Todd R. Callaway
Steven C. Ricke *Editors*

Direct-Fed Microbials and Prebiotics for Animals

Science and Mechanisms of Action

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This book is dedicated to the late Dr. Stanley Gilliland, Regents Professor and Sitlington Endowed Chair in Food Microbiology at Oklahoma State University. Dr. Gilliland had an incredible career and was a pioneer in the fields of probiotics and direct-fed microbials. He mentored many graduate students and paved the way for the further advancement of the field of direct-fed microbials through his teaching and research. Dr. Gilliland contributed to many journal articles, book chapters, and conferences during his career. He left behind a legacy of research and the inspiration for quality scientific work. Dr. Gilliland will be missed but his contributions will not be forgotten.

Without Stan's guidance and support this conference and book would not have taken place. While Dr. Gilliland was taken from us too soon, his impacts will be felt for years to come.

Preface

In recent years, the role of the microbial ecosystem in both human and animal health has become more prominent (Finegold 2008; Ley et al. 2006; Murphy 2004; Turnbaugh et al. 2009; Turnbaugh et al. 2006; Xu and Gordon 2003). The "microbial organ" is at last getting its due as a playing a part in health as well as production parameters (Lyte 2010). Though much of this research has focused on the effects of the microbial communities and cross-communication with the host in and on humans, increasing amounts of research has delved into the microbial organ of animals (Freestone and Lyte 2010). A new hypothesis has recently been advanced by Dr. Mark Lyte that probiotics may function as a drug as a delivery mechanism for neuroactive and bioactive compounds that affect the host.

In light of these changes in our understanding of intestinal microbial ecology based on new molecular and older culture-based methods, a revised vision of the role of Direct fed Microbials and prebiotics in animal agriculture was necessary. With this in mind, an American Dairy Science Association DISCOVER conference was held in 2009 on "Probiotics in Animal Agriculture: Science and Mechanisms of Action". Following discussions with Dr. Gilliland and others at that conference, it was decided that a "state of the art" book needed to be produced for the animal and DFM industries.

The practice of supplementing direct fed microbial and prebiotic additives to domestic animals during growth is becoming more widespread in food animal production. Beneficial effects particularly in cattle, pigs and poultry including improved general health, foodborne pathogen reduction, more efficient food utilization, faster growth rate and increased milk and egg production continue to be reported. The success associated with direct fed microbial and prebiotic applications in multiple species ensures their continued commercialization and widespread use of such additives. However, several fundamental questions remain. It appears that early establishment and retention of an ecological balance in the gastrointestinal tract is an important first step for an external biological additive to be effective in young animals. Therefore, it is possible that the effectiveness of direct fed microbials and prebiotics in some animal species may only be an indirect consequence of speeding up the establishment of the dominant microflora characteristic of the adult

gastrointestinal tract. Consequently an understanding of the key processes during establishment of microflora in the gastrointestinal system that lead to the subsequent fermentation characteristics and ecological balance exhibited by the highly protective microflora is needed. Identifying these processes should lead to continued improvement in the effectiveness of available commercial products. Several additional areas of future research directions are also likely needed for further development and implementation of these biologicals.

A critical area that is now becoming possible is the rapid identification *in vivo* of characteristic microbial profiles to confirm successful establishment. Such techniques involve incorporation of molecular fingerprinting of both externally introduced cultures as well as the indigenous gastrointestinal microflora. This may also potentially help to achieve a better understanding of the mechanism(s) required for successful selection and optimization of direct fed microbials and prebiotics. In addition, this will provide insight into environmental factors that may play a role in the ability of direct fed microbials to limit pathogen transmission. Other arenas in which direct fed microbials and prebiotics may be important are in limiting establishment of pathogens in older animals which possess a more mature and developed gut microflora and need removal of pathogens already colonized in animal gastrointestinal tracts. Here success will be dependent on a much more complete picture of gastrointestinal microbial ecology and may include organisms which have been overlooked when typical direct fed microflora have been identified and characterized. In addition, modeling of microbial interactions in the gastrointestinal tract may be important to identify common factors within the complex matrix of the microbial consortium which help to serve as a barrier to prevent pathogens from coexisting with these microorganisms. Continued research on direct fed microbials and prebiotics in general should markedly expand their commercial applications.

Definitions

In this book, we use an overarching definition for **probiotics** as "a preparation or a product containing viable, defined microorganisms 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"(Schrezenmeir and De Vrese 2001). **Direct-fed microbials** (DFM) are a category of probiotics that are used in the animal industry in the United States (Fuller 1989; Schrezenmeir and De Vrese 2001). Typically, DFM as a category includes: traditional "probiotics" (live bacterial, fungal or yeast cultures), non-viable bacterial, fungal or yeast cultures, or end-products of bacterial, fungal or yeast fermentations. Some of these products include cultures that utilize a mechanism of action similar to **Competitive Exclusion Cultures**, but are not included in that FDA definition (CVM 1997). **Prebiotics** are defined as non-living compounds that can be degraded by the intestinal microflora, and are often considered a "colonic food"(Collins and Gibson 1999; Crittenden 1999; Schrezenmeir and De Vrese 2001). Many of the yeast products (DFM) used

in the animal industry contain endproducts of fermentation that are prebiotics, or prebiotic like, which can explain some of the effects of those products on the microbial population of the intestinal tract.

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About the Editors

Dr. Todd R. Callaway received his B.S. Agriculture degree in Animal Health and Animal and Dairy Sciences, as well as his M.S. in Animal and Dairy Sciences from the University of Georgia. He received his Ph.D. in Microbiology from Cornell University, with minors in Biochemistry and Animal Science. Dr. Callaway is a Research Microbiologist for the Agricultural Research Service of the U.S. Department of Agriculture, and has served as a Science Fellow for the Foreign Agriculture Service and the U.S. State Department. He has adjunct faculty appointments in the Department of Agriculture at Angelo State University, the Animal and Dairy Sciences Department at Mississippi State University, and the Department of Animal Science at Texas A&M University. Dr. Callaway has a research program focused on manipulating the microbial ecology of the animal gastrointestinal tract to reduce populations of foodborne pathogenic bacteria in food animals before they enter the food chain.

Dr. Steven C. Ricke received his B.S. degree in Animal Science and M.S. degree in Ruminant Nutrition from the University of Illinois and his Ph.D. degree from the University of Wisconsin with a co-major in Animal Science and Bacteriology. He is currently holder of the Donald "Buddy" Wray Endowed Chair in Food Safety and Director of the Center for Food Safety at the University of Arkansas. He is also a faculty member of the Department of Food Science, the Department of Poultry Science and the Cellular and Molecular Graduate program. Dr. Ricke's research program is primarily focused on virulence and pathogenic characteristics of foodborne salmonellae.

Part I
Overview of Direct-Fed Microbials and Prebiotics and their Interactions with the Host

Chapter 1
The Commensal Microbiota

John A. Patterson

Abstract The commensal microbiota in the intestinal tract are important to the host, not only in relation to food digestion, but also in terms of reducing infection by pathogens (colonization resistance) and it is becoming increasingly apparent that the commensal microbiota are important in developmental programming and function of organ systems in the adult. This is not surprising as there are ten times as many microbial cells as host cells and 100 times as many microbial genes. The commensal microbiota could be considered an additional organ that not only influences function in the adult, but also development in the neonate. The commensal microbiota is an important component of the host animal's genome. During and immediately after birth, the intestinal tract is colonized by a succession of bacteria. The presence of these bacteria is important for functional development of the intestinal tract (angiogenesis, epithelial tissues, mucosal system) and more recent data suggests a role in development and function of the brain and hypothalamic pituitary axis (HPA) that last throughout life.

The commensal microbiota in the intestinal tract are important to the host, not only in relation to food digestion but also in terms of reducing infection by pathogens (colonization resistance). It is also becoming increasingly apparent that the commensal microbiota are important in developmental programming and function of organ systems in the adult. This is not surprising as there are ten times as many microbial cells as host cells and 100 times as many microbial genes (Gill et al. 2006). The commensal microbiota could be considered an additional organ that influences not only function in the adult but also development in the neonate (O'Hara and Shanahan 2006). The commensal microbiota comprise an important component of the host animal's genome.

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During and immediately after birth, the intestinal tract is colonized by a succession of bacteria. The presence of these bacteria is important for functional development of the intestinal tract (angiogenesis, epithelial tissues, mucosal system), and more recent data suggest a role in the development and function of the brain and the hypothalamic–pituitary axis (HPA) that lasts throughout life. From an ecological perspective, the intestinal tract could also be viewed as a major river running through a continent, originating in the headwaters and discharging after passing through the continent. The river ecosystem is dynamic and constantly changing and is influenced by the surrounding land, as the land is affected by the river. The intestinal tract has major (e.g., rumen in ruminants; crop, proventriculus in birds) and minor (human, mouse) differences in stomach structure that influence subsequent microbial ecosystems. There are major differences between species in the structure of the small intestine, cecum, and large intestine that also influence the dynamics of the microbial ecosystem. Individual differences in intestinal structure, pH, transit rate, water content, immune function, and expression of molecules lining the epithelium influence the unique microbiota in individual animals.

From a microbial ecological perspective, disturbances of ecosystems decrease microbial diversity and increase opportunities for invading species. For example, pasture soil may contain 3,500–8,800 bacterial species, whereas species diversity in arable land comprises some 140–350 species (Horner-Devine et al. 2004; Xavier et al. 2005). The luminal contents of the intestinal tract are constantly being disturbed, especially in the small intestine; and not only the number of bacteria but bacterial diversity is lower in the small intestine. However, estimates of bacterial diversity in the colon rarely exceed 1,000 species. From a nutritional competition perspective, high diversity in an established ecosystem is thought to inhibit invasion by new species, whether they are pathogens or beneficial microorganisms. In environmental ecosystems, the low susceptibility to invasion by high-diversity communities is due to low levels of available resources, which is because redundancy of species utilization of resources reduces the niche width. Disturbances in the ecosystem may allow invading species to overcome resource-dependent limitations to invasion (Tilman 2004; Xavier et al. 2005). In complex ecosystems, superior competitors for a nutrient may be limited by growth rate; thus, they may be unable to exploit all of the available space, and inferior competitors can exploit these gaps if they have high growth rates (Amarasekare et al. 2004). Lactic acidosis in cattle that were rapidly switched from a forage diet to a high grain diet is a good example. *Streptococcis bovis*, which is normally a minor species in the rumen, has poor affinity for carbohydrates but can grow rapidly when carbohydrates are available. *S. bovis* produces lactic acid, which rapidly decreases ruminal pH, inhibiting the normal dominant microbiota. There are a variety of ecosystems in the intestinal tract, and each exerts different ecological pressures on microbial colonization. Another example would be the growth rate or metabolic activity of different sections of the intestinal tract. Although the number of bacteria and bacterial species is lower in the small intestine, if one calculates the ATP per bacterial cell in each location, the metabolic activity of the microbiota in the small intestine is tenfold greater than that of the colon (Patterson, unpublished, adapted from Jensen and Jorgensen 1994).

The conceptual framework of microbial ecological theory may help explain the different enterotypes discussed by (Arumugam et al. 2011 and Yin et al. 2010). Additional concepts for host contribution to species/enterotype colonization (e.g., differential gene expression resulting in unique epithelial cell composition/secretions, mucosal immune status) need to be developed for host/microbiota ecological theory development.

1.1 Temporal Colonization of the Intestinal Tract

The intestinal tract is sterile at birth and becomes colonized in a series of successional steps (Dominguez-Bello et al. 2011; Ley et al. 2006; Koenig et al. 2011; Lu et al. 2003; Yin et al. 2010). Facultative microorganisms rapidly colonize the intestinal tract; and as they modulate the nutritional and environmental (oxygen, pH, host gene expression) ecosystems of the intestinal tract, more anaerobic microorganisms sequentially colonize it (Dominguez-Bello et al. 2011; Koenig et al. 2011; Ley et al. 2006; Wilkinson 2002). The early, rapid initial colonization helps protect against pathogen invasion (colonization resistance), but the climax microbial population may not become established for several years or even until the adolescent period in an animal (Marques et al. 2010). Colonization is dependent on the microorganisms in the host animal's environment, the host's physiology, and the host animal's response to the early colonizers. In adult animals, there is a gradient of oxygen from food and water consumption and a gradient from tissues into the lumen that influences species composition along the digestive tract (Wilkinson 2002). There is an increase in total numbers of bacteria along the small intestinal tract, in the cecum, and in the proximal and distal large intestine resulting in facultative microorganism being <0.01–1.0% of the total population. The ecosystems influence the types of microorganism colonizing each ecosystem and are the source of microorganisms that colonize subsequent intestinal ecosystems (see Rawls et al. 2006 and Yin et al. 2010 for a discussion of the impact of the microbiota source and the host on microbial colonization). The stability of the intestinal microbiota also changes in the elderly (Claesson et al. 2011; Spor et al. 2011).

1.2 Postnatal Programming

There is increasing information about fetal and postnatal programming, not only regarding behavior (Heijtz et al. 2011; Huang 2011; Li et al. 2009; Marques et al. 2010; Tarry-Adkins and Ozanne 2011) but also about metabolic and immune function (Badr and Mohany 2011; Leach and Mann 2011). The most detailed example of postnatal programming interactions with the microbiota was offered by Hooper et al. (1999). They described the programmed expression of fucose on epithelial cell surfaces in germ-free mice during early postnatal development and demonstrated

that fucose expression in germ-free mice lasted only briefly. However, in the presence of *Bacteroides thetaiaotamicron*, fucose expression expanded among epithelial cells and continued in the presence of *B. thetaiotamicron*. They also showed that *B. thetaiotamicron* secreted a signal molecule stimulating expression of fucose, which the bacterium could use as a nutrient source. It would be naive to assume that this was the only molecule in the intestinal tract with programmed expression, with subsequent regulation by the presence of bacterial signals.

Another example of the importance of early colonization by specific microbial populations is that children who develop allergies are colonized less frequently with bifidobacteria and enterococci but more frequently with *Clostridium difficile*; moreover, there is a correlation between nonallergic children and secretory immunoglobulin A (SIgA) levels. Early colonization with bifidobacterium species correlated with higher levels of SIgA in Swedish infants 1 month after birth, whereas there was a negative correlation between *Bacteroides fragilis* and toll-like receptor 4 (TLR4), CCL4, and interleukin-6 (IL-6) expression in peripheral mononuclear cells 12 months after birth (Sjögren et al. 2009).

The initial inoculum is primarily thought to be bacteria from the mother's rectal/vaginal microbiota, although other environmental sources may also be important. Tapiainen et al. (2006) showed that the gut microbiota varied significantly over the first few days of life and varied among individuals. The fecal microbiota resembled that of both the mothers and their nurses 6 months after birth. Other factors influencing the microbiota include mode of delivery, gestational age, antibiotic use, hospitalization, surrounding environment, and maternal infection (Adlerberth and Wold 2009; Marques et al. 2010; Tanaka et al. 2009). We (Patterson, unpublished data) have shown that chickens raised intensively versus on pasture carry *Salmonella* longer in the cecum but not the ileum. Yin et al. (2010) used two continuous culture inocula or material from adult birds or water to inoculate day-old chicks. Both continuous culture inocula contained ~36% *Bacteroides*, 61% *Firmicutes*, and 3% *Proteobacteria*; however, one inoculum was characterized by much higher levels of *Bacteroides fragilis*, whereas the second inoculum contained much higher levels of *Prevotgella albensis*, *Acidaminococcus*, and *Dorea*. Over 15 days, the chicks developed significantly different microbial populations, with the latter treatment and water inoculum having more similar populations. Gene expression in ileal samples was also different between the three inocula. Thus, certain microbial populations inoculated early have lasting effects on the subsequent microbial populations.

Early life exposure to microbes drives expansion and development of immune cells and tissues; and the diversity and specific types of microorganisms at least partially influence subsequent ability of the immune system to respond to allergens and infection (Bjorksten et al. 2001). Exposure of neonatal piglets to low- and high-hygiene environments showed a greater diversity of microbiota in low-hygiene-raised piglets, slower accumulation of dendritic cells in the intestinal mucosa, and differential production of IL-2 and IL-4 by mucosal and systemic T cells (Inman et al. 2010). Arumugam et al. (2011) found three enterotypes among humans from different cultures characterized by high levels of either *Bacteroides*, *Prevotella*, or *Ruminococcus* species that differ in functional properties. Hydrogen disposal may

also be a factor in enterotype development as the high *Bacteroides* enterotype is associated with higher levels of *Desulfovibrio*, and the high *Ruminococcus* enterotype is associated with higher levels of *Methanobrevibacter*. The newer high throughput molecular approaches may be able to identify other minor components of the microbiota that have important functions in the intestinal tract. Current information is not detailed enough to determine the host properties that dictate the different enterotypes and that may be influenced by host immune modulation (which may be influenced by early colonizers) and/or physiological factors such as transit time, dry matter content, or luminal pH. However (Spor et al. 2011) indicated some host genes associated with specific microbiota.

1.3 Impact of Stressors on the Intestinal Microbiota

It is well known that stress makes animals more susceptible to infection; and until recently it was thought that stress hormones acting through the HPA increased susceptibility through suppression of the immune system. Recent data show that catecholamines do increase growth and expression of virulence genes in gram-pathogenic bacteria (Freestone et al. 2008; Lyte 2004; Lyte et al. 2011). The central thesis from this group is that the intestinal microbiota is an organ, and the microbial species composition influences host homeostasis and disease susceptibility. Also, the host's nervous system (in conjunction with the immune system) influences the species composition, and the microbiota has its own nervous system (quorum sensing as well as the ability to sense and secrete a variety of signal compounds) (Lyte 2010). Massive release of norepinephrine caused by administration of a neurotoxin has been shown to cause a several-log-fold increase in *E. coli* within hours (Lyte and Bailey 1997). Bailey et al. (2010, 2011) have shown that mild stressor exposure disrupts the commensal microbial population and that infection levels were associated with the presence of specific microbial genera.

1.4 Conclusions

The picture that is emerging is that the sterile intestine is rapidly colonized by a succession of bacterial species and then slowly approaches its climax population with increasing numbers and diversity of bacteria. The composition of this early microbiota has implications regarding programming not only of the climax microbiota but also the development of the intestinal epithelium, immune system, and brain. Furthermore, this early developmental programming may influence how these systems respond to stress and disease during adulthood. The data also suggest that although there are unique individual differences in microbial composition, the developmental and climax microbial populations may be manipulated to enhance animal well-being and resistance to stress and disease. The intestinal microbiota

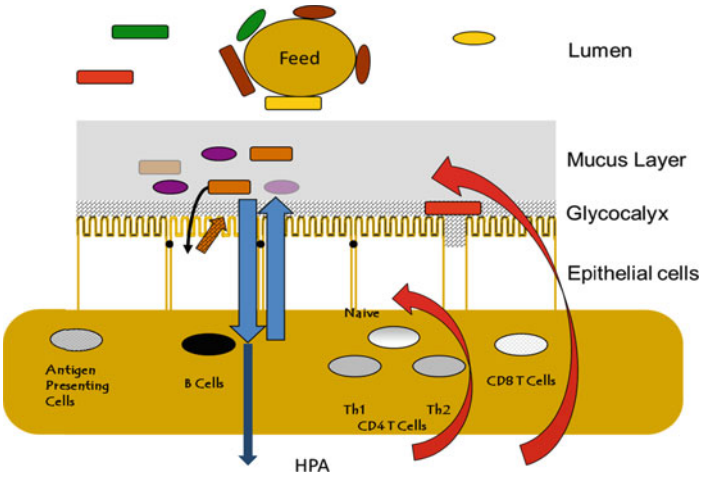

Fig. 1.1 Beneficial and pathogenic microorganisms secrete signal molecules that modulate secretion and cytoskeleton rearrangement of epithelial cells. Epithelial cells signal both luminal bacteria and mucosal immune cells, which in turn can signal the hypothalamic–pituitary axis (HPA). Signals from the HPA also influence mucosal immune cell and epithelial cell function and can influence the microbiota in the lumen of the intestinal tract

secrete signals that affect the epithelium, mucosal immune system, and brain. In turn the epithelium, mucosal immune system, and brain influence the composition of the intestinal microbiota. The interactions between these systems during development and homeostasis dictate how these systems respond to stressors and infection (Fig. 1.1).

Recent data suggest that the early microbial colonizers influence the development of subsequent microbial populations as well as host development and function. There is increasing interest in the use of probiotics, prebiotics, and other dietary additions to improve animal health and well-being. Although the efficacy of these dietary additions is variable, it may be important to address offering these dietary additions shortly after birth (Bezirtzoglou and Stavropoulou 2011; Nava et al. 2005).

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