **Gertrude Wegl**, Scientist, and Dr. **Viviana Klose**, Research Team Leader Microbiology

The spread of antibiotic resistant bacteria pose a major threat to modern medicine (WHO, 2014). Extensive antibiotic use in agriculture is one of many factors that may contribute to antibiotic resistance (Ventola, 2015). A key issue is the development of multi-drug resistance (MDR) in pathogenic bacteria found in the digestive tract.

Globally, there is a continuing need to prevent the further increase in MDR bacteria, not just in clinical, but in natural environments, too. In order to come up with successful strategies to battle the spread of antimicrobial resistance in animals, certain feed additives with competitive properties (e.g. that inhibit the growth of pathogenic bacteria) might also disrupt resistance mechanisms.

At the BIOMIN Research Center, we use several advanced analytical methods to better understand and potentially combat the mechanisms of antibiotic resistance. First, *in vitro* efflux pump inhibitor assays allow us

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At the BIOMIN Research Center, we use several advanced analytical methods to better understand and potentially combat the mechanisms of antibiotic resistance.

to identify substances as potential resistance inhibitors in the laboratory. Second, cutting-edge metagenomics technologies allow us to detect and track bacterial genes or elements in complex environments using samples sourced from farms.

How multi-drug resistance happens

Multi-drug resistance in bacteria occurs by the accumulation of resistance genes on resistance plasmids, with each gene coding for resistance to a specific agent (*Figure 1A*), and/or by the action of multidrug efflux pumps, which can pump out more than one antibiotic drug (*Figure 1C*).

Resistance plasmids are often transferred very efficiently from cell to cell (*Figure 1B*). Resistance by efflux pumps occurs by the increased expression of genes that code for these pumps. Some pumps in Gram-negative bacteria (e.g. *Acr*AB-*Tol*C in *Salmonella*) are especially important because they can pump out most of the antibiotics currently in use.

Figure 1. Important bacterial mechanisms conferring multidrug resistance (MDR). A) Multidrug resistant bacteria often carry mobile genetic elements like resistance plasmids, which can acquire many resistance genes through gene accumulation. B) Horizontal gene transfer via resistance plasmids efficiently passes resistance genes from one bacterium to another, contributing to the spread of antibiotic resistance in bacterial populations. C) Another mechanism of multidrug resistance is the active pumping out of drugs by multidrug efflux pumps.

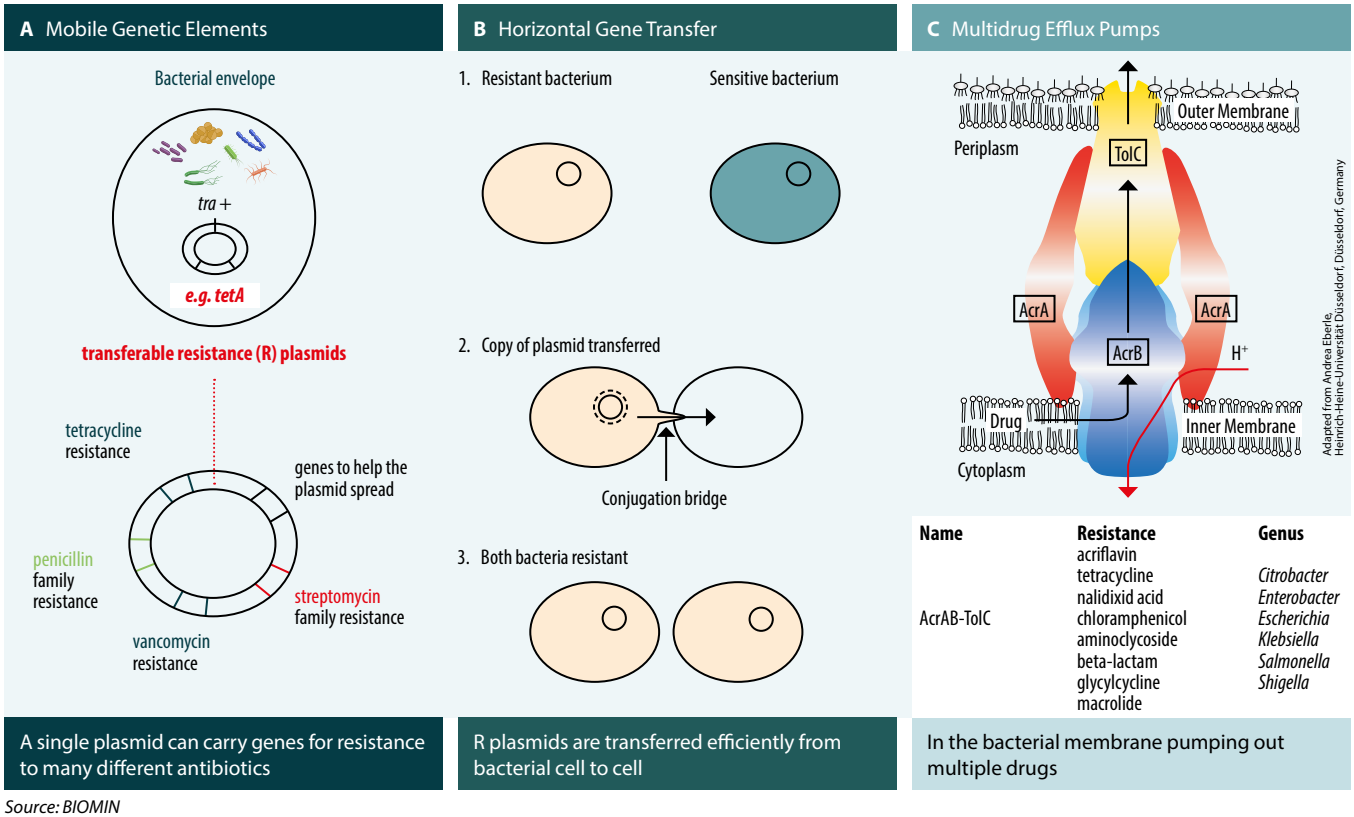

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Figure 2. Principle of efflux pump inhibitors assay. The multidrug resistant *Salmonella* strain with upregulated efflux pump displays very weak fluorescence, as the fluorescence dye is pumped out of the cells (right). The same *Salmonella* strain, treated with an efflux pump inhibitor, loses its resistances, displaying a strong fluorescence, as the dye is not pumped out (left).

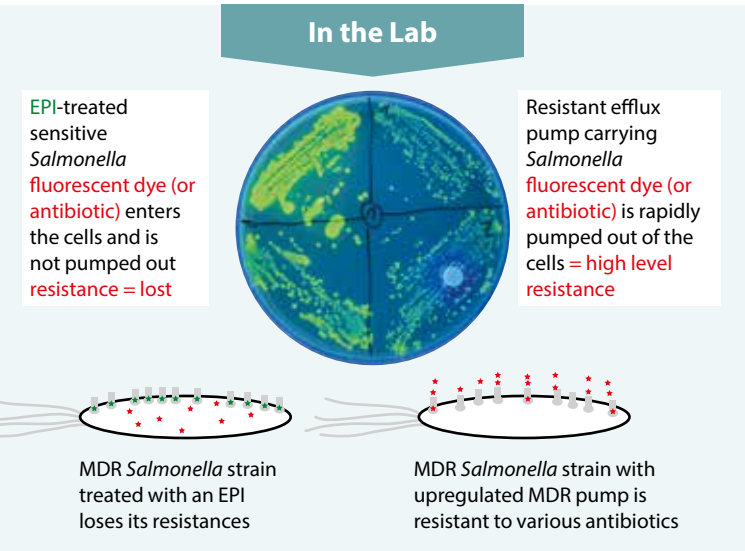

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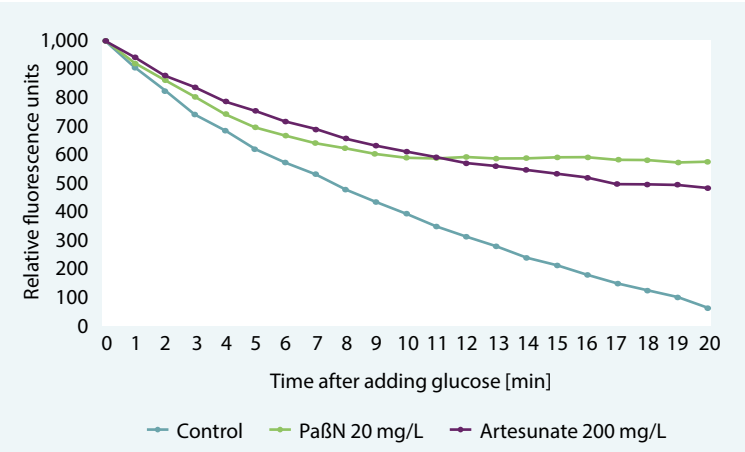
Figure 3. Release of fluorescence from cells of an efflux gene overexpressing MDR *Salmonella* strain, after treatment with two known EPIs.



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Multidrug efflux: a key target in reversing antibiotic resistance

One way of prolonging antibiotic efficiency against multidrug-resistant pathogens is by blocking their efflux pumps with efflux pump inhibitors (EPIs). Natural plant-derived substances (phytogenics) have emerged as promising candidates, capable of improving the potency of antibiotics even at low concentrations, and preventing the emergence of resistance.

Efflux activity can be directly measured by fluorescence-based assays, based on two principles. First, various fluorescent dyes will shift in color and intensity when they enter the lipophilic environment inside of bacterial cells. Second, these dyes are actively pumped out of the cells by the efflux machinery. Monitoring the shifts in fluorescence enables us to see how fast the bacteria can pump out dyes, and, if an added substance is a potential inhibitor (*Figure 2*).

Efflux pump inhibitor assay results

In experiments at the BIOMIN Research Center, a *Salmonella enterica* serovar *Typhimurium* strain carrying the *acr*AB-*Tol*C pump was brought to over-expression of the efflux gene, by adapting it gradually to higher concentrations of enrofloxacin, a commonly used veterinary antibiotic, until it was able to survive thousand times the initial concentration (0.06 to 60 mg/L).

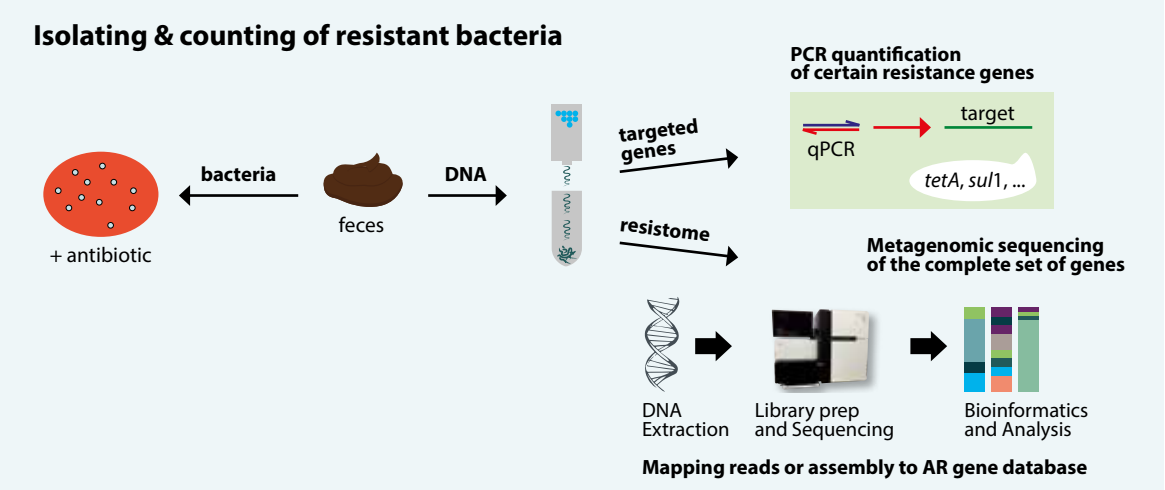
This *Salmonella* strain overexpresses the efflux gene, making it resistant to a wide range of antibiotics (e.g. tetracyclines, ß-lactams). For substance screening, the strain was stained with a fluorescent dye in the presence of potential EPIs.

After adding glucose, which induces efflux activity, the shifts in fluorescence were measured (*Figure 3*). In the untreated control, the dye was extruded and fluorescence rapidly decreased as a result. When *Salmonella* is treated with known EPIs, phenylalanine-arginine ß-naphthylamide (PAßN) and the anti-malaria drug artesunate, the efflux is clearly inhibited.

Andreas Köstelbauer, Research Associate,
Gertrude Wegl, Scientist, and
Dr. **Viviana Klose**, Research Team Leader Microbiology

The gastrointestinal tract is habitat of an enormous species diversity and density, a reservoir for thousands of antibiotic resistance genes.

Figure 4. Methodologies for assessing bacterial antibiotic resistances in complex samples at different levels. Starting from a fecal sample, the resistances can be assessed by culture through targeted detection and detection of resistance genes (by quantitative polymerase chain reaction, qPCR) or by metagenomics sequencing, in order to characterize the gut resistome.



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Gut resistome studies to assess antibiotic resistance on the farm

The gastrointestinal tract is habitat of an enormous species diversity and density, a reservoir for thousands of antibiotic resistance genes. Assessment of antibiotic resistance has long relied on traditional isolation techniques by cultivating and counting bacteria on nutrient agars with and without antibiotics.

However, these provide only information on the minority of bacteria—those that can grow under laboratory conditions.

Quantitative polymerase chain reaction

Molecular methods, targeting the genetic basis of resistance, use DNA to characterize and quantify antibiotic resistance determinants. Extraction of DNA from farm-derived samples (e.g. feces) allows reliable quantification of resistance genes in a high number of samples, by using quantitative Polymerase Chain Reaction (qPCR) (*Figure 4*). PCR is a targeted approach using synthetic oligonucleotides ("primers") that are complementary to the flanking regions of the gene of interest to amplify this particular gene fragment. It will not provide information on the presence of genes that are not targeted by the primers.

Metagenomics offers a complete view

The most comprehensive approach to exploring antibiotic resistance in complex environments uses metagenomics, which aims to assess the entire genomic information stored in a given sample ("metagenome") by using modern sequencing technologies. Current platforms, like the Illumina HiSeq, yield anywhere from 10 to 1000 GB of DNA sequences in a single lane. The sequencing datasets can be analyzed by assembly of the short reads into larger contiguous DNA fragments or by mapping to reference sequences. This method allows determination of the microbiota composition and simultaneous detection and quantification of the complete set of resistance genes ("resistome") or other genes of interest (e.g. virulence) in the microbiota.

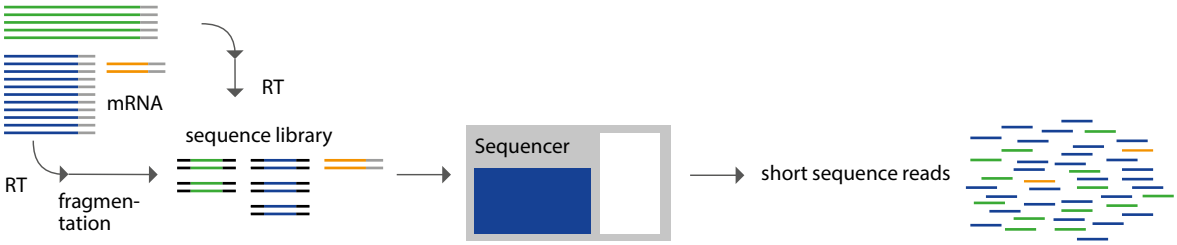
Next-Generation Sequencing Reveals Links Between Nutrition and Genes

RNA sequencing tools create new possibilities for understanding animal growth, health and performance.

By Dr. **Bertrand Grenier**, Scientist

Next-generation sequencing (NGS) enables researchers to study biological systems at a level never before possible.

Figure 1. The RNA sequencing process.



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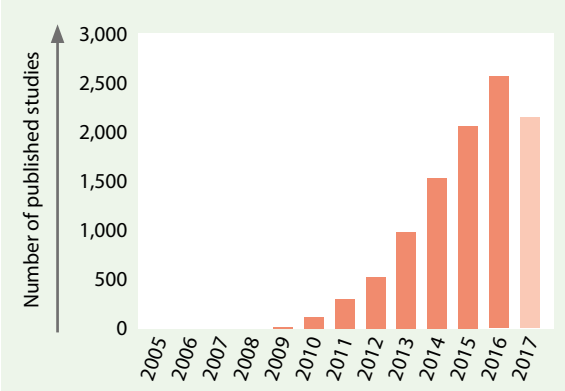
It is remarkable that sequencing the first human genome in 2003 required 13 years of work and cost nearly $US3 billion. In contrast, the HiSeqX Ten, released in 2013, can sequence over 45 human genomes in a single day for approximately $US1000 each. The critical evolution in terms of technology/chemistry is that, instead of sequencing a single DNA fragment as in the past, next-generation sequencing (NGS) today extends this process across millions of fragments in a massively parallel fashion, and enables researchers to study biological systems at a level never before possible.

The transcriptomics approach

Transcriptomics refer to the study of the transcriptome—the sum total of all messenger RNA molecules (mRNAs) being actively expressed from an organism's genes. The use of NGS technology to study the transcriptome at the nucleotide level is known as RNA sequencing (*Figure 1*). RNA sequencing is a major advance in the study of gene expression because it allows a snapshot of the whole transcriptome rather than a predetermined subset of genes. This provides a comprehensive view of a cellular transcriptional profile at a given biological moment—which entails the quantification of each product of gene expression from between 20,000 and 25,000 genes. Recent advances in RNA sequencing technology have made this high-throughput sequencing platform more accessible to researchers,

Figure 2. Scientific studies using 'RNA sequencing'.



Source: PubMed

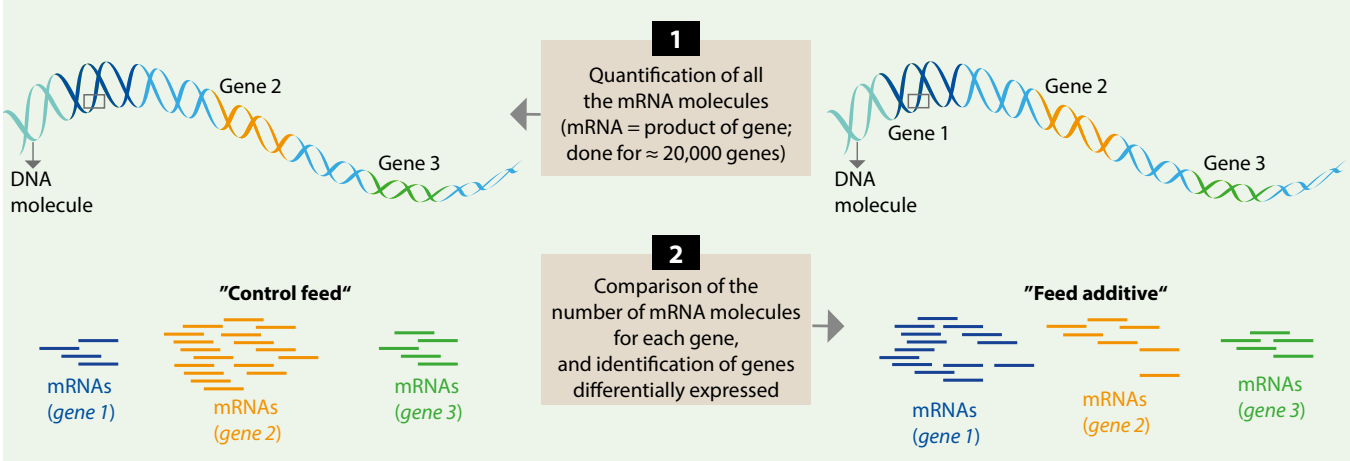
and it is expected to become the predominant tool for transcriptome analysis. As exhibited in *Figure 2*, the use of RNA sequencing in scientific studies has grown exponentially—largely due to the advantages it offers over using microarrays.

Application in animal nutrition

Rapidly developing NGS technology will play an important role in increasing our understanding of how nutrition influences metabolic and immunity pathways and enhances animal health and well-being—a field of animal science called nutrigenomics. Its principal line of

Rapidly developing NGS technology will play an important role in increasing our understanding of how nutrition influences metabolic and immunity pathways and enhances animal health and well-being.

Figure 3. Feed additives and the analysis of differences in gene expression.



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enquiry examines the direct effects of feed constituents on gene expression. As such, nutrigenomics could lead the way to develop rational means to optimize animal nutrition and achieve more sustainable, profitable agriculture. *Figure 3* presents an overview of how RNA sequencing can determine the mode of action of feed constituents and/or find potential biomarker(s).

Nutrigenomics and phytogenics in poultry

To the best of our knowledge, there are few nutrigenomics studies on the effects of phytogenics on the whole transcriptome. In 2016, a broiler experiment was conducted at the BIOMIN Research Center in which RNA sequencing analysis was done on tissue samples from the intestinal tract of birds fed with or without phytogenics.

The expression of more than 20,000 genes was determined. Preliminary results showed that 73 genes were differentially expressed between birds fed basal feed and birds fed the same feed supplemented with phytogenics. *Table 1* gives an overview of some signaling pathways associated with these DEGs (Differentially Expressed Genes), namely the acute phase response and cytokine signaling pathways related to inflammatory response. These results were obtained based on an updated version of the chicken genome (Galgal5) released in December 2016. This makes them more accurate than results using older versions (e.g. Galgal4) or technologies, such as microarray, that rely on a pre-designed sequence detection probe for hybridization that would need to be redesigned for every new update of the genome.

Limitations of NGS and transcriptomics

As with any technology, next-generation sequencing is not without its limitations and challenges. For instance, despite the superior benefits of RNA sequencing, microarrays are still the more common choice of researchers when conducting transcriptional profiling experiments.

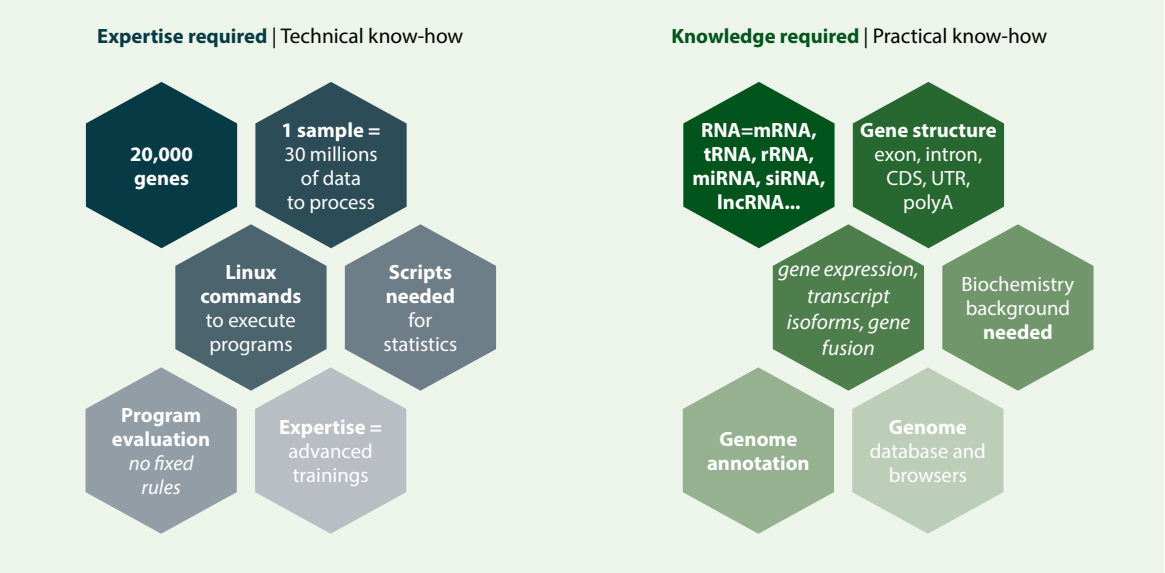
This is likely because the newer RNA sequencing technology is more expensive than microarray, data storage is more challenging and analysis is more complex. NGS platforms provide vast quantities of data (e.g. 200 GB generated from RNA sequencing for 30 biological

Table 1. Effect of phytogenics on the intestine of 35-day-old broiler chickens.

Total number of genes analyzed with Galgal5	24838
Number of differentially expressed genes compared to control birds – mapping Galgal5	73
Signaling pathways associated with these differentially expressed genes:	- Acute phase response signaling - Cytokine signaling (IL-22 and STAT3 pathway)

Source: BIOMIN

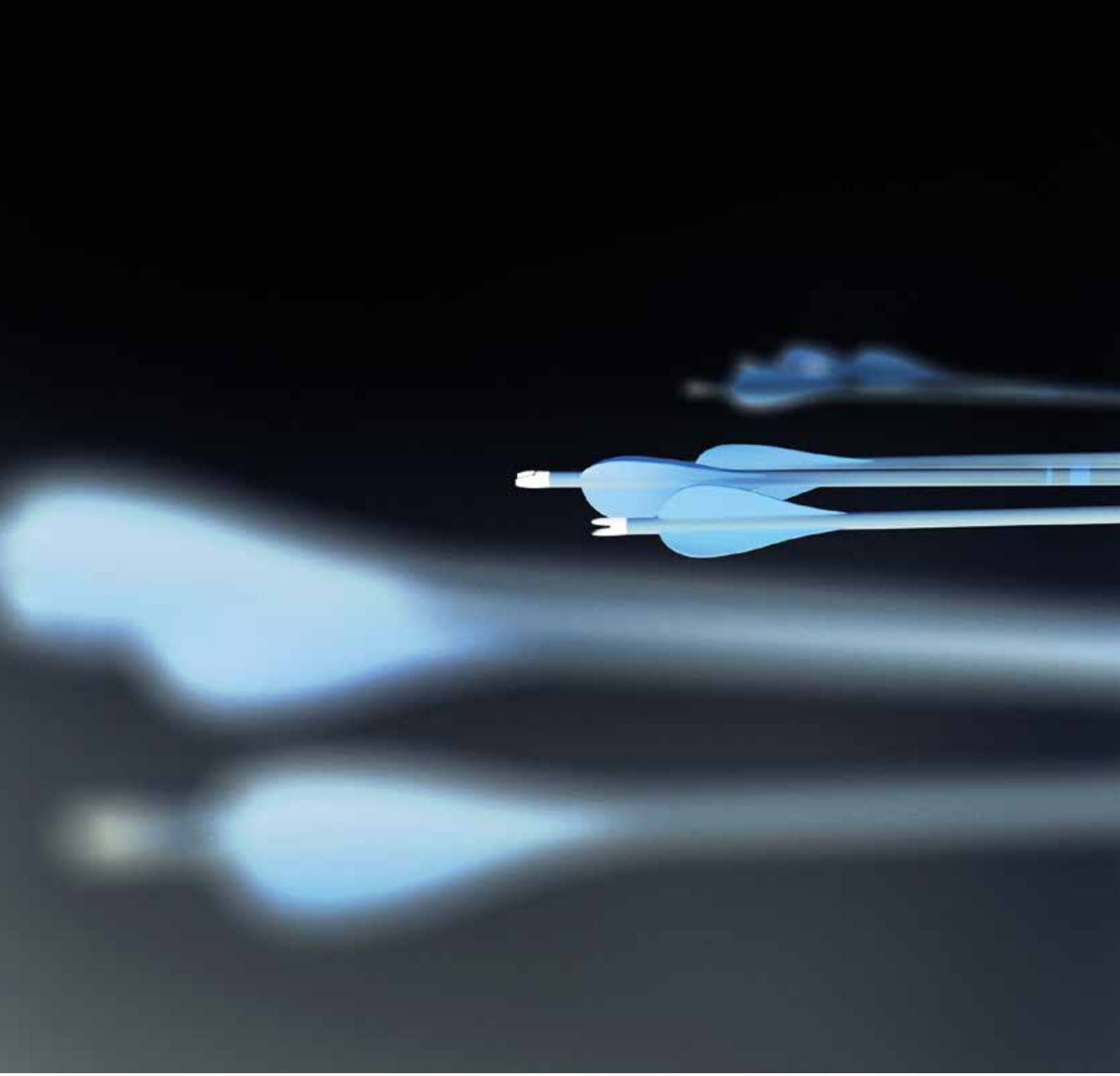
Figure 4. Requirements for RNA sequencing analysis.



Source: BIOMIN

samples), and therefore requires servers with high computational resources. There is no reference methodology for processing and analyzing NGS data: this is a growing field with continuous development of bioinformatics tools. All have advantages but also limitations, and it is necessary to evaluate them and take a consensus for data analysis. The complexity of NGS analysis and data interpretation requires both expertise and knowledge in informatics and biology (*Figure 4*).

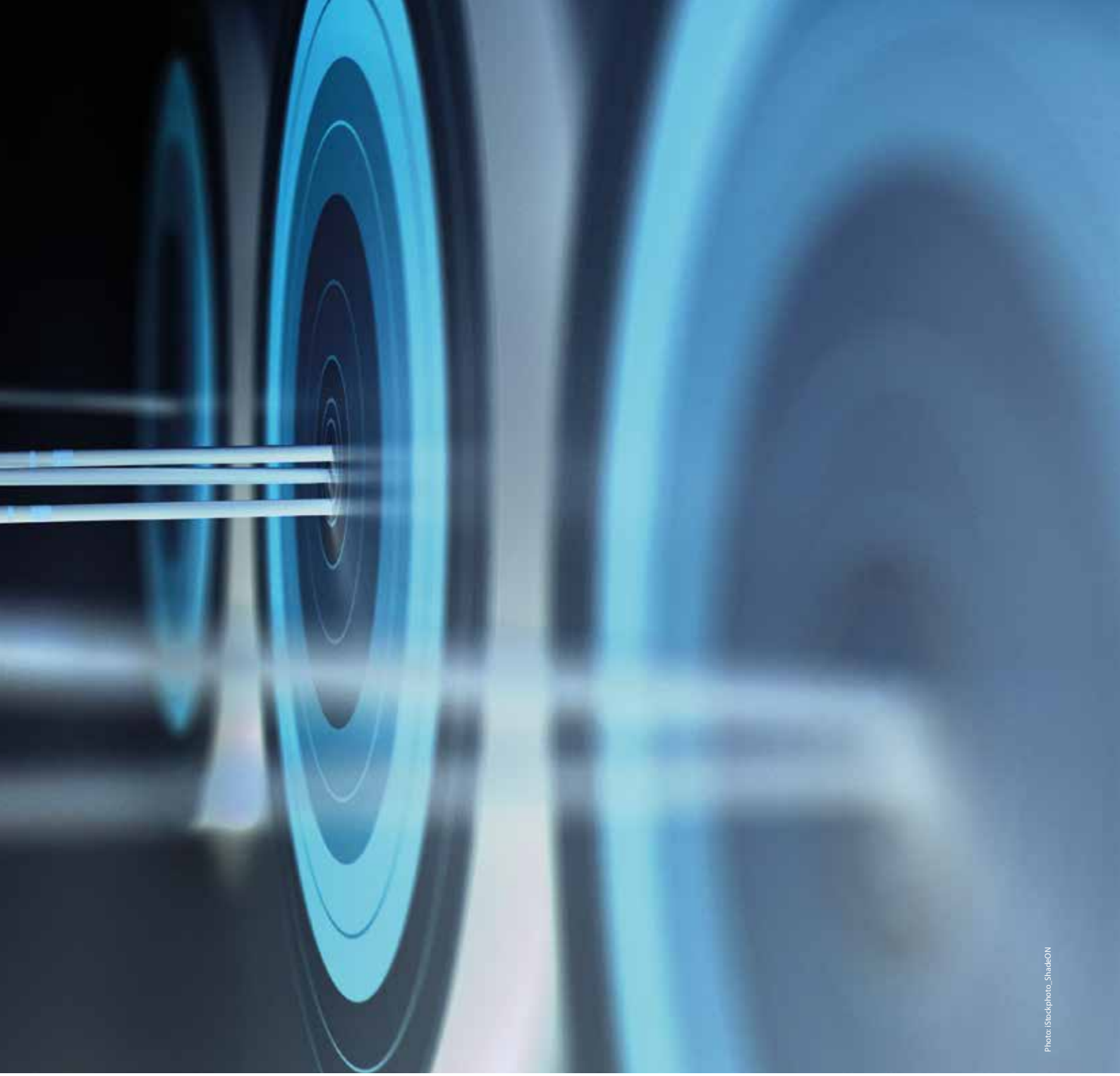
Additionally, a transcriptomics approach alone is not sufficient to fully conclude on mode of action of feed constituents. For instance, the intestinal tract environment is quite complex: the host tissue, cells and nutrients all interact with the intestinal microflora. Combining so-called '–omics' approaches, such as genomics, transcriptomics, proteomics and metabolomics, would provide an even better understanding on the mode of action and gut performance.



3 Keys to Ensuring Proper Active Substance Delivery

Achieving homogenous distribution, thermostability and controlled release are all necessary for proper formulation of an effective feed additive.

By Dr. **Stephen Cole**, Development Team Leader - Bioactive Ingredient Formulation



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The active substances contained in an animal feed additive can be a single ingredient or complex mixture of enzymes, binders, phytochemicals, live microorganisms and organic acids. Whatever their nature, they have to be fed to the target animal in a form that ensures the substance will be delivered, reliably, to the targeted site of action within the animal and still be 100% active.

A variety of techniques

Because of the diverse nature of the active substances, different formulation techniques have to be used for different products. For example, enzymes and microorganisms can be quite fragile, so it is usual to dry preparations containing enzymes and microorganisms to preserve their activity. For microorganisms, this is often performed by freeze-drying (lyophilisation) of stabilized cell cultures, while spray drying or fluidized bed drying, granulation and coating can be used for enzymes. When the active substances are volatile liquids, such as phytogenic essential oil preparations, alternative formulation techniques such as encapsulation are required to protect the volatile active substituents.

Alternatively, we may sometimes desire the feed additive to be a liquid preparation such as an enzyme for post-pelleting application. In this case, the added challenge involves stabilization of the active substance while ensuring the product can be reproducibly applied to finished feeds.

The density and size of the feed additive preparation will determine whether the active substance can be effectively and accurately distributed throughout the feed.

In general, successful formulation of feed additives must overcome three distinct challenges –homogenous distribution, thermostability and controlled release— in order to achieve the best results in animals.

Challenge 1: Homogenous distribution

The first challenge for the bioactive ingredient formulator is to ensure that the product is accurately distributed throughout the feed. Feed additives are always micro-ingredients and are typically added at an inclusion level around 100g per ton of feed. The density and size of the feed additive preparation will determine whether the active substance can be effectively and accurately distributed throughout the feed. For example, the average 4mm broiler feed pellet weighs 0.055g which equates to over 18 million pellets per ton of feed. To ensure every feed pellet received the active substance would require there to be at least 18 million particles of the active substance in a typical 100g inclusion. Such a calculation should always be performed to determine the optimum size and size distribution of the active preparation.

Challenge 2: Thermostability

The second challenge is that the active substance has to survive subsequent steam conditioning and pelleting where the feed and included additives are subjected to high temperatures (in excess of 85°C), elevated moisture content and high physical pressures. Considerable success has been achieved in protecting enzymes through this process by drying the enzyme inside a granular matrix and applying a single or multiple coatings onto the granules. Both processes were achieved by fluidized bed processing (*Figure 1*). Fluidized beds can be used to gently dry sensitive active substances but can also be used to form granular materials which makes the product much easier and safer to handle as there is reduced or no dust and the products are easier to disperse accurately in a feed matrix. Figure 1 illustrates a laboratory-scale machine with a batch size up to approximately 1kg. Larger, pilot-scale, full industrial-scale and continuous-flow machines also exist which can granulate and coat materials at ton per hour scale.

Figure 1. Laboratory-scale fluidized bed dryer, granulator and coater.

Source: BIOMIN

The formulation of a feed enzyme can even result in a product that performs better than inherently thermostable competitor products. *Figure 2* shows the recovery of an inherently thermostable feed enzyme together with a thermo-labile (regular) and coated thermo-labile enzyme which had been added to feeds and pelleted at three different temperatures. The coating of the regular enzyme resulted in a product with over 70% recovery at 95°C and clearly outperforms even the inherently thermostable, engineered enzyme.

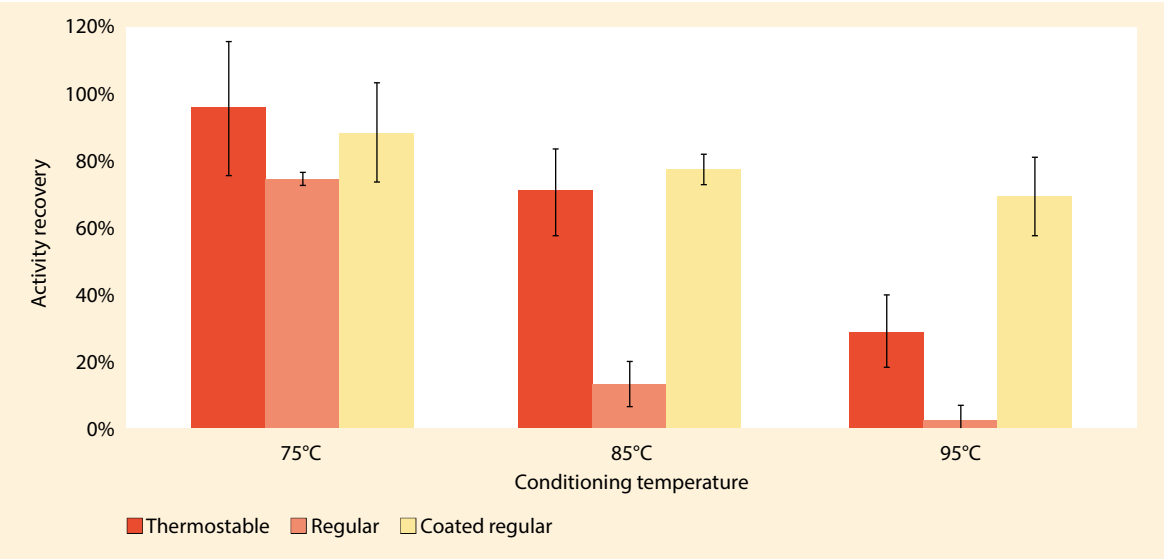
Challenge 3: Controlled release

The third challenge is to ensure the active substances are available in the desired part of the gastrointestinal

Dr. **Stephen Cole**
Development Team Leader - Bioactive Ingredient Formulation

The formulation of a feed enzyme can even result in a product that performs better than inherently thermostable competitor products.

Figure 2. Recovery of enzyme activity from feeds conditioned at different temperatures.

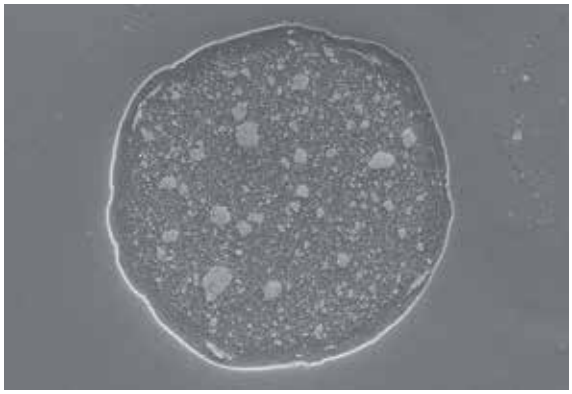


Source: BIOMIN

tract of the target animal. Care must always be taken to not encapsulate or coat a product to such an extent that availability and effectiveness in the animal is reduced. However, the formulation of an active substance can be used to direct where an active is available, or to protect a substance while passing through one part of the gastrointestinal tract—for example, gastric or rumen protection for a product that needs to work in the lower gastrointestinal tract.

The recently launched Biomin® Duplex Capsule that gives Digestarom® DC its name illustrates the power of product formulation. The inner core of the capsule contains the essential oils and substances that are active in the lower gastrointestinal tract of the animal. These are matrix encapsulated to enable their release throughout the lower gastrointestinal tract while protecting them from release in the stomach and enhancing the palatability of some components. Further palatability enhancement was achieved by applying a second essential oil substance in the coating matrix which is immediately available to the olfactory and gustatory senses of the animal. *Figure 3* is an electron micrograph of a duplex capsule clearly showing the core and coat regions.

Figure 3. Electron micrograph of a Biomin® Duplex Capsule.



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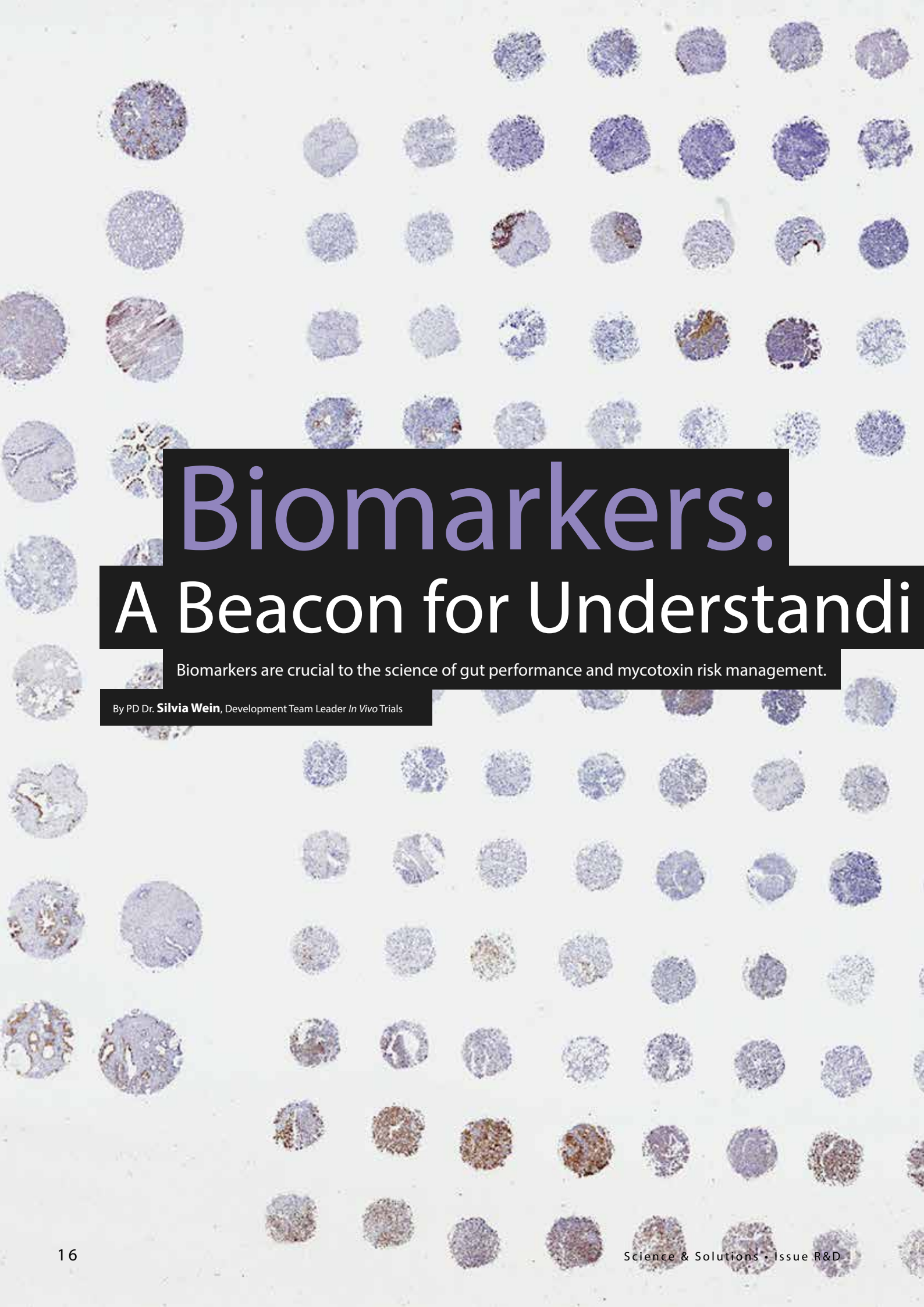


Biomarkers:
A Beacon for Understandi

Biomarkers are crucial to the science of gut performance and mycotoxin risk management.

By PD Dr. **Silvia Wein**, Development Team Leader *In Vivo* Trials

ng *In Vivo* Processes

In practice, biomarkers can be used

a) **for diagnosing or staging a disease,**
b) **for monitoring the response to a therapy or**
c) **to indicate the exposure to environmental factors in living organisms.**

Though often referred to as a protein in the blood stream, a biomarker might be any molecule in body fluids, tissue or excreta that serves as an indicator for health, disease, exposure or effect. There are several overlapping definitions of biomarkers found in the literature.

One of the early sources defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."

3 key applications

The identification and use of valid biomarkers substantially contributes to clinical research. Aside from validity, the ideal biomarker should possess the following features: safe and easy to measure, cost efficient to follow up, consistent across gender and modifiable due to treatment.

In practice, biomarkers can be used a) for diagnosing or staging a disease, b) for monitoring the response to a therapy or c) to indicate the exposure to environmental factors in living organisms. Thus, the descriptions under a) and b) are often summarized as "biomarkers of effects" and the latter as "biomarkers of exposure". Selected biomarkers with high relevance for swine health and mycotoxin exposure are depicted in *Table 1*.

Feeding strategy investigations

In experimental feeding trials, biomarkers can be a powerful tool to explore the effect of an investigated feeding strategy and accordingly determine its potential for commercial application at an early stage. It also ideally serves to investigate mycotoxin exposure and effects. Many investigations using biomarker analysis require urine and/or feces sampling. Obtaining proper samples requires the use of specially equipped facilities, such as the BIOMIN Center for Applied Animal Nutrition (CAN) in Tulln, Austria – one of seven such sites globally in the Center for Applied Animal Nutrition network.

Table 1. Selected biomarkers in swine.

Process	Biomarker	Sample
Inflammation	Acute phase protein: haptoglobin	Serum
	Total sialic acid (TSA)	Serum
Small intestinal inflammation	Neopterin	Feces
	Myeloperoxidase (MPO)	Serum/Feces
Small intestinal enterocyte damage	Intestinal fatty acid binding protein (I-FABP)	Serum/Feces
Oxidative stress	Catalase	Serum
	Superoxide dismutase	Serum
	Glutathione reductase	Serum
Fumonisin B_1 effect	Sphinganin/Sphingosin-ratio	Blood/Tissue
Fumonisin B_1 exposition	Fumonisin B1	Blood/Urine/Feces
Zearalenone effect	Glucuronic acid conjugates	Urine/Feces
Zearalenone exposition	Zearalenone and respective metabolites	Blood/Urine/Feces

Adapted from Niewold (2015), Kataria (2012) and Baldwin (2011).

PD Dr. **Silvia Wein**
Development Team Leader Feeding Trials

Figure 1. Metabolic pens used at the Center for Applied Animal Nutrition in Tulln. A) Front and side view with feeders and drinkers in the front door B) Inside view with slatted floor, collection area with screens and trays.

Source: BIOMIN

BIOMIN CAN Tulln

The site in Tulln comprises a feeding station, two rooms each equipped with 12 metabolic pens and one additional room fitted with four plastic coated slatted floor pens. Depending on the housing concept, criteria of trial and size of animals used, a maximum of 96 pigs can be housed in the two rooms with the metabolic pens. In the floor pens, the maximum capacity is 24 pigs up to a body weight of 30kg.

Figure 1 shows the principle set-up of a metabolic pen used at our CAN in Tulln. The pigs are able to move freely on a plastic coated slatted floor. Underneath the floor are two screens that will collect all the fecal material voided by the pig. Under the screens is a stainless steel tray that drains into the center and allows for the collection of all the urine produced during the day.

Knowledge generated

These metabolic pens allow for a large variety of state-of-the-art investigations in living organisms, including biomarker analysis, nutrient digestibility and nutrient retention. By collecting urine and feces separately, metabolism and excretion of molecules (e.g. mycotoxins and respective metabolites) can be studied. The impact of a feeding strategy on inflammation or oxidative stress can be monitored by analyzing the respective biofluid (blood, saliva, urine) or feces.

Knowing the amount of feed given each day and the nutrient composition of the feed, total nutrient intake can be calculated. The digestibility and the amount of nutrients retained in body tissues or lost nutrients can be calculated as well. Total nutrient excretion in feces and urine can be estimated and nutritional strategies evaluated.

6 Tools to Measure Gut Microbiota Modulation and Gut Performance

Gut microbiota and gut integrity determine an animal's gut health and are important to evaluate the overall health status.

By **Gertrude Wegl**, Scientist, **Theresa Kaschubek**, Research Associate, and Dr. **Elisabeth Mayer**, Research Team Leader Cell Biology

The importance of a healthy gut is increasingly recognized as key to modern production systems—particularly as it relates to the reduction of antibiotics and improvement in animal welfare. The gut is the first line of a body's defense: a sufficient balance of beneficial intestinal microbiota and a tight gut barrier both protect animals against pathogens and toxins. The gastrointestinal tract acts as an interface between diet, host, and gut microbiota, and plays a clear role in an animal's health status (*Figure 1*). Diet, including feed and feed additives, constitutes a major factor that affects the composition and the activity of the gut micro-organisms and gut peformance.

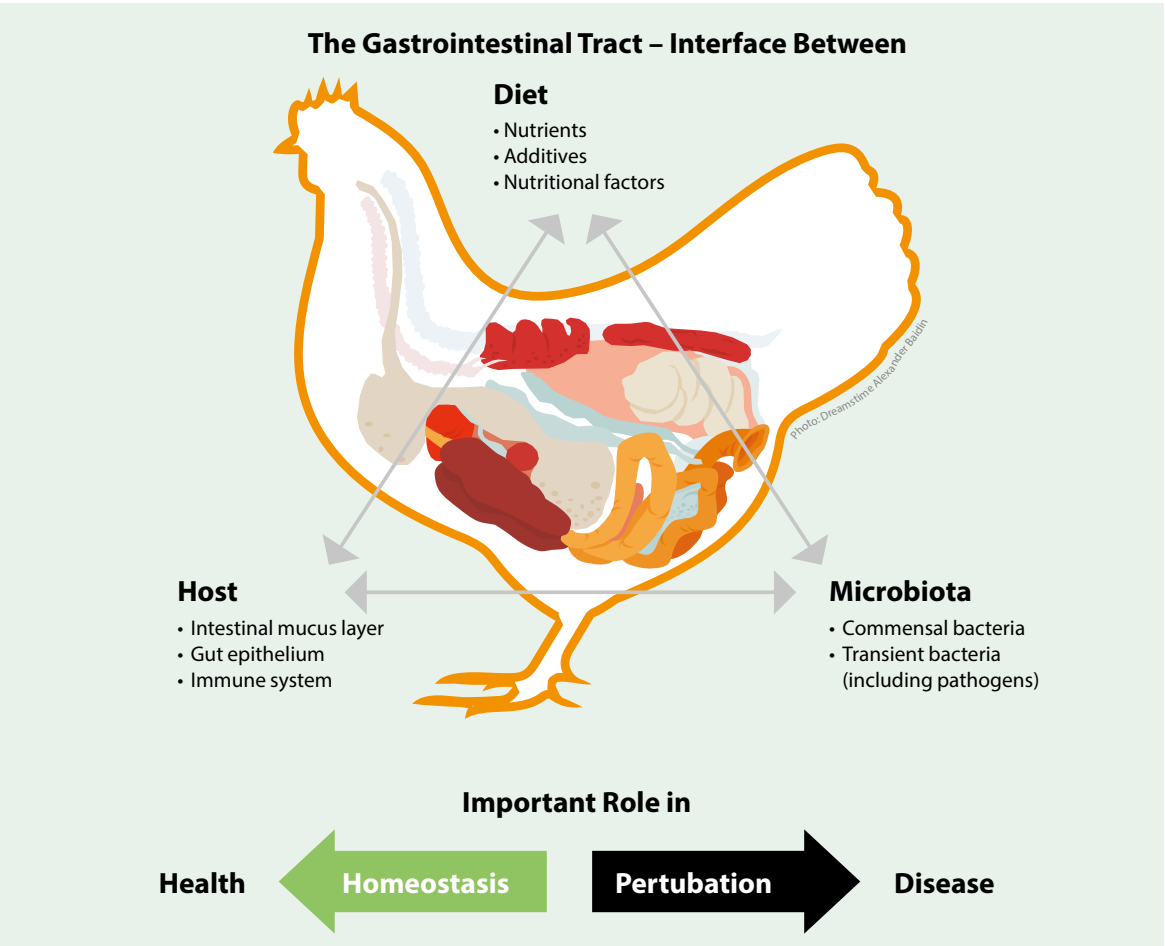
Our research

Researchers and scientists at the BIOMIN Research Center utilize a range of different methods to characterize the intestinal microbiota and substances that increase the overall gut health. Here, we examine three molecular methods to assess gut microbiota that allow analysis of samples *ex vivo*, and three cell culture based models for gut performance that simulate the gut intestine/epithelia *in vitro*. The former constitute a major improvement to analyze bacterial communities and allow us to take a closer look on microbiota composition and diversity as well as quantity of particular bacterial groups. The latter allow for mimicking of infections and screening of beneficial substances without harming animals by using animal specific cell lines, e.g. the intestinal porcine epithelial cell line (IPEC-J2).

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) takes an overall profile of the microbial community, and can quickly process a large number of intestinal or fecal samples.

Figure 1. Interplay between diet, host, and gut microbiota - factors influencing the gut ecosystem and contributing to health and disease.



Source: Adapted from Conway, 1994

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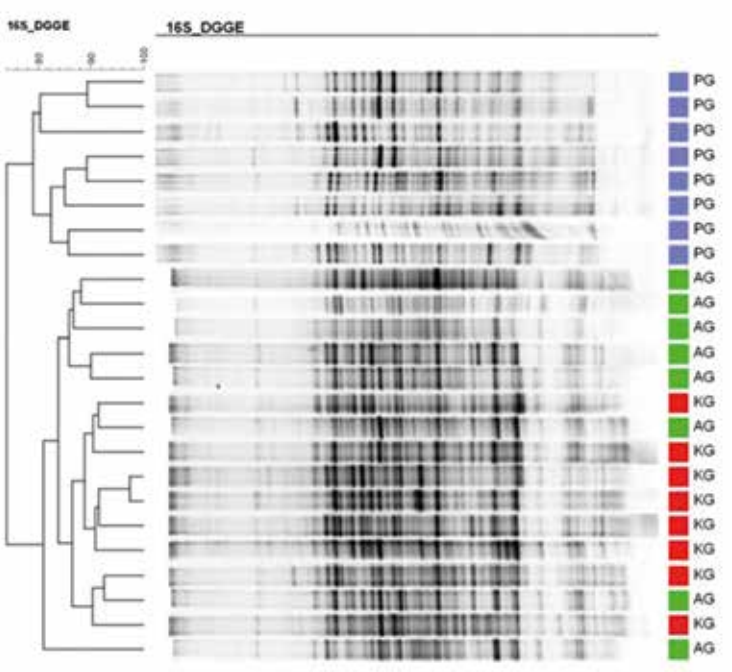
Figure 2. Cluster analysis showing similartiy of *16S* rRNA DGGE community profiles of fecal samples from pigs fed different additives.



Source: BIOMIN

DGGE is based on amplification of a specific gene, typically *16S* rRNA used as a molecular marker, and separation of the different variants of the gene in the community sample by electrophoresis in a denaturing gel. After staining, differences in the gene sequence result in the appearance of characteristic band patterns in the gel, so-called 'fingerprints'. DGGE allows comparison of microbial communities by cluster analysis of these fingerprints, which can be further used to monitor the effects of feed additives on the diversity and dynamics of fecal microbiota of animals (*Figure 2*).

Sequence based Gut Microbiome Profiling

For detailed information on the bacterial composition, *16S* rRNA based amplicon sequencing enables identification of the entire microbial community within a sample up to the species level. Total sample DNA is first amplified by PCR using *16S* rRNA oligonucleotides and using specific adapters and barcodes, many samples can be combined in one sequencing run. PCR amplicons are therefore coupled to spherical particles and loaded on disposable sequencing chips.

Using e.g Illumina Miseq as sequencing platform, out of complex samples 5 to 10 GB of sequence data (approx. 10 to 15 million sequencing reads, depending on sequencing depth) can be expected. Bioinformatic evaluation includes processing of raw reads and clustering of related sequences. These clusters of similar sequencing reads are referred to as operational taxonomic units (OTUs). Microbial identification is accomplished by comparison to sequences in *16S* based reference databases (e.g. RDP II or Silva).

Quantitative polymerase chain reaction

For quantitative information on bacterial group or species level, real-time polymerase chain reaction (qPCR) using specific oligonucleotides (targeting the *16S* rRNA gene or other marker genes) can be used. It allows direct identification of dietary effects on beneficial and harmful bacteria. Furthermore, it can be used to specifically detect probiotic strains, like *Lactobacillus reuteri*, within the gastrointestinal tract.

Anti-oxidative assay

The anti-oxidative potential of phytogenics in IPEC-J2 is assessed by using 2', 7'- dichlorodihydrofluorescein diacetate (DCFH-DA) which is able to incorporate into cells and become fluorescent upon exposure to reactive oxygen species (ROS). ROS are induced by stimulation with H_2O_2 and are determined via measurement of fluorescence, directly proportional to the amount of intracellular ROS. Potent phytogenic test substances can counteract the ROS production, indicated by a decreased fluorescence (*Figure 3A*). The reduction of oxidative stress supports animal performance.

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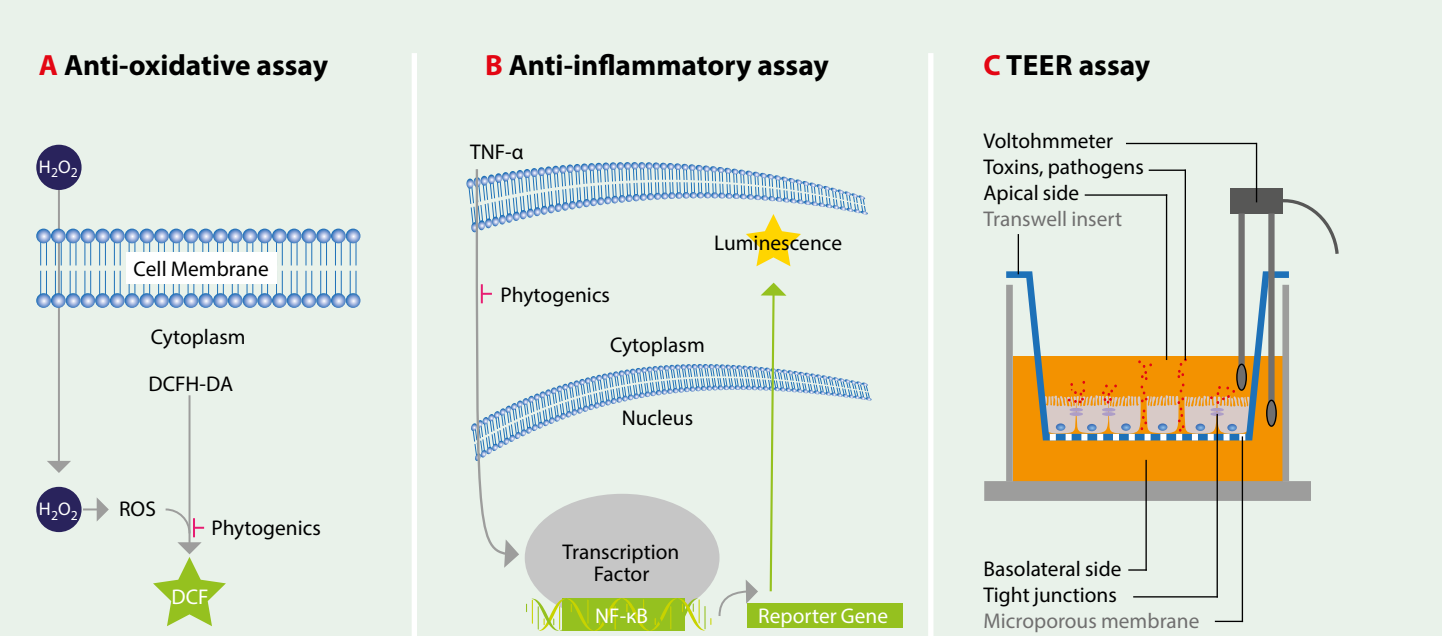
Figure 3. Three cell culture assays used to investigate the anti-oxidative (A) and anti-inflammatory (B) properties of phytogenic test substances as well as to study the epithelial barrier integrity (C) in an intestinal porcine epithelial cell line (IPEC-J2).



Source: BIOMIN

Anti-inflammatory assay

To screen for anti-inflammatory activity of phytogenic test substances in IPEC-J2, levels of the pro-inflammatory transcription factor NF-κB are monitored via the luminescence based NF-κB reporter gene assay. Cells are transfected with the NF-κB reporter vector and are pre-incubated with phytogenics. Followed by the activation of NF-κB by stimulation with the pro-inflammatory cytokine TNF-α, the potential of test substances to attenuate TNF-α-induced inflammation is determined via measurement of luminescence, directly proportional to the amount of activated NF-κB (*Figure 3B*). By reducing inflammation, animals have more energy to put towards growth.

TEER assay

The transepithelial electrical resistance (TEER) assay (*Figure 3c*) is a cell culture model to assess gut barrier function *in vitro*. Therefore, IPEC-J2 are seeded in transwell-inserts with a porous membrane imitating the apical (luminal) and the basolateral (blood) side of the gut. After 8 days of differentiation, the ohmic resistance between the two compartments is measured, indicating intactness of the intestinal epithelium. A reduction of the TEER value is an early marker for disruption of the epithelial barrier. The TEER model offers the opportunity to assess the effect of mycotoxins on gut barrier integrity and to screen for bioprotective substances that are able to counteract these negative effects.

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