DFM DEVELOPMENTS – WHAT THE FUTURE CAN HOLD

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INTRODUCTION

The use of direct-fed microbials (DFM) is widespread with increasing demand for new DFMs with improved activities and functionalities. DFMs are used as feed additives for the following reasons:

1. To maintain the balance of intestinal microorganisms
2. To enhance average daily weight gain
3. To improve the nutritional value of diets
4. To improve animal health
5. To replace subtherapeutic antibiotic growth promoters
6. To readjust animal physiology during periods of stress
7. To reduce environmental waste
8. To address food safety issues

Traditionally, DFM development was hampered by technological limitations and lack of knowledge concerning the mode-of-action of DFMs. As a result, an empirical approach was used for DFM development which typically involved *in vitro* screening of microbial cultures for desirable attributes, followed by testing of promising candidates in animal field studies for efficacy and consistency. This approach carried significant risks, because of the limited *in vivo* data supporting development of the DFM, and the expense of performing animal trials. In recent years, the advent of new molecular technologies has improved *in vivo* detection, quantification, and visualization of DFMs, and determination of their mode-of-action with the host and other gut microbiota components. The objective of this paper is to introduce these new technological approaches that allow more efficient and targeted DFM development.

GUT MICROFLORA

At birth, the gut of the newborn animal begins to be naturally colonized by microorganisms from the parents and the environment. These microorganisms consist of beneficial, commensal bacteria as well as undesirable bacteria (Hume et al., 2004). Under most conditions, the beneficial, commensal bacteria will dominate the microbial population in the gastrointestinal tract and establish a variety of symbiotic relationships with the host. The coexistence and balance of these bacteria is an important factor for the general health and performance of the animal. If the balance is upset, then the animal's health and growth potential will be at risk, because decreased levels of beneficial bacteria

have been correlated with increased levels of undesirable bacteria and potential pathogens (Jin et al., 1997). To prevent and/or overcome this risk, DFMs are typically fed to animals when they are most susceptible to colonization or when they are subjected to increased metabolic activity by these undesirable bacteria, such as after birth, and with dietary changes, periods of stress, and therapeutic antibiotic treatment (Jin et al, 1997).

The gut of the mature animal is densely populated (10^9 to 10^{12} cells per gram of digesta contents in the colon) with a diverse microflora (approximately 500 different species identified to date) (Zhu et al., 2002; Xu et al., 2003). Many more species are being identified by genetics-based, culture-independent methods. The gut microflora is dynamic. It is in a constant state of flux throughout the animal's life cycle due to, for example, dietary changes relating to production, transport, as well as a variety of environmental and host-associated factors. Factors that affect the gut microflora include host age, diet, digestive functions, drug administration, environmental and management changes, and disease challenges, among others (Jin et al., 1997). The complex and dynamic nature of the gut environment and its microflora present unique challenges for developing new DFMs that are efficacious and consistent at enhancing animal performance. As a result, new approaches are needed to address these challenges that have historically confounded new DFM development.

DIRECT-FED MICROBIALS

Efficacious DFMs have activities and properties that augment and complement those of the host's physiology and the host's gut microflora. They can stimulate both the gut microflora as well as host functions. Interactions between the DFM, the commensal gut microflora, and host can facilitate systemic effects that improve animal health and performance. DFMs have been shown to enhance animal health and performance through the following modes of action:

1. Competitive exclusion: Beneficial microorganisms that outcompete potential pathogens and undesirable bacteria for attachment sites on the gut epithelium and thus preclude them from colonizing the intestinal tract (La Ragione and Woodward, 2003). Competitive exclusion DFMs are most effective when administered to newborn animals.
2. Antimicrobial activity: Production of bacteriocins, hydrogen peroxide, and other compounds, which can inhibit similar, closely related bacteria as well as potential pathogens and other undesirable bacteria (Lindgren and Dobrogosz, 1990; Sanders et al., 2003).
3. Organic acid production: Production of lactic, formic, acetic, propionic, butyric, and other acids can inhibit potential pathogens and other undesirable bacteria, and favor growth and establishment of beneficial bacteria (Van Der Wielen et al., 2000; Zampa et al., 2004).
4. Host immunomodulation: Beneficial microorganisms influence angiogenesis, development of gut-associated lymphoid tissue (GALT), induction of oral tolerance, mucosal immunity, and diversification of the preimmune antibody repertoire (Rhee et al., 2004; Vinderola et al., 2004).

5. Enzyme production: Beneficial microorganisms, especially *Bacillus*, produce a variety of enzymes that can contribute to the enzymatic capacity of the gut (Ferrari et al., 1993). Many of the hydrolases produced by these microorganisms can improve digestion and absorption of nutrients by the host (Jin et al., 1997; Omogbenigum et al., 2004).
6. Toxic metabolite reduction: Beneficial microorganisms can suppress or modify production of toxic metabolites, such as from bile acid metabolism, by undesirable bacteria (Jin et al, 1997; Zampa et al., 2004).

A large number of DFM organisms exist, and new ones are being developed. The Food and Drug Administration (FDA) and Association of American Feed Control Officials (AAFCO) maintain a list of approved microbial species for use in DFM products. Expectations are that this list will continue to expand as the properties and safety of (new) DFM organisms are demonstrated.

CONSIDERATIONS FOR NEW DFM DEVELOPMENT

Targeted, rational DFM product development

To develop a new DFM or to improve an existing product, strategy dictates that a specific target be selected. But even with a specific target in mind, the gut environment and microflora is so complicated that new technologies are required to provide reasonable expectations of success.

Possible targets for DFM candidates

1. Production of a growth factor or an effector molecule that supports the growth or stimulates the activity of indigenous commensal bacteria.
2. Generation of a substrate that can selectively support growth of beneficial bacteria in gut microflora (Jaskari et al., 1998; Konstantinov et al., 2003).
3. Fermentation of host indigestible poly- or oligosaccharides to either assist the host's nutrient uptake or to produce short chain fatty acids, such as acetic, propionic, and butyric acids to inhibit undesirable and pathogenic bacteria, and reduce the pH of the gut (Van Der Wielen et al., 2000; Zampa et al, 2004).
4. Delivery of DFMs to regions of the gut in which the cells lyse, releasing their complement of intracellular enzymes.

Requisite Bacterial Attributes

In order for a DFM to function in an efficacious and consistent manner, there are some properties and characteristics that they should possess. Among them are:

1. To maintain activity in the anaerobic gut environment
2. To associate with the epithelial lining, if needed for activity and/or to trigger a response
3. To tolerate the pH, digestive enzymes and other gut conditions

4. To metabolize (continue activity), or to replicate with the gut
5. To be recognized as "self" by the host animal

TOOLS FOR NEW DFM DEVELOPMENT

With the advent of molecular technologies such as immunochemistry, molecular biology, and fluorophore chemistry, multiple molecular probing systems have been developed that specifically track, detect, and quantify microorganisms in complex ecological systems, as well as measure their physiological status and response *in situ*. Probes are labelled chemical compounds, such as oligonucleotide probes, antibodies, and metabolizable chemicals, which have specific target molecules or sequences, and are used to bind to these targets to measure an effect under a given set of conditions being studied. Probes labelled with specific reporters can be combined to measure several parameters in a single experiment (multiplexing). The following paragraphs provide examples of use of these probing systems and how they can help develop DFMs based upon a targeted approach.

An important challenge to developing new DFMs is demonstrating their effect on the indigenous gut microflora. This is made particularly difficult because current microbiological methods support the growth of only a fraction of the total bacterial diversity present in the gut, and cannot identify and/or track other components of the bacterial community that are not cultivated. These "not yet cultivated" microorganisms can be identified and monitored by cultivation-independent molecular methods that combine specific oligonucleotide probes and Polymerase Chain Reaction (PCR). A cultivation-independent approach typically used for surveying gut microflora involves phylogenetic analysis of 16S ribosomal DNA (rDNA) sequences obtained directly from gut content samples by PCR amplification, random cloning, and nucleotide sequencing of the clones. The 16S ribosomal RNA gene is common to all bacteria and has distinctive features that permit microorganisms to be distinguished from one another at the genus and species levels. Combination of this method with more traditional cultivation-dependent methods has resulted in more complete surveys of the biodiversity of the gut microflora. These approaches have significantly improved knowledge of the gut microbiota in humans (Suau et al., 1999), pigs (Leser et al., 2002) and chickens (Zhu et al, 2002).

Although random cloning of PCR-amplified 16S rDNA is very powerful for assessing the biodiversity of a bacterial community, it is not appropriate for detecting changes in the composition of the gut microflora resulting from addition of a DFM or other factors. To analyze population shifts and/or changes in the predominant microflora, techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), Fluorescent *In-Situ* Hybridization (FISH), and Quantitative/Real-Time PCR are more appropriate. DGGE is a genetic fingerprinting technique that can be used to qualitatively evaluate the predominant microflora and identify changes in the composition and balance of the microflora in response to addition of a DFM or other factors. DGGE is a high-resolution chromatographic method that separates amplified 16S rDNA molecules on polyacrylamide gels containing a linear gradient of denaturing agents. The separated 16S

rDNA molecules appear as a series of bands of defined intensities. The bands correspond to specific bacterial constuents and the band intensities reflect the relative predominance of the bacteria in the community. This method has been used to document changes to the gut microflora of pigs in response to dietary changes and aging (Simpson et al., 1999; Konstantinov et al., 2003).

FISH is a hybridization technique that can quantitatively determine specific groups of bacteria in complex microbial communities through selective binding of fluorescently labelled oligonucleotide probes to 16S rRNA of fixed whole cells. The probes can be designed to be highly selective for closely related bacteria (sub-species-level specificity) or less selective to be more inclusive of remotely related bacteria (domain-level specificity). Several probes labelled with specific fluorophores can be used simultaneously to allow multiplex detection and counting of bacteria. This method has been used to quantify changes in the levels of specific bacterial groups within the gut of chickens in response to aging (Zhu and Joerger, 2003).

Another technique that can be used to facilitate tracking and quantification of DFMs and other bacteria is Quantitative or Real-Time PCR. This method is similar to FISH, because it combines specifically designed probes (targeted for 16S rDNA or other genes) and fluorescent labels to detect and quantify bacteria. However, this technique is different from FISH, because it uses PCR to amplify the target sequence. Amplification of the fluorescently labelled product is dependent upon the initial level of target sequence, which can be used to quantify specific bacteria present in the microbial community. This method has been used to detect and quantify changes in the gut microflora of humans in response to hospitalization and antibiotic treatment (Bartosch et al., 2004).

DGGE, FISH, and Quantitative PCR can be used to assess compositional changes of the gut microflora in response to typical stresses imposed by commercial production. Evaluation of these changes will enhance the knowledge needed for a targeted and rationale approach to new DFM development in response to these changes. These methods can also be used to determine what effect the DFM has on both the diversity and specific bacterial levels of the gut microflora. The information obtained using these techniques will improve knowledge of the potential mode-of-action of the DFM as well as responses by the host to the DFM and other factors. These methods can also be used to track the DFM in the gut and to determine predominance and/or persistance of the DFM during and after treatment.

Another important challenge to developing a new DFM is understanding gene expression that occurs in both the DFM and the gut epithelium. The advent of microarray technology for evaluating and understanding gene expression has improved understanding of gene expression and their environmental change or stress. Microarrays work by exploiting the ability of a given mRNA molecule to specifically bind or hybridize to the DNA template from which it originated. By using an array containing many DNA samples, scientists can estimate, in a single experiment, the expression levels of hundreds or thousands of genes within a cell by measuring the amount of mRNA or

cDNA bound to each site on the array. With the aid of a computer, the amount of mRNA or cDNA bound to the spots on the microarray is precisely measured, generating a profile of gene expression in the cell. Microarray technology can be used to determine which genes of a potential DFM are expressed when subjected to environmental conditions of the host animal's gastrointestinal tract. Also, microarrays can be used to determine how the animal host's immune system is stimulated by interaction with a DFM, and can help determine the mode-of-action of the DFM in the host.

While microarray data is used to evaluate the expression of the entire genome of an organism, Quantitative PCR allows more precise quantification of gene expression and over a wider range of expression level, for a specific subset of genes. Together, these techniques provide information about the physiological response of the organisms and which genes are mediating this response. This information can be used to determine the mode-of-action of the DFM as well as the specific genes responsible for this beneficial effect.

Another important challenge in developing a DFM is to determine if the microorganism has the right activities and properties to generate a beneficial effect in the gut. Working with animal models or conducting animal field trials can be costly and is not a practical approach for screening DFM candidates. Although animal studies will ultimately be required to validate the efficacy and consistency of a DFM, model systems can be effective for screening microorganisms and developing candidate DFMs, while reducing costs and risks.

Simple systems that simulate selected conditions of the gut can be used to evaluate the effects of anaerobic conditions, high concentrations of volatile fatty acids, bile salts, and the presence of indigenous microorganisms on potential DFMs. Then, more sophisticated models can be employed that accurately mimic the fluid dynamics (passage rate) of the GI tract, pH, bile salts, and digestive enzyme regimen (Arkbage et al., 2003; Blanquet et al., 2004; Van Der Werf and Venema, 2001; Verwei et al., 2004). These model systems (e.g. TIM-1, TIM-2) can be used to determine if the potential DFM being evaluated can grow, and express the activities and properties important for DFM functionality. These systems can also be used to determine the effect of the DFM on a gut microflora established in the system to determine if they promote beneficial bacterial growth and inhibit undesirable bacteria. Additionally, the DFM could be challenged with undesirable or pathogenic bacteria to determine the degree to which the DFM inhibits these bacteria. Although *in vitro* models have their limitations, they can be validated and are subject to less variability than animal models. Also, sampling may be easier from a simulation model rather than from an animal.

Evaluation of the metabolic potential of a complex bacterial community often reveals specific metabolic activities originating from a subpopulation that hold promise for DFM development. A new technique called Stable Isotopic Probing (SIP) has been developed that permits identification of bacterial sub-populations responsible for specific metabolic activities without cultivation (Jeon et al., 2003). Specific substrates labelled with stable isotopes are provided and metabolized only by the subpopulation expressing the desired

metabolic activity. The isotopes accumulate in the DNA of these bacteria. After harvesting cells from environmental samples (no cultivation bias) and extracting the genomic DNA, the DNA with incorporated isotopes is separated from the rest of the DNA based on differentiating properties. The identities of the bacteria are determined from this DNA by 16S rDNA sequencing. Phylogenetic classification of the bacteria will likely provide insight on cultivation requirements for the bacteria, which can be used to selectively grow and isolate these bacteria from the bacterial community.

In DFM development, it is important to determine viability of cells under different environmental test conditions as well as challenge by other bacteria. Flow Cytometry is a technique that permits direct counting of bacteria in a liquid sample without the bias associated with cultivation techniques. By using different combinations of genetic, immunological, and/or chemical probes, Flow Cytometry can be used to distinguish live/dead cells and can be used to specifically count a DFM within a mixed population of bacteria. In a similar manner, Flow cytometry can also be used to evaluate cell counts and viability of specific undesirable bacteria in the presence of DFM. This technique may be useful for evaluating candidate DFM viability under gut-like conditions, and facilitate selection of DFMs that are robust under these conditions. For example, flow cytometry has been used to assess the viability of bifidobacteria and lactic acid bacteria after bile salt stress (Bunthof et al., 2001; Amor et al., 2002)

SUMMARY

In recent years, DFMs have shown exciting potential for improving animal health and growth performance. However, development of efficacious DFMs that provide consistent results has been hampered by the complexity of the gut environment and insufficient knowledge about DFM mode-of-action. Technological advances have provided new tools and approaches for studying complex microbial communities and gene expression of constituent organisms. By using these tools, scientists have a better understanding of both the gut environment and DFM mode-of-action. As a result, a more targeted approach can be used to develop more efficacious and consistent DFM products.

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