MODERN PROBIOLOGY - DIRECT FED MICROBIALS AND THE AVIAN GUT MICROBIOTA

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Summary

Direct fed microbials (DFM - microorganisms which when fed exert beneficial effects on poultry performance, health, and immunity) routinely demonstrate efficacy in enhanced feed conversion and growth performance that is comparable to that obtained with subtherapeutic antibiotic usage. The mechanistic basis of the probiotic is largely unknown. Our laboratory has used a microbial ecology approach to understanding gut microbial communities, and host immune response from DFM feeding in broilers, layers and turkeys. A unique DFM strain selection and formulation process is presented which is based on an understanding of the genetic diversity and levels of *C. perfringens* and avian pathogenic *E. coli*. Changes in microbial diversity and profile or balance of the avian gut are associated with disease and poor performance; examples of this are the cases of clostridial dermatitis, and focal duodenal necrosis and the concomitant changes in gut microflora. Probiology is the study of probiotics and their interaction with the host. The probiotic concept is evolving and a new generation of DFMs, for different feedstuffs, climates and genetic lines of poultry is potentially on our horizon.

I. INTRODUCTION

Reduction or elimination of subtherapeutic antibiotics in poultry production is a formidable issue with the potential for significant losses in production efficiencies. Considering alternate rearing strategies, Bedford (2000) emphasized how antibiotic withdrawal, and high variability in feedstuffs will require nutritional control to help mitigate negative impacts of increased intestinal pathogens and parasites. Feed ingredient variation greatly influences gut microstructure, microflora, and tissue enzyme activities which are linked to performance (Amerah et al , 2009; Shakouri et al, 2008). Dietary and management practices for clostridial and *Eimeria* control will include multiple technologies, in combination and on rotation (Dahiya et al., 2006).

Progress and growth of the field of probiotic biology is strongly tied to the development of molecular microbiological techniques and bioinformatics which have greatly freed the biologist from the once arduous tasks of microbial community assessment. DFM use has increased in the last ten years and is growing in light of what is becoming a post-antibiotic era of food animal production. DFM usage will increase as more poultry scientists are trained in the use of DFMs and the scientific and economic basis for their inclusion in feeds.

Probiotic biology is a two-sided system of probiotic microorganisms intertwined with the host. The gut microbiome and its host conduct active chemical communications within and between each other (Corthesy et al., 2007; Deplancke and Gaskins, 2001; O'Flaherty and Klaenhammer, 2009). Knowledge of the between the gut microflora and its host provide a framework from which to understand how controlling and manipulating this system by DFM feeding will be understood at the biochemical level.

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II. A PRIMER ON DIRECT FED MICROBIALS

Probiotics for livestock, are termed direct fed microbials or DFMs. Three definitions of probiotics are [i] 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO, 2002, ftp://ftp.fao.org/docrep/fao/009/a0512e/a0512e00.pdf), [ii] 'A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989) and this has been expanded to [iii] 'A preparation or a product containing viable, defined micro-organisms in sufficient numbers, which alter the micro-flora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host' (Callaway et al., 2008). DFMs can be bacteria, yeast, fungi, or even viral agents, this paper is limited to bacterial DFMs, mainly thos ewhich are *Bacillus*-based. A comprehensive list of DFMs can be found at www.efsa.europa.eu/en/efsajournal/doc/2497.pdf.

Which bacteria are accepted for use and can be generally recognized as safe (GRAS) differs across countries. In the United States, the US-Food and Drug Administration Center for Veterinary Medicine regulates the usage of DFMs in feeds. In Australia, the pertinent regulatory for DFMs is the Australian Pesticides and Veterinary Medicines Authority (www.apvma.gov.au/publications/guidelines/gl9_microbial.php). Within the European Union, DFMs are regulated by the European Commission (2011) with scientific input from EFSA (European Food Safety Agency).

Bacterial DFMs are either sporulating (spore-forming) or non-sporulating (asporogenous) Sporulating DFMS can be administered through the watering system or in heated extruded feed, whereas non-sporulating DFMs are limited to water delivery or non-heated feeds.

Sporogenous DFMs administered as spores are bacteria confined to the bacterial genus *Bacillus*. Bacilli are grown in large-scale industrial fermentors to where sufficient proportions of cells have developed endospores (spores and endospores are synonymous). Then, a concentrate of bacterial spores is made by concentrating the cell/spore mass with centrifugation, and spray-drying or freeze-drying then milling into the "pure culture form" ranging from ~ 10^{10} to 10^{12} cells or colony forming units (CFUs) per gram of powder. At this point, *Bacillus* spore DFMs are stable for many years when held under dry conditions and ambient temperatures; refrigeration of *Bacillus* spore preparations is not required. The spore concentrate is subsequently blended into a pre-mix with carrier materials such as soy hulls or limestone to ~ 10^6 to 10^{11} CFU/g and supplied to mills for blending into feeds. At his point, feed mills generally will add the pre-mix at an inclusion rate of ~1 lb per treated ton of feed giving ~ 10^5 CFU per gram of feed. The standard in broiler and turkey production is extruded pelleted feeds, Extrusion subjects the DFM to temperatures of 82 to 88˚C for 30 to 40 seconds. This treatment does not significantly affect viable *Bacillus* DFM cell counts.

Proficient manufacturers of *Bacillus*-based DFMs conduct quantitative microbial assays for viable cell counts and purity at two stages of production; the concentrated product/pre-mix and the final feed. In addition, genetic fingerprint analysis of the *Bacillus* product assures identity and is an analytical step which, though not required (e.g. in the United States), is used by some manufacturers of *Bacillus* DFMs., Due to the environmental stability of Bacillus spores, fermentation facilities that produce *Bacillus* on a large fermentation scale are usually dedicated facilities which do not produce other types of DFMs outside of the *Bacillus* genus.

Within the current US guidelines *Bacillus* species allowed for feed use in poultry are: *Bacillus subtilis, B. lentus, B. licheniformis, B. pumilus*, and *B. coagulans*. Others with EFSA

qualified presumption of safety (QPS) are: *B. amyloliquefaciens, B. atrophaeus, B.clausii, B. coagulans, B. fusiformis, B. megaterium, B. mojavensis, B. vallismortis* and *Geobacillus stearothermophilus (EFSA, 2011.)*

Asporogenous DFMs include several genera of bacteria mainly classified as lactic acid-producing bacteria or "lactics" (LABs or LAB). This group is not confined to the *Lactobacillus* genus. *Lactobacillus*, for example, is produced in fermentation-requiring complex production media. Following fermentation, cells are concentrated and separated from the spent fermentation medium and either freeze dried or spray dried and stored frozen or under refrigeration until used. Bacuase LAB are heat labile, they must be fed in non-extruded feeds or through water. . Much like techniques for feed vitamin protection, encapsulation and coating have all been proposed for DFMs. Yet, efficacious and cost effective protective technologies for asporogenous DFMs have not been developed. This is an area in need of continued research.

The genus *Lactobacillus* is generally a major constituent of the healthy gut within the maturing animal. The nemesis group of gut bacteria largely consists of type A *Clostridium perfringens(Cp)* and avian pathogenic *Escherichia coli* (APEC) groups. In the diseased or dysbiotic avian gut, Cp and APEC can be at levels directly inverse to the resident lactobacilli. The DFM user should understand the significance of taxonomic subtypes also called strains, , within the genus and species nomenclature. First, bacteria are generally classified and named according to an accepted system of classification based roughly on Linnaean taxonomy (www.bacterio.cict.fr/). Within a single species are different subtypes each with distinct genetic and functional traits. Subspeciation occurs within all species of bacteria. A good example is *Bacillus subtilis*, a species commonly used in DFMs. At the species level, all are classified as *B. subtilus*, yet within the species are a collection of different subtypes commonly known as strains. The same holds true for species within the DFM genera of *Lactobacillus*, *Enterococcus*, *Pediococcus, Bifidobacterium, Propionibacterium* and others. Pulsed field gel electrophoresis (PFGE) is a technique to separate different strains by a DNA fingerprinting. In specific cases, PFGE has been accepted by the European Food Safety Agency (EFSA) for registration of DFMs.

From country-to-country there are differences among DFM registration processes. Also, for the DFM user, it is worth stating that the naming of bacterial species based on classical systematics (*Kingdom, Phylum, Class, Order Family, Genus, Species, Subtype*) is still in use and is the accepted nomenclature. However, as subtyping data accrue, the naming will eventually be modified to accommodate new names. Here, the regulatory and DFM production sectors will have to convene to harmonize with current microbiological taxonomy.

Some questions common to DFM users arebriefly presented in the following paragraphs.

Does the DFM have to be alive or viable to work or can it work as a dead cell? There is evidence that nonviable cell DFMs can have biological effects (Adams, 2010); however at this stage, most probiotic biologists still assume viability is required. Feeding components of dead bacterial cells can result in immune stimulation. This concept is not covered in this review. Dead cell DFMs and components might offer superior stability to live cells. By current definition, probiotics are live and viable cells.

Are *Bacillus* normal inhabitants of the gut? Yes, they are frequently isolated from poultry. Species of the genus *Bacillus* are found at high levels in the intestines of animals and poultry (Cutting, 2008).

What are traits of an ideal probiotic? Patterson and Burkholder (2003) summarized as follows: probiotics should be of host origin, non-pathogenic, resist gastric pH and bile, resistance to processing, stable in storage, adherent to gut epithelium, persist in the gastrointestinal tract,

produce inhibitory compounds, modulate immune response and alter other microbial activities in the gut. Some of these apply more to some DFMs than others, they are a good generalized set. As caveats, it is important to state that not all DFMs persist or colonize and therefore require continual feeding.

Will feed antibiotics, both subtherapeutic and therapeutic impact DFM efficacy? This would depend on the antibiotic, its dosage or inclusion rate, and the DFM itself. In some cases, DFMs can be inhibited by antibiotics *in vitro,* but it would appear that DFM efficacy on performance is equivalent whether subtherapeutic growth promoting antibiotics are used or not. The potential for a synergism between DFMs and subtherapeutic antibiotics is possible. The current reality is that, with the exception of the EU and Korea, growth promoting antibiotics are still widely used. Again, as probiotic biology is taught to new generations of nutritionists, veterinarians, and poultry scientists DFM usage will rise.

Once ingested, do spores germinate in the bird's gut? Cartman et al. (2008)(1) demonstrated germination of *Bacillus subtilis* spores in the avian chick gut and, post spore ingestion, vegetative forms of the *Bacillus* DFM outnumbered spore forms. This is evidence that *Bacillus* DFMs function by mechanisms which are linked to their metabolic activity.

How do DFMs work? DFMs are capable of one or all of the following activities; better nutrient conversion, lower mortality immune stimulation, anti-inflammatory action and protection from enteric pathogens. To accomplish these duties, DFMs make or are a source of volatile fatty acids, antimicrobials and bacteriocins, competitive exclusion, cell wall components, , small molecular weight antimicrobial and bioactive metabolites, enzymes, bile salt deconjugation enzymes, mycotoxin inactivation, mucin stimulation and others (Patterson and Burkholder, 2003; Rehman et al, 2007). Herein, I will present a unique DFM selection process with a synopsis of probiotic research, and future speculations on this exciting field of biology. Much of what is presented from our laboratories is the outcome of fundamental and applied scientific research conducted in controlled and production conditions over the last decade. The perspective is from the standpoint of the gut microbial community in health and disease as well as functional impacts of DFM usage in poultry immunity.

III. CUSTOMIZING DFMS FOR POULTRY - THE MULTI STRAIN APPROACH

The complexities of poultry production and the interconnectivity of all of the contributing factors is well described by Williams (2005), who penned the term the 'intercurrent coccidiosis-necrotic enteritis syndrome', abbreviated here as ICNES. As gut microbial ecologists, it has been our working hypothesis that the ICNES is a major driver and that, specifically, the levels of *C. perfringens* as well as avian pathogenic *E. coli* (APEC) often are a major driver of health and performance. It is obvious that these factors do not function in isolation of all others, but it is becoming generally accepted that, in the post-subtherapeutic antibiotics era, subclinical and clinical levels of Cp and APEC with a coccidial overlay can dictate routine bird performance. Necrotic enteritis (NE) is the final end of a spectrum of symptoms generally derived from ICNES. More the norm is subclinical NE and the continuous synthesis of several toxins in the gut including Cp α and NetB toxins (Keyburn et al, 2010; Cooper and Songer, 2010) all of which are a likely source of gut leakage and pathogenesis (Lovland et al., 2004).

In our lab we routinely use RAPD (Randomly Amplified Polymorphic DNA) as a tool to genotype and generate pathogen profiles of the populations of Cp and APEC in poultry intestinal tracts taken from farms and whole operations. From each intestinal tract, it is possible to isolate

hundreds of different isolates, and using RAPDs we create family trees of isolates and pick representative members that are highly related. These selected isolates are representative of the dominant genotypes of pathogens in an operation. It became evident that the levels of Cp and APEC differ widely in operations as do their genotypes or genetic fingerprints. We concluded that, not only were there resident populations of APEC and Cp, but that subtypes differed from operation to operation and farm-to-farm and within the sane operation over time (Gebert et al., 2006).

How does pathogen fingerprinting relate to DFMs and specifically *Bacillus*-based DFMs? The answer seems to be the difference in susceptibility of different genotypes of pathogens to the inhibitory effects of different strains of *Bacillus*-based DFMs; also that pathogen subtype populations are not static. Finally, based on those data, a set of *Bacillus* strains is formulated from the mixture of bacilli offering the highest level of inhibition for a specific set of pathogens obtained from a specific farm. This entire process (pathogen isolation, genotyping, subtype selection, susceptibility to *Bacillus* DFMs and selection of combination of strains to formulate a custom DFM) is known as CSI (Customer Specific Inoculant) and serves as a core basis for formulating DFMs which are not generic but tailored to pathogen populations. A typical outcome of a CSI-derived *Bacillus* DFM on the levels of toxigenic C. perfringens in the broiler gut can be seen in Table 1.

Antimicrobial activities of *Bacillus* strains *in poultry* have been previously documented against Cp, APEC and *Salmonella* in pen-reared broiler trials (La Ragione and Woodward, 2003). *Bacillus coagulans* as a poultry DFM, resulted in performance comparable to that of virginiamycin supplementation (Cavazzoni et al., 1998) and Wu et al. (2011), in feeding *B. subtilis* to broilers rates of 10^9, 5×10^9, and 10^{10} bacilli/kg of feed reported increases in lactobacilli and concomitant decrease in *Escherichia coli* versus controls.

A typical Cp reduction control response from a CSI-derived *Bacillus* DFM used in commercial broiler production is presented (Table 1). Final performance outcomes however are perhaps the more important measures of multi-strain *Bacillus* DFM efficacy. An example will be provided which illustrates a successful application in a US broiler flock experiencing high levels of gut *E. coli* as well as a viral disease challenge. CSI-DFM *Bacillus* usage in this operation resulted in regaining performance goals as well as reportedly shorter time to final weight. Achieving target weights sooner permitted the producer longer inter-flock down periods and 2-3 days more litter drying time. Increased drying times result in lower litter water activity (A_w) and subsequently lower litter *Salmonella* isolation rates (Hayes et al., 2000).

Bacillus DFMs are commonly applied as a single strain of probiotic, however multi-strain *Bacillus* DFMs are not only warranted, but essential for broader pathogen control of highly diverse Cp and APEC populations (Gebert et al., 2006).

IV. GUT MICROFLORA IN HEALTH AND DISEASE

What is the normal composition of the intestinal microflora of domestic poultry? Is there actually a core microbiome of the domestic chicken? In a healthy state, the microbiome is a community of hundreds of microbial genera and species which changes from birth to death, and is influenced by diet , age, environment and genetics. In comparative terms, diet is perhaps the single factor contributing the most to the profile (Apajahlati et al., 2001). In a healthy state, the relative proportions of major groups will change, but, are dominated by bacteria classified as lactobacilli, enterobacteriaceae, clostridia, bacteroides, enterococci and many other groupings

both anaerobic and facultative (Ewing, 2008). In a healthy or balanced state lactobacilli dominate, but under conditions of disease or imbalance, termed dysbacteriosis, other populations will dominate and relative proportions change. The case of NE is a suitable example; here the proportion of *C. perfringens* and clostridia enlarges with a concomitant decrease in the proportion of the lactobacilli. Such an inverse relationship appears, from consistent reports in the scientific literature, to be a repeated theme in the avian gut system (Bjerrum et al., 2006). Using techniques ranging from microbiological culture (Barnes et al., 1972) to molecular biology (Apajalahti et al., 2001; Lu et al., 2003; Torok et al, 2008; Wise and Siragusa, 2006; Nordentoft et al, 2011; Qu et al 2008; Zhu and Joerger, 2003), the resulting microbial profiles are remarkably similar.

Attempts to correlate gut microbiota with high levels of performance have been attempted (Apajahlati et al, 2004; Torok et al, 2008). Here are presented examples of avian gut microbial ecology and observations from our labs applied to problems of poultry production over several years using different techniques of assessment. All of these methods rely upon using a species specific bacterial signature gene known as the 16S-rRNA-DNA (16S) ribosomal RNA-DNA gene.

A. Microbial profiling of turkeys with clostridial dermatitis - cloning and sequencing

Clostridial dermatitis (a.n.a. gangrenous dermatitis, or turkey cellulitis) is a lethal condition of intestinal origin caused by the histolytic clostridia *C. septicum* and *C. perfringens.* (Clark et al., 2010). Our lab characterized the gut microflora of turkeys in the same flock, symptomatic and asymptomatic for clostridial dermatitis. Gastrointestinal tracts (GITs) were harvested from approximately 12-15 week old turkeys fed a standard corn-soy diet, from a flock afflicted with clostridial dermatitis. DNA was isolated from the duodenal, illeal, and jejunal sections of the GIT mucosa. Derived 16S sequences were analyzed and profiles constructed. Figure 1 (top) displays community assessments obtained by using 16S cloning and sequencing of DNAs from healthy turkeys and the same number from diseased flock mates. In the diseased turkey samples, the major portion (82%) of the diseased bird microbial profile were clostridia, whereas microbes comprising 85% of the profile of the healthy bird belonged to the lactobacillus group.

B. Microbial profiling of turkeys with clostridial dermatitis - TRFLP

Terminal restriction length polymorphism (TRFLP) profiling is a powerful technique useful for assessing overall microbial community structures. The same DNA samples used in the above section were subjected to TRFLP. Each TRFLP peak corresponds to a group of bacterial species, or in some cases, a single species. It can be seen (Figure 1, bottom) that the general pattern of the avian gut microbial community is again evident. Comparing the healthy to the diseased GIT profiles, we observe several lactic acid bacterial peaks not present in the diseased GIT, and vice versa for the levels of clostridia and enterobacteriaceae (the group containing *E. coli*).

Another finding from our lab is the first description of the putative clostridial origins of the layer condition termed focal duodenal necrosis or FDN. Denaturing gradient gel electrophoresis or DGGE was used in conjunction with TRFLP to substantiate the hypothesis that the putative causative agent in FDN was *Clostridum colinum* and, conversely in FDN-negative flockmates, a TRFLP peak specific to *Lactobacillus* was identified. (Baltzley et al., 2008).

C. Community assessment of healthy broilers – Pyrosequencing

Litter is a significant part of the total mass intake by broilers. An experiment to profile the gut microbiomes of broilers reared on used litter from either diseased or healthy flocks (Neumann et al., 2011) was conducted. Over time, GITs were harvested at points day 14, 28,and 42. Mucosal DNA was isolated and, following 16S gene amplification, the amplicons were subjected to high throughput automated DNA sequencing using pyrosequencing technology (Hume et al., 2011). Each community profile is represented in a pie chart format (Figure 2). Each profile is based on at least 3,000 bacterial signature sequences per mucosal DNA sample. Again we observe the progression of the gut bacterial community from mainly clostridial to a more lactobacilli dominated population. It is noteworthy that the dominant sequence reported at day 14 is a clostridial organism known as *Candidatus* Arthromitus, also known as segmented filamentous bacteria or SFBs. This group of bacteria are members of the clostridial group and, to date, are non-culturable. *Candidatus* Arthromitus is reportedly the most potent stimuli of the gut immune system (Talham, et al., 1999).

Analysis using high throughput pyrosequencing offers the greatest range of resolution to date in the shortest return time. Using the same technique, a study was recently initiated and preliminary data reported on differing microbial community profiles of healthy heavy and light flock mate turkeys (Benson, 2011).

V. DIRECT FED MICROBIALS AND AVIAN IMMUNE EFFECTS

The gut microbiota exerts a significant influence on the host's immune function starting at birth and throughout life (Corthesy et al., 2007; Klasing, 2007). DFM feeding has been demonstrated to influence the gut, systemic, and tissue adaptive and innate immune responses. In a series of experiments Lee et al reported *Bacillus*-based DFMs enhanced humoral antibody in response to *Eimeria* infection and lower lesion scores, macrophage augmentation, differences in cytokine gene expression and lymphocyte profiles, and subdued acute phase protein levels in broilers fed *Bacillus* based DFMs, singly and combined, through 22d (Lee et al., 2010a, 2010b, 2011a, 2011b) Also from our lab, a study of farm-reared, ionophore and antibiotic-fed turkeys, Novak et al. (2007) reported differences in $CD4^+$, $CD8^+$ and dual $CD4^+CD8^+$ peripheral blood lymphocytic cells in turkeys pulse fed *Lactobacillus brevis* for three days compared to vs controls not fed the DFM. These same authors found that in addition to altering immune development, in the early life stage (up to 16d) a growth enhancement effect was reported which was not observed at 37d. In this instance and as previously reported, there is a distinct difference in immune effects between different subtypes of the same species of DFM.

VI. NEXT GENERATIONS OF DFMS

Using molecular microbial ecology techniques for DFM strain selection, we have applied DFMs to a portion of the US poultry population. Our strategy was to acquire knowledge of the microbiology specific to individual operations but which could be extrapolated to production in general. From that knowledge there are several areas which should be a focus of further research and development for the next generation of DFMs.

Understanding the microbial succession which occurs in the avian gives a more coherent understanding of gut microbiomes across production systems (see *Big Science* below). DFM strain selection can be for the level of gut maturity at feed change points. In general, broiler production feeding regimens give six points for different DFM addition; maternal flock, hatchery, starter, grower, finisher, withdrawal. Multi-functional DFMs will mimic the heterogeneity of the natural gut microbiota. Just as the gut microbiota is a widely diverse community with each member serving some function, multi-functional DFMs, and mixtures thereof, would ideally have greater species strain diversity, heterogeneity in metabolic products and enhanced ability to induce a favorable immune response relative to inflammation. Promoting a balanced and diverse core gut microbiome should be an over-riding goal for the probiologist. Whether through using probiotic mixtures with multiple activities or through feeding prebiotics or botanicals to achieve such a profile; multi-function DFMs will function in the specific region of the avian gut to which they are adapted, and, as per above, are tailored for the specific age of the poultry species.

DFMs should be selected for pharmacological activities in the host bird including hormone release, and neuroactive activities. For example, a specific strain of *Lactobacillus acidophilus* induced expression of intestinal opioid and canabanoid receptors in the mammalian gut, thereby lowering the neural pain threshold and imparting an analgesic effect similar to that of morphine. (Rousseaux et al., 2004). Feeding a probiotic–mannan combination to broilers reduced the effects of high levels of cortisol in birds under heat stress (Sohail et al., 2010). Hypothetically, DFMs specifically selected for anti-inflamatory properties could be applied to the avian in face of stress-induced hormonal levels and chronic inflamation. Chronic inflammation and elevated hormone levels outside of overt disease, shunt energy from growth and development (Klasing, 2007) and hurt flock performance.

VII. OPPORTUNITIES TO ELUCIDATE PROBIOTIC MECHANISMS

Current tools readily available to the microbial ecologist allow the study of microbial communities and their activities in ways unprecedented both in speed and low costs. Our ability to achieve high throughout rapid gene sequencing for gut profiling and analysis of those datasets using open source software has eliminated a major hurdle to understanding DFM effects. Several paths for progress are suggested.

Linking gene expression to metabolic response. Metabolomic analysis through GC-mass spec and NMR; coupled with gene-expression microarrays could provide an understanding of the host's response to gut microflora and DFM induced effects not only at the gene expression level, but at the biochemical expression level by identifying previously unknown chemical markers and signals resulting from DFM feeding.

Bacterial communications in situ. Understanding the chemical languages of resident gut bacteria, through cell-cell signaling molecules (also known as quorum sensing), are potential targets for interruption of signals that would otherwise enhance pathogen growth and, conversely, promote high density growth of beneficial intestinal organisms.

Probiotic lifestyle in the gut. Understanding the details of probiotic bacterial lifestyles in situ and how host stress hormone levels influences gut microflora and pathogen excretion (Humphrey, 2006; Lyte, 2011) will direct efforts to discover and develop neuroactive probiotics to mitigate the stress response of the host (Rousseau et al., 2004). Bacterial gene expression is

can now be studied at the epigenetic level (Veening et al, 2008) an approach well suited for *Bacillus* DFMs in situ.

Selectable markers for poultry breeding. Using gene markers derived from expression analysis of DFM fed high performing birds will give selectable markers for geneticists for use in breeding programs. Ample evidence exists that the composition of the gut microflora is highly correlated with MHC and other heritable factors (Khachatryan et al., 2008; Vaahtovuo, 2003).

Big science. The time has come for a multi-national project based on high throughput DNA sequencing to establish a repository and reference database of core microbiomes and microviromes of the avian in reference to life stage, feedstuffs, genetic line, and climate. Similar to the human genome project, such an effort will require government-academic and industry cooperation.

VIII. POULTRY PROBIOTICS – MYTH NO MORE

Although used for over a century (Vila et al., 2010), it was not long ago that DFMs were frequently disparaged by skeptics. From these very humble beginnings has evolved a scientific foundation to understand the biological mechanisms and improve the technology of probiotics for livestock. Used properly, the efficacy of modern DFMs is rarely debated. Probiotics are not antibiotics, they are a different approach to achieve efficient poultry production. Through a close and continued partnership of producers, and scientific research, we will begin to change the previously skeptical perceptions of DFMs. This author considers the myth that probiotics could not replace subtherapeutic antibiotics to be no more, i.e. *myth busted*. Much remains to be understood about this technology and that knowledge will only be derived from a multi-disciplinary scientific approach. DFMs offer great potential as a technology for producing poultry meat in the post-antibiotics era, and assure a sustainable livelihood for farmers and poultry producers.

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Table 1. Levels of intestinal mucosal *C. perfringens* in commercial broilers fed a CSI-derived multi-strain *Bacillus* DFM compared to control (Pre-CSI) fed no DFM. Means (top) and categories analysis (bottom) bare log_{10} CFU/g of *C. perfringerns* mucosal homogenate. Means separation indicated by different superscripts , P<0.05.

Parameter	**Untreated**	***Bacillus*-DFM Fed**
mean	2.85^{a}	0.88^{b}
n	52	16

	Frequency		**Proportion (%)**	
log_{10} CFU/g	**Untreated**	***Bacillus*-DFM**	**Untreated**	***Bacillus*-DFM**
0-1	18	11	34.6	68.8
1-2	3	2	5.8	12.5
2-3	5	1	9.6	6.3
3-4	7	1	13.5	6.3
4-5	9	0	17.3	0.0
5-6	4	1	7.7	6.3
6-7	3	0	5.8	0.0
>7	3	0	5.8	0.0
Total	*52*	*16*	*100*	*100*

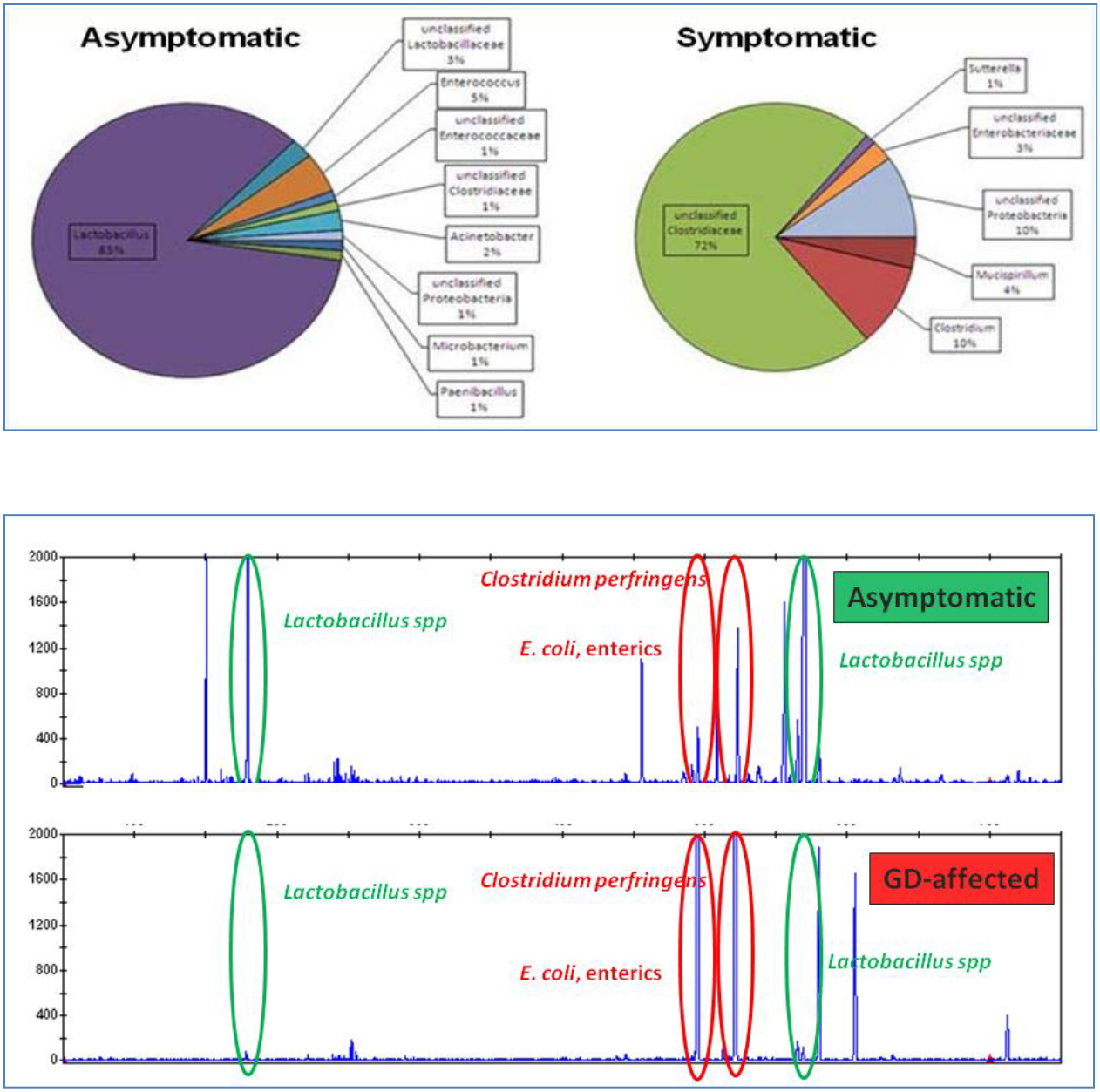

Figure 1. Microbial (bacterial) community profiles of asymptomatic and clostridial dermatitis afflicted flockmate turkeys. Top panel: 16S cloning and sequencing library (n=96 clones per sample). Bottom Panel: TRFLP analysis of the same samples.

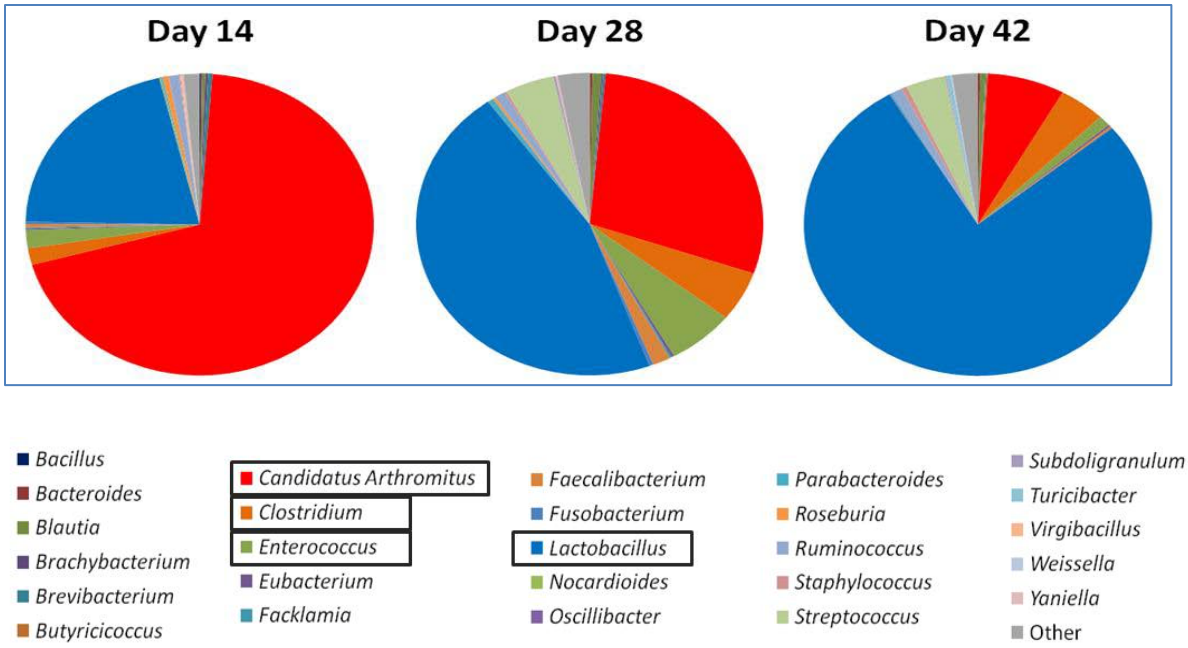

Figure 2. Mean relative abundance of major bacterial genera in non-cecal mucosa DNA extracts during broiler maturation as determined by pyrosequencing determined over time. All genera reported are > 0.1% of the total 16S bacterial signature gene sequences.