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Commensal microbiome effects on mucosal immune system development in the ruminant gastrointestinal tract

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Abstract

Commensal microflora play many roles within the mammalian gastrointestinal tract (GIT) that benefit host physiology by way of direct or indirect interactions with mucosal surfaces. Commensal flora comprises members across all microbial phyla, although predominantly bacterial, with population dynamics varying with host species, genotype, and environmental factors. Little is known, however, about the complex mechanisms regulating host–commensal interactions that underlie this mutually beneficial relationship and how alterations in the microbiome may influence host development and susceptibility to infection. Research into the gut microbiome has intensified as it becomes increasingly evident that symbiont–host interactions have a significant impact on mucosal immunity and health. Furthermore, evidence that microbial populations vary significantly throughout the GIT suggest that regional differences in the microbiome may also influence immune function within distinct compartments of the GIT. Postpartum colonization of the GIT has been shown to have a direct effect on mucosal immune system development, but information is limited regarding regional effects of the microbiome on the development, activation, and maturation of the mucosal immune system. This review discusses factors influencing the colonization and establishment of the microbiome throughout the GIT of newborn calves and the evidence that regional differences in the microbiome influence mucosal immune system development and maturation. The implications of this complex interaction are also discussed in terms of possible effects on responses to enteric pathogens and vaccines.

Keywords: bovine, gut microbiome, innate immunity, mucosal immune system, genetics, diet, ecology

Introduction

Mammalian species have evolved over millions of years to flourish in demanding and diverse environments, with various pressures driving adaptation through interactions with the surrounding environment. These adaptations can range from gross anatomical changes that drive structural speciation to genetic polymorphisms and transfers that profoundly impact phenotypic traits. One such pressure throughout mammalian evolution has been the constant interaction with diverse microbial species. Although the potential for pathogenic interactions exists, mammals have evolved to form symbiotic relationships with a variety of microbes, where mutualism provides numerous nutritional, developmental, and physiological benefits (reviewed in Walter and Ley, 2011). The predominant site for this interaction is mucosal surfaces that form ecological environments facilitating microbiota establishment while maintaining a trophic exchange interface. It is important to consider that hosts maintain this homeostatic relationship during constant exposure to billions of

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foreign microbes of varying pathogenic potential, interspersed among dietary and environmental macromolecules, throughout the various stages of host development and immune competence.

Maintenance of the commensal microbiome is a tightly regulated process that tolerates symbionts, while maintaining defense mechanisms to identify and respond to pathogenic microbes (reviewed in Macpherson *et al.*, 2012). The mucosal immune system comprises various organized tissues, cell types, and macromolecules specialized in surveillance and coordination of both inductive and suppressive immune responses. Surveillance at mucosal surfaces occurs predominantly through specialized antigen sampling cells, M-cells, and genetically encoded pattern recognition receptors (PRRs) expressed by mucosal epithelium and leukocytes constituting the mucosa-associated lymphoid tissue (MALT). Signaling downstream of ligand-based PRR activation depends on many factors, including the specific agonist and receptor involved, the molecular milieu of the cell, adjacent cell populations, and the overall immune competence of the individual. Effector mechanisms induced from host–commensal flora interactions lead to a state of active tolerance whereby the mucosal immune arsenal facilitates close proximity habitation of the epithelium by influencing microbial colonization, adherence, and invasion. The full complement of the mucosal immune system includes physical barriers, such as the mucosal epithelium and lumenal secretions, working in close coordination with the underlying innate and adaptive arms of the mucosal immune system. Cells constituting the MALT are positioned within and basal to the epithelium. This proximity to lumenal contents facilitates both sampling and appropriate responses to the commensal microbiome and pathogens, which is critical for maintaining homeostasis and the health of the gastrointestinal tract (GIT).

Mammals lack the enzymatic repertoire to efficiently harvest energy and nutrients from plant structural carbohydrates. This deficiency has been compensated by GIT anatomical compartments and architecture, which promote colonization by microbes that have the capacity to degrade plant material. This is best exemplified in ruminants that have an herbivorous diet lacking the nutritional density of omnivores and carnivores. The microbiome in the ruminant GIT aids digestion of plant material, allowing the use of fermentation products for up to 70% of total dietary energy (Flint and Bayer, 2008). Digesta leaving the rumen travels throughout the remaining GIT where nutritional components are absorbed or further fermented. Microbes surviving the ruminal environment have the opportunity to autochthonously colonize GIT compartments depending on the fitness of the species for a given environment. In contrast, allochthonous microbiota interact with the host in a more transient fashion and are unable to colonize mucosal epithelium as stably as autochthonous biota. Compartmentalization of the GIT creates specific environmental conditions for the breakdown and absorption of dietary nutrients, and each compartment varies in terms of ecological and immunological structure. This variability creates niches that are selective for microbes tailored to each environment, and specifically, those that have the capacity to colonize in close proximity to the mucosal epithelium.

Breakthroughs in the 1940s and 1950s led to the identification of a wide range of previously uncultivable flora, requiring strictly anaerobic conditions, and was a transition toward a more complete characterization of microbial populations of the GIT. Presently, a shift toward the use of culture-independent analysis, made possible by advances in PCR-based molecular biology techniques, has allowed researchers to more fully characterize the presence, abundance, diversity, and functions of GIT communities (Tannock, 2005). These techniques have been applied to diverse research topics across numerous species to investigate the role of commensal flora in normal development and disease, but many questions remain regarding the effect of the microbiome on the development and function of the ruminant mucosal immune system. Recent evidence regarding the diversity of the microbiome in the bovine GIT and possible interactions with the mucosal immune system will be reviewed. The implications of these potential interactions will also be discussed within the context of animal health and potential interactions with pathogens or therapeutic strategies, such as vaccination.

Microbial colonization of the GIT in newborn calves

The fetal calf develops in the absence of exogenous antigen with both the systemic and mucosal immune systems developing to deal with the inevitable antigenic deluge that begins at birth. The thymus, spleen, and lymph nodes begin to develop during the first 9 weeks of gestation (Schultz *et al.*, 1973). MALT develops later during gestation with mesenteric lymph nodes, diffuse lymphoid tissues, and Peyer's patches (PPs) fully formed by birth (Beyaz and Asti, 2004; Chase *et al.*, 2008). The innate arm of immunity develops gradually throughout gestation and is characterized by the presence of functional phagocytes within the periphery, exemplified by their capacity to respond to stimulation through interferon (IFN) production (Charleston *et al.*, 2001). Progression toward birth is characterized by an increasing abundance and functional capacity of these leukocytes, although their activity is diminished just prior to birth by maternal hormonal signaling (Barrington and Parish, 2001). The adaptive immune system has also developed in fetal calves with a full complement of peripheral T-cell lineages (Wilson *et al.*, 1996), encompassing proliferative capacity if stimulated *ex vivo* or *in utero* (Wyatt *et al.*, 1983; Jensen *et al.*, 1988; Bartley *et al.*, 2004). B-cell populations are also present in a naive state since the

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absence of exogenous antigen results in a failure to produce antibody throughout gestation (Johnston *et al.*, 1977). These studies indicate that newborn calves have a functional immune repertoire that can respond to the microbial bombardment that begins with birth. During this early period, immune education by the microbiome must occur without disrupting the functional homeostasis of the GIT. A pivotal period for the postpartum calf is directly after birth when pioneering microbes rapidly colonize the GIT and the establishment of the beneficial bacterial population is of utmost importance in animal health and immune development. The establishment of a calf's microbiome is influenced by diet, host genetics, and a variety of maternal factors.

Maternal influences on the gut microbiome

The establishment of the GIT microbiome in the newborn calf is mediated by both successional and host-mediated factors. The first bacterial encounter occurs during the birthing process where *Firmicutes* and *Bacteriodetes* are the predominant phyla in the vaginal cavity (Zambrano-Nava *et al.*, 2011). Studies in mice and humans have shown the importance of this first exposure to maternal flora, as infants delivered via the birth canal acquired gut microbiota closely resembling populations of the vaginal mucosa. In contrast, the GIT flora of infants delivered by Caesarian-section more closely resembled maternal epidermal flora (Dominguez-Bello *et al.*, 2010). Similar results were reported from studies with mice (Ley *et al.*, 2005). An interesting outcome from a natural birthing process is a superior-functioning mucosal immune system, both in terms of resisting infection and proper immune education with respect to tolerance (Dominguez-Bello *et al.*, 2011). It is also interesting to note that pregnant sows were found to have a predominantly *Firmicutes* phylotype within isolated cervical mucosal secretions, and this trend was also observed with heifers. The correlation between maternal and neonatal microbiomes needs further attention as the authors characterizing the vaginal flora utilized culture-dependant techniques to primarily characterize the presence of pathogenic microbiota (Ata *et al.*, 2010). Repetition of this study with a more thorough characterization of all commensals within the birth canal could strengthen the importance of the maternal microbiome, especially during natural delivery, in defining the early neonatal microbiota profile. The current understanding of pioneer bacteria, acquired vertically during birth, is that they play a critical role in neonatal immune development with maternal flora having long-term effects on both GIT health and mucosal immune development. This succession may provide the correct environment for the macrobiotic shifts observed as aging progresses.

A second method by which maternal microflora is transferred to the neonate is through colostrum. Maternal colostrum is the nutrient-dense first milk of mammals containing numerous antimicrobial proteins, immunoglobulins, cytokines, growth factors, and leukocytes that confer passive immunity to the immunologically naive neonate (Pakkanen and Aalto, 1997; Liebler-Tenorio *et al.*, 2002; Stelwagen *et al.*, 2009). Apart from the obvious beneficial immunologic components of colostrum, bacteria have also been identified within colostrum. A recent study found bovine colostrum relatively dilute in terms of bacterial abundance but bovine colostrum was dominated by a *Lactobacillus* and *Bifidobacterium* phylotype, which is in accordance with studies showing a similar prevalence in colostrum-fed calves (Kehoe, 2006; Lindner *et al.*, 2011). The results reported by Lindner *et al.* are intriguing, but the characterization of microbiota abundance and diversity in colostrum was again limited by the reliance on culture-dependant techniques with abundance and diversity data produced from a low-resolution PCR technique using a 16S rRNA library generated from cultivable bacteria. This study also lacked sufficient DNA isolation to fully characterize colostrum diversity by length heterogeneity-PCR. This study was significant, however, in showing a trend toward homogeneity at higher taxa, with a predominance of certain phyla, and was corroborated by Poroyko *et al.* (2010) in a study with pigs. A metatranscriptome-based approach was used to determine that maternal fed versus formula fed piglets shared relatively similar cecal bacterial profiles at the level of phyla, but interestingly, significant differences were observed at the level of lower taxa (Poroyko *et al.*, 2010). These observed differences may play a role in GIT and mucosal immune system development, but this study provided a single-compartment snapshot of the microbial differences immediately postpartum, as well as the sow's influence on GIT colonization. Collectively, these results support the hypothesis that the commensal microbiome present in various maternal tissues and fluids contributes substantially to development of the newborn's GIT. Further studies are required, however, to determine the immune competence of calves that have acquired the maternal microbiome versus a microbiome influenced by formula diets.

Genetic influences on the gut microbiome

Host genetics is another factor affecting the acquisition and maintenance of microbial populations in the neonatal GIT. There is evidence that an individual's allelic composition affects the microbial composition of the GIT, even with concurrent dietary and maternal pressures. These variations may provide functional benefits or deterrents to the host in terms of mutualistic relationships with commensals, the ability to digest and absorb nutrients, and interactions with pathogens. There is little research investigating genotypic factors driving commensal establishment in cattle and studies with humans and mice have just begun to examine this question (Kunz and

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Rudloff, 2008; Spor *et al.*, 2011). Bacterial fingerprints of monozygotic twins showed a similar richness and diversity in comparison with dizygotic twins, although the differences were quite minor (Zoetendal *et al.*, 2001). These findings contrasted with several other human twin-based studies where bacterial communities were very similar within and between identical and fraternal twins. The results from the latter trials may be more reliable in that more sensitive techniques such as metagenomic or metatranscriptomic profiling were employed (Turnbaugh *et al.*, 2008; Greenblum *et al.*, 2012). Murine studies provide further evidence that genotype influences microbiome composition. An investigation of the microbiome structure of three genetically distinct and germ-free mouse strains, inoculated with identical fecal samples, found little difference before 4 weeks and after 8 weeks except the commensal flora of one strain differed greatly when compared to the other two strains at these two time points (Loh *et al.*, 2008). The results from this trial suggested that host genetics may contribute to commensal discrimination, but these effects may be overcome in later development by environmental and dietary factors. A recent study using a qualitative PCR method across multiple mouse lines, and particularly an outbred strain, found that genotype also has a major influence on the microbiome (Kovacs *et al.*, 2011). Furthermore, a quantitative trait loci (QTLs) approach, using hundreds of genetically defined mice, identified a conserved group of bacterial taxa present in all animals that correlated with variability in 18 genomic regions (Benson *et al.*, 2010). Interestingly, host genetics seemed to discriminate commensals at lower-order genus or species taxa. The translational products of QTLs correlating with these bacterial populations play predominant roles in mucosal immunity and included IFN-γ, interleukin (IL)-22, and IL-1 receptor-associated kinase 3 which is involved in downstream signaling of certain Toll-like receptors (TLRs). Although the extent to which host genetics contribute to commensal selection in cattle has yet to be explored, these powerful techniques have been applied in cattle to study immune responses (Leach *et al.*, 2012) and other traits (Maltecca *et al.*, 2009; Komatsu *et al.*, 2010; Casas *et al.*, 2011). A study by Zhou *et al.* revealed significant variation in the gut microbiome when comparing young calves and this individual animal variation applied to all regions of the GIT (Zhou *et al.*, submitted). Therefore, substantial insight into animal health may be gained by investigating how cattle breeding and selection influences development of the GIT microbiome and subsequently function of the mucosal immune system.

Dietary influences on the gut microbiome

The greatest influence on GIT microbial structure, especially with increasing age, is environmental factors, and more specifically the diet of individual animals. A predominant environmental effect on GIT microbiota populations was observed in humans where ileal effluent was sampled over a 3- to 28-month period following ileostomy. A dramatic shift toward facultative aerobes was observed following the ileostomy procedure, but upon closure, populations returned to 'normal' profiles dominated by obligate anaerobes (Hartman *et al.*, 2009). Although ileostomy reflects a biologically abnormal situation, postpartum feeding has a great influence on pioneering colonization of the GIT which is pronounced when comparing maternal versus formula fed individuals. The diversity exhibited in the neonatal GIT is predominately derived from maternal sources, but major shifts in microbial populations occur when significant changes in dietary intake occur, such as the transition period of weaning. The shift from a liquid, nutrient dense diet toward a solid, plant-based diet confers greater reliance on enterobiota capable of digesting the plant fibers in this diet.

A groundbreaking study into the dietary effects on the microbiome found the murine GIT microbiome to be highly susceptible to manipulation. Turnbaugh and Gordon (2009) found that significant microbiome restructuring occurred within 24 h following an abrupt transition from a low-fat plant-based diet to a high-fat, high-sugar 'western' diet. Amazingly, fecal transplants from humanized mice with 'westernized' microbiomes were transmissible to gnotobiotes (Turnbaugh *et al.*, 2009). This change also had a profound effect on both the genetic abundance and functionality of the microbiome. While these results pertain to mice, similar effects of diet on microbiome dynamics were observed in studies on humans. Schwartz *et al.* (2012) reported similar results when employing a metagenomic approach, but also extended their study to identify variable host expression profiles between 3-month-old, exclusively maternal or formula fed infants. Their results corroborated previous human and animal studies where the microbiome of animals varied based on diet, but this study also correlated maternal feeding with the expression of immunoprotective genes. Harmsen *et al.* (2000) conducted a study to determine how bacterial populations of infants were affected by breast milk or formula diets. The authors found that although postpartum infants shared similar 'adult-like' fecal microbiomes, populations changed quickly after birth. Microbiome populations diverged significantly when comparing differently fed infants, with *Bifidobacterium* dominating the GIT of suckling infants, while similar proportions of *Bifidobacterium* and *Bacteroidetes* were present in formula fed infants.

Studies in cattle have also identified a significant dietary effect on GIT colonization. Newborn calves fed colostrum for 14 days postpartum had 108 less days per treatment group of gastrointestinal distress, as monitored by diarrhea, than calves fed milk replacer (Berge *et al.*, 2009). Furthermore, calves grazing pastures had

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significantly different immunological profiles, exemplified by lower IFN-γ production from ConA stimulated leukocytes and significantly different cytokine profiles from small intestine tissue, when compared to indoor-housed heifers fed a grain-based diet (Lejeune *et al.*, 2010). Furthermore, when Callaway *et al.* (2010) used bacterial tag-encoded FLX amplicon pyrosequencing to define diversity in rumen and faeces, they observed that as dietary supplementation with dried distiller grains increased there was a decreased *Firmicutes*:*Bacteroidetes* ratio within ruminal fluid, an increased proportion of *Acinetobacter* in feces and that pH decreased on higher grain diets. The altered rumen ecology at lower pH actively selected for acidophilic biota. Next generation sequencing technology of a 16S rRNA library was used to track changes in the ruminal microbiome structure after a transition to a grain-based diet from Bermuda grass hay. As the diet changed, significant microbiome differences were observed based on operational taxonomic unit estimates, and enrichment of certain phyla in grain-fed animals (Pitta *et al.*, 2010). Many other studies have shown that diet plays a significant role in defining ruminant microbiome structure and overall GIT health (Kreikemeier *et al.*, 1990; Foote *et al.*, 2005; Jeyanathan *et al.*, 2011). A better understanding of how the neonatal microbiome is established and maintained will help clarify its function in development of the mucosal immune system.

Regional ecology of the GIT

Several distinct ecological environments are found throughout the GIT, each with characteristics that influence bacterial growth and persistence. At a macro-scale, the bovine GIT can be divided into rumen, small intestine (duodenum, jejunum, and ileum), large intestine, cecum, and colon. GIT environmental differences can be further refined by denoting ecologies defined by microbes associating closely with the mucosa or with ingesta passing through the lumen. Lee (1985) also defined three different mucosal regions: the unstirred mucus layer covering the GIT epithelium, the deep mucus layer found in intestinal crypts, and the apical surface of the epithelium. Although Lee described the digesta as being a location where most allochthonous bacteria are found, it should be noted that numerous studies have found consistent communities associated with luminal contents, and therefore this may be considered as another microniche within the GIT. Characterizing microbial populations associated with each mucosal microniche is limited by sampling difficulties and a more effective approach may be to sample either mucosa-associated or digesta-associated microbiota. Direct bacterial attachment to mucosal epithelium is not observed under homeostatic non-infectious conditions, but rather bacteria attach on the surface and within the mucous covering the epithelium (Van Der Waaij *et al.*, 2005). A recent review by Sansonetti (2010) redefines this symbiotic relationship from the host perspective with an emphasis on actions by the mucosal immune system to maintain the distance between commensals and the epithelial cell surface. Dissymbiosis or pathogens can disrupt this microbiome-mucosal epithelial cell cross-talk and allow microbial access to the mucosal surface (Sansonetti, 2010).

The extent to which biota preferentially colonize specific GIT compartments is still very debatable, and in large part, may be reliant on the key factors discussed previously. Taking this into account, researchers continue to characterize microbiome profiles for individual GIT compartments and ecologies to better understand homeostatic biology and the biota's role in disease. A full characterization of the mucosa-associated microbiome throughout GIT compartments of the broiler chicken was conducted by Gong *et al.* (2007). Individual animal variation was eliminated by pooling samples from each site prior to DNA extraction. Substantial variability was observed throughout the GIT, even in close-proximity compartments (crop and gizzard), and microbiome variation throughout the GIT was exemplified by a predominant species in one compartment being absent from another. Furthermore, the microbiome in the distal GIT displayed greater abundance and diversity when compared to the small intestine, which was dominated by *Lactobacillus* phylotype. Although the results from this study agree with previous less-sensitive studies using culture-dependent analysis, diet may account for these differences since antibiotics were present in the diet (Lev and Briggs, 1956; Barnes, 1979).

Microbiome regionality was also observed when comparing GIT compartments in newborn and weaned calves. Vlková *et al.* (2006) found trends of varying microbial proportions within various tissues of the GIT over 7 weeks, but notably, fluorescence *in situ* hybridization revealed a greater bacterial abundance and diversity within the rumen, cecum, and colon of calves, whereas tissues from the small intestine displayed opposite trends. These results relied on culture-dependent techniques and therefore under-estimate microbial abundance and diversity. A more extensive study was recently completed to analyze the effect of age on microbiome abundance and diversity throughout compartments and microniches of the bovine GIT (Malmuthuge *et al.*, 2011). PCR-DGGE (Denaturing Gradient Gel Electrophoresis) and 16S rRNA analysis were used to analyze bacterial diversity associated with ingesta and the mucosa throughout the GIT of newborn milk-fed and weaned calves. Regional diversity was influenced by both age and dietary changes. Bacterial richness in newborn calves was similar throughout all regions of the GIT, and digesta phyla richness trended toward a decreased richness in distal GIT regions. Interestingly, after weaning significant regional diversity in both mucosa-associated and ingesta bacterial diversity and abundance was observed throughout the GIT. In

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weaned calves, selected PCR-DGGE bands associated more closely with the mucosa than with ingesta, and the association of these specific bands with the mucosa varied throughout the GIT. Only 'predominant species' were probed for, and therefore this observation does not include an assessment of the total microbiome. The studies conducted by Guan's group revealed regional microbiome differences within the bovine GIT that were age-dependent and furthermore, there was segregation of bacterial communities between the mucosa and ingesta (Malmuthuge *et al.*, 2011). Recent studies by Nava *et al.* (2011, 2012) corroborate the variation observed between ingesta and mucosal populations in the colon of humans and mice. Microbiome regionality with differences between mucosa and digesta bacterial populations was observed previously, with *Fermicutes* dominant in both sample types but enrichment of *Bacteroidetes* in the digesta (Salzman *et al.*, 2002; Eckburg *et al.*, 2005; Hill *et al.*, 2009; Tanikawa *et al.*, 2011). Discrepancies among studies regarding enrichment of specific phyla are most likely explained by different GIT ecologies in various species, and possibly due to differences in experimental design, such as housing environment, diet, and sampling techniques. Key findings in these studies strengthen the hypothesis that microbial communities form close associations with mucosal epithelium and these mucosa-associated communities tend to be more stable, or autochthonous, in nature. The autochthonous microbiota is markedly different than that of the community associating more closely with digesta which tends to be allochthonous, or more transient within the GIT. The presence of a more stable mucosa-associated bacterial community may have significant implications for animal health in terms of both homeostatic processes that maintain trophic capabilities of mucosal surfaces, as well as development of the mucosal immune system. It will be important to determine whether autochthonous or allochthonous microbiota have a major influence on mucosal immune system development and function.

Evidence for a core mucosa-associated microbiota

A recent study conducted with Hydra defined a stable and relatively constant endosymbiotic relationship with microbiota over the course of three decades (Fraune and Bosch, 2007). The concept of a relatively stable microbial community within the GIT has been termed a 'core' microbiome, being defined as a set of microbial features present across individuals within a given species. These features were originally thought of in terms of distinct taxa of organisms that colonize specific regions, but a seminal study by Turnbaugh *et al.* (2008) reported a broad range of fecal microbiota diversity between animals of the same species, and a lesser – but significant – amount of diversity between related animals. This study and subsequent studies by Gordon's group limited GIT microbiome analysis to fecal samples, and therefore failed to properly sample the mucosal-associated bacterial community (Turnbaugh and Gordon, 2009). It was subsequently proposed that the core microbiome should be redefined at a functional level, placing greater emphasis on the processes carried out by the resident biota, and less emphasis on the absolute or relative abundances of individual organisms. The above findings and conclusions were further supported by observations made by using a pyrosequencing-based approach to analyze rumen contents in cattle (Jami and Mizrahi, 2012). The authors found greater than 80% phylogenetic similarity among the ruminal contents of 16 animals under identical feeding and housing conditions. Although these results substantiate the concept of a core microbiome, further studies comparing the various GIT compartments, as well as lumenal versus mucosal-associated biota, and the plasticity of these communities over time and under various environmental conditions are required. Although this hypothesis seems logical from a metabolic perspective, it may also be consistent with the requirement to maintain mucosal immunity and tolerance. Maintaining mucosal tolerance may require the presence of and interaction with a relatively consistent biota. For example, many autoimmune conditions, such as inflammatory bowel disease and Crohn's disease, have been associated with changing microbial populations (Karlsson *et al.*, 2008; Wlodarska and Finlay 2010). It has been proposed that a more extensive characterization of GIT microbiomes in individuals would allow researchers to discriminate between constituents associated with health or disease.

Microbiome effects on neonatal mucosal immune system development

The previous sections reviewed factors influencing the development of microbial populations throughout the GIT of the neonate. Knowledge of exogenous factors affecting microbial constitution and evolution is important to understand how these populations drive development of mucosal immunity, and, more specifically, drive responses to commensal flora and pathogens. As stated previously, the newborn calf has a fully developed but naive mucosal immune system that undergoes rapid changes in response to enteric antigens which vary greatly in their potential danger. Immediate and sustained exposure to diverse microbial constituents is of utmost importance in imprinting the correct cytokine profile upon mucosal T-cells in the GIT. Studies with gnotobiotic animal models revealed that an absence of microbial stimulation results in severe developmental and immunological consequences. Mucosal epithelial architecture is altered, mucosal cytokine profiles are biased, the response to environmental antigens changes, and innate immune responses are altered (Falk *et al.*, 1998). The gut microbiome has been implicated in many different

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aspects of homeostasis and neonatal mucosal immune development, including angiogenesis (Stappenbeck *et al.*, 2002), quorum sensing and biofilm production (Zimmermann *et al.*, 2006), and host defense peptide secretion (Bevins and Salzman, 2011). The microbiome is critical for maintaining mucosal barrier function and education of the mucosal immune system during the rapidly changing neonatal period.

Maturation of epithelial barrier function

The GIT mucosa of the perinate is extremely permeable, as evidenced by its ability to take up proteins and leukocytes (Jain *et al.*, 1989). This permeability is critical for the uptake of maternal-derived immune factors during a time of decreased immunocompetence, but fortification of the GIT epithelium is critical for survival and development of endogenous mucosal immunity. Permeability of the mucosal epithelium in newborn calves decreases within 24 h after birth and was correlated with a rapid generation of new epithelial cells (Jochims *et al.*, 1994). There is also evidence that exposure to bacteria, such as lactobacilli, decreases mucosal barrier permeability through a direct influence on the expression of tight junction proteins (Isolauri *et al.*, 1993). Furthermore, a murine study also found beneficial effects of *Bifidobacterium infantis* on mucosal barrier function. IL-10 knock-out mice are prone to GIT epithelium dysfunction but when treated with *B. infantis* conditioned media, containing bacterial soluble components, GIT inflammation, and IFN-γ secretion were decreased and colonic permeability was reduced (Ewaschuk *et al.*, 2008). Further work is required to determine if specific commensal bacteria promote or sustain mucosal barrier function and whether this function is performed by different bacteria throughout the GIT. Regional differences in both the ingesta- and mucosa-associated microbiome (Malmuthuge *et al.*, 2011) suggest that this function may not be limited to a single bacterial species.

Generation of mucosal immunoglobulin A

Although many components of the innate immune system impact the composition of the GIT microbiome, emerging evidence suggests a prominent role for secreted immunoglobulin, particularly secretory (s)IgA. The predominant role of sIgA is to bind lumenal biota and prevent adherence to host epithelial receptors thereby blocking direct interaction with the mucosal barrier. This interaction also promotes bacterial transit and removal which controls bacterial numbers (Hansen *et al.*, 2010). The passive transfer of maternal immunoglobulin to the neonate is critical in protecting newborn calves both systemically and mucosally. Uptake of immunoglobulin is rapid and can be identified in large quantities in blood (Stott *et al.*, 1979). Fetal B-cells begin developing in ruminant PPs during the last half of gestation with immunoglobulin receptors undergoing somatic hypermutation (Griebel and Hein, 1996). Phenotypic differences indicative of maturation steadily increase throughout gestation until full term, when ileal PP B-cells are significantly different from B-cells circulating in blood (Griebel *et al.*, 1992). Further regional differences in mucosal B-cell development were evident when mucosal immune responses in ileal and jejunal PP were compared. Ileal PP provides a site for antigen-independent B-cell selection, but jejunal PPs function as a site for the generation of sIgA (Mutwiri *et al.*, 1999). Yasuda's group found similar results, where up to 95% of ileal PP B-cells become anergic when exhibiting self or environmental reactivity, and surviving cells exit the PP to populate peripheral B-cell compartments. Jejunal PPs persist throughout the lifespan of the animal and are a major site for generating IgA producing B-cells but ileal PPs involute at sexual maturity (Yasuda *et al.*, 2006). These fascinating regional and age-related differences in MALT function are intertwined with the commensal microbiota. Experiments by Reynolds and Morris (1984) demonstrated that the ileal PP underwent rapid involution within gnotobiotic ileal segments. This involution could be reversed by re-connecting the ileal segment to the GIT and exposing the PP to normal ingesta. Exposure to the GIT microbiome appeared essential to maintain even antigen-independent B-cell development. Antigen-dependent B-cell responses are also influenced by certain species of bacteria. For example, exposure to bacteria increased pIgA receptor expression and increased secretion of mucosal sIgA (Majamaa and Isolauri, 1997). Also, certain segmented filamentous bacteria, related to *Clostridium* spp., are known to induce Transforming growth factor beta (TGF-β) expression in mucosal epithelium and this cytokine regulates IgA class-switching in B-2 plasma cells (Thurnheer *et al.*, 2003). Thus, while sIgA plays a major role in regulating host interactions with commensal microflora it also appears that sIgA production is also regulated by commensal microflora.

Regulation of mucosal lymphocyte development

A key factor in mucosal immune development, and specifically 'correct' development, is the acquisition of a correct helper T-cell program following birth. A bias toward T-helper (TH)2 mucosal responses in the neonate is characteristic of a poorly educated immune system with a predisposition for incorrect responses to environmental and commensal antigens and chronic inflammatory conditions. Wyatt found that intraepithelial lymphocytes, as well as lamina propria lymphocytes expressed IFN-γ and tumor necrosis factor (TNF)-α, TH1 type cytokines, in the absence of infection (Wyatt *et al.*, 1997). Previously, Wyatt *et al.* (1996) also reported that although

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T-lymphocytes were scarce within the ileum of newborn calves, their abundance rapidly increased over a 3-week period, and interestingly, $\gamma\delta$T-cells were prominent among the T-cell subsets. Murine studies have shown that $\gamma\delta$T-cells are strong producers of the TH1 cytokine IFN-γ and these cells may be important in protection in conjunction with maternal-supplied passive immunity during immune development (Ramsburg *et al.*, 2003). This is interesting as the animals were kept under enteric-pathogen-free conditions from birth, and a description of diet was not published. Although further details of animal housing and diet may further elucidate these findings, it seems that calves have a strong mucosal TH1 bias immediately postpartum. A study by Fries *et al.* (2011) also found enrichment of ileal $\gamma\delta$T-cells when comparing 6-month-old calves with 3- to 5-week-old calves. An interesting finding was that the increase in $\gamma\delta$T-cells in older calves was specific for the ileum, and a similar regional and age-dependant enrichment of CD4 T-cells was observed in the jejunum, which has been shown to be a major region for the development of TH3-type inducible regulatory T-cells (Treg) (Sun *et al.*, 2007; Round and Mazmanian, 2010). A recent review by Veenbergen and Samson (2012) sheds light on the different mechanisms by which specific commensal microbiota may induce Treg development. Multiple interactions between the commensal microbiome and the mucosal barrier may be involved in controlling Treg development.

Neonatal exposure to certain enteric bacteria may also influence immune responses at other mucosal surfaces, such as the lung. Björkstén *et al.* (2001) conducted a study with participants from two countries to determine if GIT microflora played a role in allergy development in infants. Using fecal samples and culture-dependent methods, the authors observed that the presence of a number of phyla was not significantly different between allergic and non-allergic infants, but rather proportional representation of individual phyla was distinct between the two groups. The findings of this study support the hypothesis that specific bacteria do not play a role facilitating immune education in isolation, but rather that bacterial proportions during succession and microbiome evolution may drive either beneficial or detrimental immune tolerance establishment. There is increasing evidence in humans that the GIT microbiome plays a critical role in Treg development but there is currently limited evidence regarding a similar role in cattle. It may be important to pursue similar investigations in cattle to further understand the pathogenesis of chronic enteric infections, such as Johne's disease.

Regulation of TLR expression

TLRs are a family of PRRs that play a key role at mucosal surfaces by acting as sensors to detect pathogen-associated molecular patterns (PAMPs) expressed by both pathogenic and commensal flora alike. Mucosal epithelial cells throughout the GIT express TLRs on their apical and basal surfaces and within their cytoplasm. Furthermore, various combinations of TLRs are expressed by leukocytes and lymphocytes located within MALT (Abreu *et al.*, 2005). TLR binding with the appropriate bacterial or viral agonist induces signaling through predominantly MyD88 and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) kinase pathways which results in the transcription of pro-inflammatory cytokines and chemokines, as well as host defense peptides (Iwasaki and Medzhitov, 2004; Sansonetti, 2006).

A seminal study by Rakoff-Nahoum *et al.* (2004) found that TLR stimulation was absolutely necessary for maintenance of the mucosal barrier. Dextran sodium sulfate (DSS) was delivered orally to promote intestinal injury in mice deficient in TLR-2, TLR-4, or MyD88 (a downstream mediator of TLR signaling) to test their ability to cope with microbial products in the absence of TLR signaling. There was 100% survival in *wild-type* (*wt*) mice, but survival in either TLR-2 or TLR-4 knock-out mice was reduced to 50% and complete abrogation of TLR signaling in MyD88 knock-out mice resulted in 100% mortality. Further characterization of mucosal responses in the TLR-knock-out mice following DSS treatment revealed a severe reduction in colonic pro-inflammatory cytokines (IL-1 and IL-6) and heat shock proteins when compared to *wt* mice. The effect of microflora in protecting mice from fatal inflammation was determined by depleting commensal flora with antibiotic treatment prior to DSS feeding. Mice devoid of microflora did not survive DSS treatment but mice harboring a severely reduced flora still survived. This study supports the conclusion that commensal–host cross-talk through TLR signaling is required for proper regulation of mucosal inflammation.

Cattle express a similar repertoire of TLRs as other species, as TLR1 10 have been identified and characterized (McGuire *et al.*, 2006). A recent study analyzed TLR expression in the GIT of newborn and 6-month-old calves to determine if age and microflora affect mucosal immune surveillance (Malmuthuge *et al.*, 2012). The authors found TLR expression to be comparatively greater in newborn than older calves, with the exception of TLR-1 and TLR-3. An interesting similarity between the two age groups was high TLR-10 expression in the ileal region. B-cells characteristically express high levels of TLR-10 (Krug *et al.*, 2001) and have been found to densely populate developing ileal PP in calves and sheep (Griebel and Hein, 1996; Alitheen *et al.*, 2003). Sampling of individual cell populations within MALT would be required to clarify if TLR expression is specific to epithelium or B-cells. Decreased TLR expression throughout the GIT of older calves was also interesting since it suggests the level of TLR expression may be actively regulated. *In vitro* studies

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with intestinal epithelial or dendritic cells that were continuously stimulated with TLR agonists induced hyporesponsiveness due to decreased TLR expression (Abreu *et al.*, 2001; Otte *et al.*, 2004; Cerovic *et al.*, 2009). Age-related decreases in TLR signaling may be the result of changes in the microbial structure toward a more stable and less diverse microbiome (Malmuthuge *et al.*, 2011). These results indicate that the mucosal immune system may mature toward a state where inflammatory responses may be more muted or controlled. Future studies will be required to determine what role individual bacterial species or the collective microbiome may play in this process and whether sudden shifts in the microbiome may alter the pattern of TLR expression.

Conclusions

Research into the intimate relationship between host and enterobacteria is being accelerated by new technologies that provide a more complete picture of the diversity and abundance of individual species within the microbiome. Furthermore, recent advances in our understanding of innate immunity and the regulation of acquired immune responses is providing a deeper understanding of mammalian evolution and development and the role that the GIT microbiome plays in health and disease. An ever-growing appreciation for the microbiome has led to substantial interest and investment to address knowledge gaps in the field, which is exemplified by the funding of the 5-year, $157-million USD Human Microbiome Project (NIH HMP Working Group, 2009). Furthermore, the importance of the microbiome is not lost on the research community, as some advocate that it be classified as an organ (Possemiers *et al.*, 2011). While progress in our understanding of the complex interaction between host and microbiome has accelerated, many questions remain unanswered regarding the extent to which this microbiomic relationship shapes immune system development and regulation.

Current research efforts on host–microbiome interactions tend to be directly or indirectly human-centric in nature and knowledge pertaining to other members of animalia is still very limited. While human-centric studies have received a great deal of attention, our reliance on livestock, and therefore microbiome is of vital importance. Further understanding of the bovine microbiome has the potential to increase the efficiency of animal production, reduce costs for both the producer and consumer, and to have a significant impact on animal health by decreasing susceptibility to infection by species-specific or zoonotic pathogens. Furthermore, understanding the function and development of the bovine microbiome may have a significant environmental impact through possible changes in methane production.

Advancement of our understanding of host–microbiota interactions in regulating the development and function of the bovine immune system will require methods to manipulate or alter specific components of the microbiome or study interactions at specific sites within the GIT. The use of isolated intestinal segments has been beneficial for studying GIT immunology within large ruminants since intestinal segments can be functionally similar to the adjacent intestine when inoculated with normal or limited flora (Gerdts *et al.*, 2001; Charavaryamath *et al.*, 2011). Sterile intestinal segments develop marked reductions in leukocyte, lymphocyte, and myeloid abundance, with atrophic villus architecture that is somewhat analogous to gnotobiotic intestinal tracts (Rothkötter and Pabst, 1989). The use of such models may be more applicable for the analysis of pathogens or immune responses when isolated segments are re-constituted with a full or limited microflora (Charavaryamath *et al.*, 2011). Alternatively, it may be possible to identify significant relationships between the microbiome and immune responses by profiling both the gut microbiome and mucosal immune responses in cattle subjected to specific production systems or disease conditions. These studies may be complicated, however, by significant regional differences in the microbiome throughout the GIT (Malmuthuge *et al.*, 2011) and microbiome diversity within individual animals (Zhou *et al.*, submitted).

There is increasing interest in the use of mucosal vaccines to enhance immunity in newborn calves and circumvent vaccine interference associated with passive transfer of maternal antibody (Hill *et al.*, 2012). Induction of protective immune responses following oral or intranasal vaccination of the newborn will be influenced by the state of mucosal immune system maturation and activation at the time of vaccination. There is increasing evidence that the neonatal mucosal immune system is functional and responds to vaccination in a variety of species (Mutwiri *et al.*, 2000; Van Immerseel *et al.*, 2002; Jacobs *et al.*, 2007). Microbial colonization of the upper respiratory tract and the GIT may influence the induction of acquired immune responses following immunization. The impact of the microbiome may vary with the type of vaccine delivery system and mucosal adjuvants incorporated into the vaccine. For example, there is considerable interest in using bacterial vectors for oral vaccine delivery but interactions between the vaccine vector and the microbiome may influence vector persistence and the amount of vaccine antigen expressed in the GIT. Furthermore, the activity of adjuvants incorporated into mucosal vaccines may be altered as the microbiome modulates TLR expression by the mucosal immune system (Malmuthuge *et al.*, 2012). Therefore, when developing vaccines for mucosal immunization of the newborn it may be important to consider the microbiome in the GIT and whether the co-administration of specific microbial species can

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enhance the magnitude or duration of mucosal immune responses.

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