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Review article

Role of the microbiome in swine respiratory disease

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ABSTRACT

Microbiome is a term used to describe the community of microorganisms that live on the skin and mucosal surfaces of animals. The gastrointestinal microbiome is essential for proper nutrition and immunity. How the gasterointestinal microbiome impacts primary respiratory or systemic infections is an emerging area of study. Porcine reproductive and respiratory syndrome (PRRS) is caused by a systemic virus infection with primary lung pathology and continues to be the most costly disease of swine worldwide. Recent studies have demonstrated that improved outcome after experimental infection with PRRS virus and porcine circovirus type 2 (PCV2) is associated with increased fecal microbiome diversity and the presence of non-pathogenic *Escherichia coli*. In this review, we will discuss the factors that influence microbiome development in swine, associations of the microbiome with growth and immunity during infection with respiratory pathogens, and the role of the microbiome in PRRS. Taken together, modulation of the microbiome may be an alternative tool in the control of PRRS due to its intricate role in digestion of nutrients, systemic immunity, and response to pulmonary infections.

Contents

1. Introduction

The term microbiome is defined as the "ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (Lederberg and McCray, 2001). The majority of these microorganisms live within the gastrointestinal tract, including thousands of bacterial, viral, fungal, and protozoan species. Birth route, diet, environment, and pathogens all play a role in shaping the microbiome during early life. The relationship and balance between these microorganisms in health and disease is complex and not well understood. However, there is growing evidence indicating the important role that microbiome diversity and composition plays in the regulation, elimination, and potentiation of infectious disease. The NIH Human Microbiome Project (Peterson et al., 2009), focused on understanding several microbiome sites in human health and disease, and the recently announced National Microbiome Initiative (Bouchie, 2016), focused on understanding microbiomes in various ecosystems, are programs intended to increase capacity and study in this area.

For many years, the focus of the gastrointestinal microbiome and its relationship with disease has been centered on enteric pathogens, due to the inherent relationship and proximity of

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pathogenic and nonpathogenic microorganisms in the digestive tract. Outcome following enteric infections with norovirus, rotavirus, *Clostridium difficile*, and *Salmonella* Typhimurium have all been found to have associations with the gastrointestinal microbiome (Baldridge et al., 2015; Kandasamy et al., 2016; Ross et al., 2016; Schieber et al., 2015; Theriot et al., 2014). For example, enteric bacteria were found to contribute to persistent norovirus infections in a murine model, a phenomenon reversed by the administration of antibiotics (Baldridge et al., 2015). Similarly in swine, associations have been found between the microbiome and response to the enteric bacterial pathogens, enterotoxigenic *Escherichia coli*,*Brachyspira hampsonii*, *Brachyspira hyodysenteriae*, and *Salmonella* Typhimurium (Bearson et al., 2013, 2016; Costa et al., 2014; Durmic et al., 1998; Messori et al., 2013). For example, decreased bacterial counts in swine feces were associated with the development of mucohemorrhagic diarrhea following *Brachyspira hampsonii* challenge (Costa et al., 2014).

However, an emerging field of study is understanding the significant role of the gastrointestinal microbiome on response to infections outside of the gastrointestinal tract, such as respiratory or systemic infections (Denny et al., 2016; Samuelson et al., 2015). Outcome following respiratory infections with influenza virus, *Burkholderia thailandensis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Aspergillus fumigatus*, and *Klebsiella pneumoniae* have all been shown to have associations with the enteric microbiome composition (Fagundes et al., 2012; Gauguet et al., 2015; Iwabuchi et al., 2011; Kawase et al., 2010; McAleer et al., 2016; Schieber et al., 2015; Schuijt et al., 2016; Waki et al., 2014b; Wu et al., 2013). For example, the presence of segmented filamentous bacteria in the microbiome of mice significantly improved mortality and pathology in the lungs after challenge with methicillin-resistant *Staphylococcus aureus* (Gauguet et al., 2015). Unfortunately, similar reports investigating this relationship in swine are limited; studies include *Mycoplasma hyopneumoniae* by others (Schachtschneider et al., 2013) and co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) by us (Niederwerder et al., 2016).

Since PRRS was introduced in the late 1980s, it has been considered the most costly disease of swine production worldwide (Chand et al., 2012). In growing pigs, economic losses due to PRRS are associated with reduced weight gain and increased morbidity and mortality due to respiratory disease (Holtkamp et al., 2013). Decades of research into disease control have failed to produce a broadly protective vaccine or develop programs capable of long-term virus elimination from swine-dense regions. Moreover, modified live virus vaccines developed over 20 years ago are still used in modern swine production, albeit incomplete protection and potential side effects. The challenges underlying PRRS control are due to several factors, including 1) extreme genetic diversity of isolates, 2) efficient horizontal and vertical transmission and 3) sophisticated immune evasion strategies. It is for these reasons that it has become necessary to consider alternative strategies for the control of PRRS in swine production.

Two of the most commonly studied roles for the commensal and symbiotic bacteria present in the gastrointestinal tract are in the development and regulation of the immune system and digestion of nutrients (Hooper and Gordon, 2001; Maslowski and Mackay, 2011; Ohnmacht et al., 2011; Palm et al., 2015). Considering this in the context of PRRS, it is clear that both roles of the microbiota may play an important part in disease control. This leads to questions such as 1) Are there certain microbial populations that improve the immune response against diverse strains of PRRSV? and 2) Are there certain microbial populations that improve nutrient digestion and weight gain in the presence of PRRSV? This review will describe the factors that impact microbiome development in swine, how the microbiome influences growth and infections of the respiratory tract, and recent work on the role of the microbiome in a PRRSV co-infection model with PCV2.

2. Microbiome development in swine

Development of the microbiome in swine is impacted by several factors, such as diet composition, genetics, environment, antibiotic exposure, and infection by viral or bacterial pathogens. Fig. 1 outlines the factors that impact microbial diversity and abundance during the early life of a growing pig. Although microbiome development does not seem to be impacted by gestational age of the pig (Ostergaard et al., 2015), the mode by which the pig is delivered (vaginal vs caesarean) does significantly alter initial microbial colonization (Wang et al., 2013). Initial colonization is due to exposure to the sow vaginal, fecal and skin microbiomes as well as exposure to environmental microorganisms. Microbial exposure in the pig's first few days after birth is a significant determinant of the microbiome maintained during the first few weeks of life (Jansman et al., 2012).

While lactating, the diet and gastrointestinal microbiome of the sow impacts the gastrointestinal microbiome and immunity of their suckling piglets (Baker et al., 2013; Heim et al., 2015; Starke et al., 2013). For example, pigs nursing sows being fed an immunomodulatory dietary supplement had down-regulation of pro-inflammatory cytokines after lipopolysaccharide challenge (Heim et al., 2015). Several studies in which the pig is used as a model for human neonatal nutrition have shown that the pig microbiome and immune system are significantly impacted by nursing (Lavallee et al., 2016; Wang et al., 2013; Yeruva et al., 2016). Compared to formula-fed piglets, nursing piglets have larger lymphoid follicles, higher levels of IL-10, and 5 times the number of *Lactobacillaceae* spp. in their microbiomes (Yeruva et al., 2016). Dietary supplementation of the sow continues to impact piglet microbiome and immunity even after weaning (Heim et al., 2014; Leonard et al., 2011).

Routine processing of piglets, including procedures such as clipping needle teeth, tail docking, treating with prophylactic antibiotics, and weighing, have both short and long-term effects on the gastrointestinal microbiome and immunity (Janczyk et al., 2007; Schokker et al., 2015, 2014). Schokker et al. (2014) reported that pigs administered parenteral tulathromycin at 4 days of age with or without routine processing had increased microbial diversity at 8 days of age when compared to untreated, unhandled pigs. When antibiotic-treated pigs were compared to antibiotic-treated pigs that underwent routine processing, the relative abundance of 24 bacterial genera were significantly different, indicating the importance of stress in shaping the early life microbiome (Schokker et al., 2014). In another study, a one-time amoxicillin injection administered on the day after birth resulted in significant alterations to the microbiome 5 weeks later (Janczyk et al., 2007). These practices, even when administered in the first few days of a pig's life, can impact the microbiome for months, and have the potential to last the entire lifespan of a production animal (Schokker et al., 2015).

Abundance of microbial exposure early in life can depend on environmental conditions and has a major impact on development of the microbiome and immunity in piglets (Lewis et al., 2012; Mulder et al., 2011, 2009; Schmidt et al., 2011). Mulder et al. (2009) found that pigs raised outdoors had a higher relative abundance of the Firmicutes phylum, dominated by the family *Lactobacillaceae*, when compared to conventionally-raised indoor and isolator-reared pigs. Environmental extremes during rearing also correlated with several differences in gene expression associated with metabolism and immunity, such as cholesterol biosynthesis and Type I interferon signaling (Mulder et al., 2009). In another study

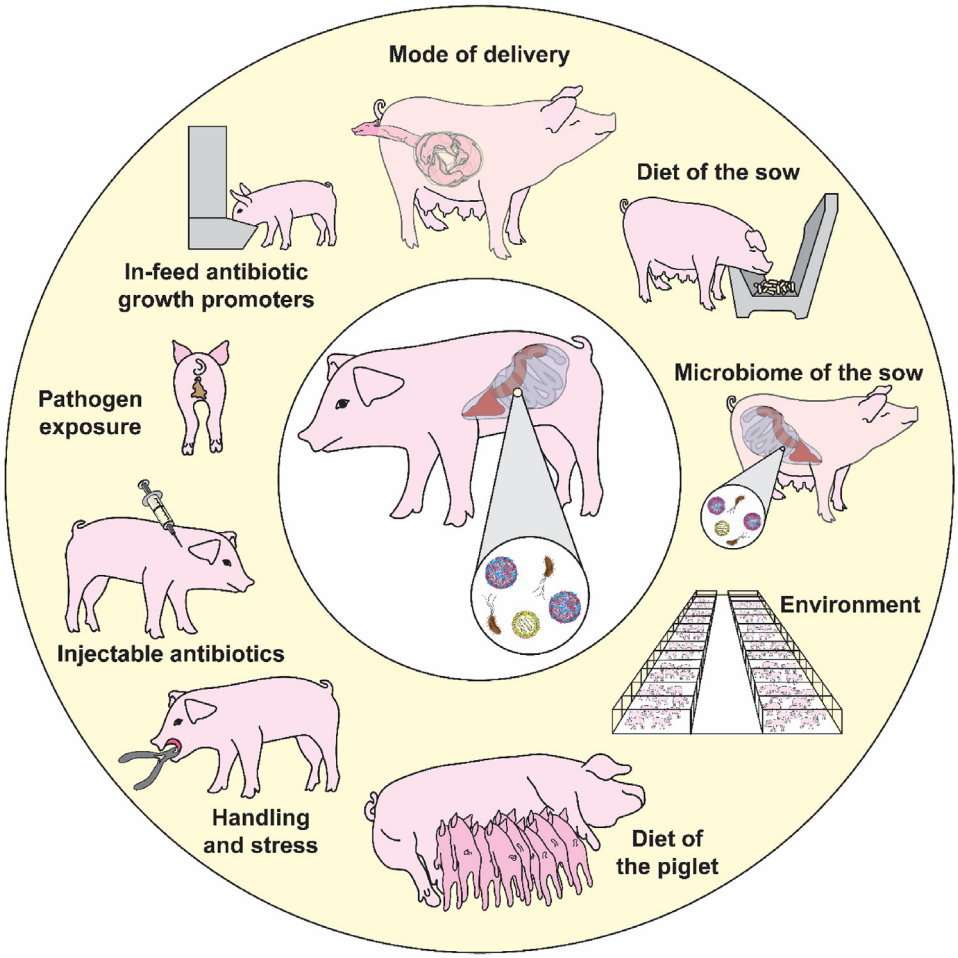

Fig. 1. Factors affecting the piglet microbiome in early life. Mode of delivery determines if initial microbial exposure is with the skin or vaginal microbiomes of the sow. After farrowing, the diet and microbiome of the sow, environmental housing and conditions, and diet of the piglet play major roles in microbial colonization. As the piglet ages, episodes of handling and stress, such as teeth clipping, tail docking, and prophylactic antibiotic administration, shift microorganism populations in the gastrointestinal tract. Pathogen exposure, in particular enteric diarrheal pathogens, and in-feed antibiotic growth promoters continue to shape the piglet's microbiome in early life.

comparing isolator-reared pigs to farm-reared pigs, the isolator-reared pigs had greater numbers of $CD4^+$ and $CD4^+CD25^+$ effector T-cells and lower numbers of $CD4^+CD25^+Foxp3^+$ regulatory T-cells in the intestine (Lewis et al., 2012). However, it should be considered that the diets between these two groups were also significantly different (i.e., farm-reared pigs were nursed and isolator-reared pigs were fed formula); as such, it is likely that diet also played a major role in shifting the immune cell populations.

Weaning and the transition to solid food is arguably one of the most pivotal factors impacting the microbiome composition of pigs (Bian et al., 2016; Frese et al., 2015). In a recent study by Bian et al. (2016) in which genetics, nursing sow, and diet were evaluated as factors influencing microbiome development, weaning and the transition to solid feed was found to be the major determinant of microbiome composition (Bian et al., 2016). The age at which weaning occurs does not seem to play a role in bacterial colonization post-weaning, despite impacts on intestinal development and immunity (Garcia et al., 2016). At the time of weaning, major microbial shifts occur in response to the transition to solid-feed, dependent on factors such as fiber and carbohydrate source (Jiao et al., 2015; Zhang et al., 2016). After weaning, *Enterobacteriaceae* and *Lactobacillus* spp. decrease in abundance whereas other species, such as *Streptococcus suis,* increase in abundance (Garcia et al., 2016; Su et al., 2008).

Enteric infections with pathogens such as porcine epidemic diarrhea virus (PEDV), *Brachyspira hampsonii*, *Salmonella enterica*, and *Lawsonia intracellularis* can also play a major role in shifting microbial colonization and causing dysbiosis (Bearson et al., 2013; Borewicz et al., 2015; Costa et al., 2014; Koh et al., 2015; Liu et al., 2015). In pigs naturally infected with PEDV, microbial diversity was significantly reduced and the majority of microbiome bacteria were from the phylum Fusobacteria. This is in contrast to PEDV-negative pigs in which microbial diversity was high and most of the microbiome bacteria were from the phylum Firmicutes (Koh et al., 2015). To combat bacterial infections or promote growth, the presence of antibiotics in feed or water, such as chlortetracycline, tylosin, sulfamethazine, and penicillin, will also shift microbiome composition in the growing pig (Kim et al., 2012, 2016; Looft et al., 2014, 2012; Rettedal et al., 2009).

3. Microbiome and weight gain

Metabolism of nutrients and metabolic diseases are closely associated with the composition of the gastrointestinal microbiome. Microorganisms in the gastrointestinal tract play a vital role in the access, degradation, and storage of nutrients (Sonnenburg and Backhed, 2016). Metabolic diseases, such as obesity and diabetes, are considered to be regulated, at least in part, by the gastrointestinal microbiome (Burcelin, 2016; Harakeh et al., 2016).

In addition, studies have discovered that the gastrointestinal microbiome plays a role in preventing cachexia associated with cancer and infectious disease (Bindels et al., 2015; Schieber et al., 2015).

Certain populations of microorganisms are associated with an obese phenotype in humans and mouse models. Specifically, greater abundance of the Firmicutes phylum and lower abundance of the Bacteroidetes phylum are relatively consistent microbiome characteristics of obese individuals (Ley et al., 2006; Ridaura et al., 2013; Turnbaugh et al., 2006). Turnbaugh et al. (2006) described the microbiomes of obese individuals as having "an increased capacity to harvest energy from the diet" (Turnbaugh et al., 2006). Further, the close relationship between weight gain and the microbiome was proven through experiments demonstrating that obese phenotypes were transmissible through microbiota transplantation (Ridaura et al., 2013; Turnbaugh et al., 2006). Although obesity is an undesirable outcome in the human population, it is possible that some of the microorganisms associated with obesity, and thus increased nutrient availability, may parallel the necessary pathways for increasing weight gain in growing pigs. Similar to obese humans, obese mini-pigs have decreased members of the Bacteroidetes phylum and specifically less bacteria of the *Bacteroides* spp. when compared to lean individuals (Guo et al., 2008).

In feed antibiotic growth promoters modulate the gastrointestinal microbiome of swine, which likely contributes to the improvement of average daily gain. For example, a combination antibiotic feed additive known for enhancing growth performance increases the relative abundance of bacteria in the Proteobacteria phylum, specifically the number of *Escherichia coli,* in feces compared to non-medicated pigs (Looft et al., 2012). Tylosin, another common in-feed antibiotic, shifts the microbiome to having an increased abundance of bacteria in the Firmicutes phylum (Kim et al., 2016). The microbiome characteristics associated with weight gain and increased nutrient availability may prove extremely valuable in a production system, particularly in the presence of PRRS.

4. Microbiome and immunity in respiratory infections

Early colonization of microorganisms in the gastrointestinal tract is critical to the development and regulation of an effective immune system (Kelly et al., 2007). Gastrointestinal microbiota can influence several aspects of immunity, such as development of innate immunity, T cell differentiation, modulation of inflammation, and regulation and homeostasis of the adaptive immune response (Honda and Littman, 2016; Ivanov et al., 2008; Kelly et al., 2004; Palm et al., 2015; Thaiss et al., 2016). The importance of the microbiome in developing and stimulating the immune system has been shown in germ-free or antibiotic-treated mice, where immunological tissues are less developed and immune molecules have reduced expression (Ichinohe et al., 2011; Round and Mazmanian, 2009). Key mediators of inflammation and immunity are modulated by the microbiome, such as regulatory T cell induction by *Clostridium* sp. and Th17 cell induction by segmented filamentous bacteria (Atarashi et al., 2011; Ivanov et al., 2009).

Although initial reports describing the close relationship between the gastrointestinal and respiratory mucosal immune systems were published decades ago (McDermott and Bienenstock, 1979; McDermott et al., 1980), there is still vast amounts of knowledge necessary to understand how these two systems function together in immunity. Much of the research in the field of the gut-lung axis have focused on diseases considered non-infectious, such as asthma, allergies, idiopathic pneumonia syndrome, and chronic obstructive pulmonary disease (Cooke et al., 2000; Fujimura and Lynch, 2015; Tulic et al., 2016). However, increasing evidence has emerged on the relationship between the microbiome and infectious diseases of the respiratory tract (Table 1). Bacterial, viral, and even fungal infections of the pulmonary system have been shown to have associations with microbiome composition or diversity. As pneumonia continues to be a leading cause of morbidities and mortalities in humans and swine, the microbiome provides a new avenue for case management and an alternative approach for disease control. The studies summarized in Table 1, all of which have been published within the last 5 years, demonstrate this emerging and exciting area of study.

Several studies in germ-free or antibiotic-treated animals have shown that the presence of endogenous gastrointestinal microbiota are beneficial for pulmonary infections. In some cases, fecal microbiota transplantation has been a successful tool to modulate this response. For example, Schachtschneider et al. (2013) evaluated the effects of orally administering the fecal microbiota from a healthy adult boar to nursery pigs prior to challenge with *Mycoplasma hyopneumoniae.* Fecal microbial transplants were delivered orally for 7 consecutive days, increasing the microbiome diversity when compared to non-transplanted littermates. After challenge, transplanted pigs had a more rapid antibody response, decreased gross lung lesions and a significant reduction in coughing. However, there were no differences in cytokine level, bacterial load, or weight gain between the two groups (Schachtschneider et al., 2013). Similar benefits were detected in a study evaluating the effects of fecal microbiota transplants and endogenous microflora on response to *Streptococcus pneumoniae* infection. Compared to mice lacking gastrointestinal microbiota, mice with healthy and diverse microbiomes had less *S. pneumoniae* present in lung, enhanced alveolar macrophage phagocytosis, lower mortality, and less interstitial pneumonia. This response was in part mediated by increases in pulmonary IL-10 and TNF-α (Schuijt et al., 2016).

Lactic acid bacteria also play a role in modulation of the systemic immune response and outcome after respiratory infection. Specifically, *Lactobacillus* sp. have been shown to increase natural killer cell activity, reduce pro-inflammatory cytokine production, enhance the antiviral immune response, upregulate cell-mediated cytotoxicity, and increase mucosal antibody production (Fukui et al., 2013; Goto et al., 2013; Maeda et al., 2009; Takeda et al., 2011; Waki et al., 2014b; Youn et al., 2012). Response to infections with either influenza A virus (IAV) or respiratory syncytial virus (RSV) is improved by microbiome colonization of *Lactobacillus* sp. For example, *Lactobacillus johnsonii* colonization associated with exposure to house dust from pets reduced the severity of pneumonia caused by RSV in mice (Fujimura et al., 2014). In an influenza model, oral administration of *Lactobacillus brevis* increased IFN-α in serum and increased mucosal IgA production in the lungs of mice after IAV challenge. Although this correlated to reduced weight loss and improved overall health after infection, there were no differences in virus replication between the control and *Lactobacillus* fed groups (Waki et al., 2014b). In a large population of schoolchildren ($n = 1783$), ingestion of this same *Lactobacillus brevis* strain significantly reduced the incidence of influenza virus infection, particularly in non-vaccinated individuals (Waki et al., 2014a). Probiotic *Lactobacillus* spp. have also been shown to reduce general clinical symptoms of respiratory infections in various populations of people, such as children 3–5 years of age, shift workers between the ages of 18 and 65, and elderly individuals at least 70 years of age (Guillemard et al., 2010a,b; Leyer et al., 2009).

Other microbiome bacteria have also proven beneficial in the response to influenza virus infection. Iwabuchi et al. (2011) evaluated the effects of administering live *Bifidobacterium longum* prior to and during influenza virus infection in mice. Clinical signs of influenza, such as lethargy and abnormal respiration, were

Table 1
Associations between the gastrointestinal microbiome and outcome in infectious respiratory disease.

Pathogen or disease	Species	Beneficial microbiome characteristic (s)	Outcome	Reference
PRRSV and PCV2 co-infection	Pig	Increased microbial diversity, *Escherichia coli*	Decreased virus replication, reduced clinical disease, increased weight gain, reduced lung pathology	Niederwerder et al. (2016)
Mycoplasma hyopneumoniae	Pig	Fecal microbiota transplant[a], increased microbial diversity	Earlier seroconversion, decreased coughing, reduced gross lung pathology	Schachtschneider et al. (2013)
Rhodococcus equi	Horse	None detected	Compared foals with clinical and subclinical pneumonia to healthy foals	Whitfield-Cargile et al. (2015)
Respiratory syncytial virus	Mice	*Lactobacillus johnsonii*[a]	Decreased airway inflammation, reduced IL-4, IL-5, IL-13 and IL-17 expression, decreased lung pathology	Fujimura et al. (2014)
Influenza virus	Mice	*Lactobacillus brevis*[a]	Decreased weight loss, improved overall condition, increased IgA antibody production, increased IFN-α	Waki et al. (2014b)
Influenza virus	Mice	*Lactobacillus acidophilus*[a]	Reduced virus titers in lung, reduced lung pathology, increased IFN-α	Goto et al. (2013)
Influenza virus	Mice	*Bifidobacterium longum*[a]	Decreased weight loss and clinical disease, reduced viral replication, lower histopathological lung scores	Iwabuchi et al. (2011)
Influenza virus	Mice	Endogenous microbiota, neomycin-sensitive bacteria	Increased antibody titer and CD4 T-cell response, increased cytokine expression and cytotoxic T-cell activity, reduced virus in lung	Ichinohe et al. (2011)
Influenza virus	Mice	Endogenous microbiota, neomycin-sensitive bacteria	Reduced lung pathology, increased IFN-γ and IL-17 expression	Wu et al. (2013)
Burkholderia thailandensis	Mice	*Escherichia coli*[a]	Reduced wasting of skeletal muscle and fat, decreased weight loss	Schieber et al. (2015)
Streptococcus pneumoniae	Mice	Endogenous microflora, fecal microbiota transplant[a], increased microbial diversity	Reduced bacteria in lung, decreased lung pathology, decreased mortality, increased alveolar macrophage function	Schuijt et al. (2016)
Methicillin-resistant *Staphylococcus aureus*	Mice	Segmented filamentous bacteria	Decreased bacteria in lung, decreased bacterial dissemination, less severe pneumonia, decreased mortality, increased IL-22 in lung	Gauguet et al. (2015)
Mycobacterium tuberculosis	Mice	Lack of *Helicobacter hepaticus*	Reduced IL-10 expression, decreased bacterial load in lung, reduced lung pathology	Arnold et al. (2015)
Klebsiella pneumoniae	Mice	Endogenous microbiota, Fecal microbiota transplant[a]	Decreased mortality, decreased systemic pathogen dissemination, increased TNF-α and CXCL-1	Fagundes et al. (2012)
Escherichia coli	Mice	Endogenous microbiota	Decreased bacterial dissemination, reduced mortality, decreased bacteria in lung, enhanced alveolar macrophage activity, increased neutrophil activity, decreased lung pathology	Chen et al. (2011)
Pseudomonas aeruginosa	Mice	Endogenous microbiota	Decreased mortality, increased TNF and IL-1β in lung,	Fox et al. (2012)
Aspergillus fumigatus	Mice	Vancomycin-sensitive bacteria, segmented filamentous bacteria	Increased IL-17 response in the lung	McAleer et al. (2016)

[a] Microorganism(s) administered to animal prior to pathogen challenge.

significantly reduced in mice administered the probiotic. In addition, these mice had significantly less weight loss, lower virus replication in the lungs, and reduced lung pathology. However, the differential response was not explained by significant shifts in cytokine production (Iwabuchi et al., 2011). In a study performed by Ichinohe et al. (2011), response to influenza virus infection was compared between antibiotic-treated mice and mice with endogenous microflora. In antibiotic-treated mice, pulmonary viral titers were increased and antibody titers, T-cell responses, and cytokine expression were decreased. Upon further experimentation, the beneficial endogenous microflora were characterized as sensitive to neomycin, an aminoglycoside antibiotic which eliminated most of the gram-positive bacteria from the microbiome (Ichinohe et al., 2011). Similar findings were reported by Wu et al. (2013) in which the beneficial activity of neomycin-sensitive bacteria in response to influenza virus infection were investigated. These bacteria were found to increase the toll-like receptor 7 (TLR7) signaling pathways, which are important for recognition of single-stranded viral RNA and inflammasome activation (Wu et al., 2013).

One respiratory pathogen that stands out due to the lack of association between microbiome and outcome is *Rhodococcus equi* in foals. Whitfield-Cargile et al. (2015) found no fecal microbiome differences between those foals that went on to develop clinical Rhodococcal pneumonia, subclinical Rhodococcal pneumonia, and those foals that remained healthy. In this case, microbiome diversity prior to development of disease was not predictive or associated with outcome. However, this study was completed on a farm with a history of *R. equi* pneumonia and variation in exposure to the pathogen (i.e., route, dose, age of exposure) may have played a role in the outcome between foals (Whitfield-Cargile et al., 2015).

Although most studies focus on how the microbiome impacts respiratory infections, other studies have demonstrated that pathogen replication in the lung can impact microbiome composition through various signaling pathways. For example, Deriu et al. (2016) recently demonstrated that type I interferons produced during pulmonary influenza virus infection results in a reduced population of anaerobic bacteria and increased populations of Proteobacteria in the gastrointestinal tract. The resulting dysbiosis increases the likelihood of gastrointestinal disorders secondary to influenza virus infection of the respiratory tract (Deriu et al., 2016). In Wang et al. (2014), Th17 cells were recruited to the small intestine secondary to influenza virus infection in the lung; these cells and the resulting cytokine production were found to be the cause of intestinal injury and symptoms of diarrhea (Wang et al., 2014). It is likely that similar effects are seen after respiratory infection with PRRSV in swine; i.e., infection with PRRSV causes shifts in the gastrointestinal microbiome. After experimental infection with PRRSV and PCV2 or infection with PRRSV alone, clinically relevant diarrhea occurs in approximately 5–6% of nursery pigs (Niederwerder et al., unpublished results). However, studies detailing how PRRSV affects the microbiome when compared to uninfected individuals are lacking.

In additional to the gastrointestinal microbiome associations, several studies have demonstrated that the normal flora of the nasal cavity also plays a role in respiratory pathogen susceptibility

(Table 2). For example, Correa-Fiz et al. (2016) recently published a study evaluating the nasal microbiomes of pigs prior to weaning as a predisposing factor in the development of respiratory disease associated with *Haemophilus parasuis*. The nasal microbiomes of pigs from farms without respiratory disease had higher microbial species richness and diversity when compared to farms with Glässer's disease (Correa-Fiz et al., 2016). In feedlot cattle, similar associations were found between increased diversity of the nasopharyngeal microbiome and reduced occurrence of clinical respiratory disease (Holman et al., 2015). In a mouse model, the presence of endogenous nasal microbiota resulted in decreased susceptibility to *Bordetella pertussis* colonization of the upper respiratory tract. Further, reintroduction of *Klebsiella* or *Staphylococcus* species into microbiota-depleted nasal cavities of mice significantly reduced colonization of *B. pertussis,* indicating the roles that both diversity and individual species may play in pathogen susceptibility (Weyrich et al., 2014).

5. Role of the microbiome in PRRS

Studies investigating the direct role of the microbiome in PRRS are limited. In 2013, Tsuruta et al. published a study investigating the effects of oral administration of the lactic acid bacteria, *Enterococcus faecalis*, on antiviral cytokine expression in weaned pigs. After administering the killed bacteria to pigs for 10 days, ileum, mesenteric lymph node and spleen were collected to evaluate the response of the tissues ex vivo to several immunostimulants, including PRRS modified live virus (MLV) vaccine. When compared to the control pigs, tissues from pigs administered *E. faecalis* had increased expression of IFN-γ in the lymph node and increased expression of TNF-α in the splenocytes. However, these increases were independent of immunostimulation and it was concluded that microbiome modulation had broadly increased baseline cytokine production (Tsuruta et al., 2013). In a recent review by Amadori and Zanotti (2016), the microbiome was listed as an important tool for immunoprophylaxis against production-related diseases, such as PRRS, due to its immunomodulatory properties (Amadori and Zanotti, 2016).

In an effort to increase the knowledge base on the role of the microbiome in PRRS and investigate alternative tools in disease control, we have focused our research on the role of the microbiome in co-infection with PRRSV and PCV2. This co-infection model is utilized for several reasons. First, co-infections involving PRRSV and PCV2 are common on a global basis and PRRSV is frequently isolated with PCV2 in U.S. field cases of porcine circovirus associated disease (Opriessnig and Halbur, 2012; Pallares et al., 2002). Second, co-infections enhance pathogenesis and contribute to a wide range of polymicrobial and multisystemic disease syndromes (Niederwerder et al., 2015a; Trible et al., 2012). Third, infections with PRRSV and PCV2 result in a number of immunological outcomes, increasing susceptibility to other pathogens (Gomez-Laguna et al., 2013; Opriessnig and Langohr, 2013). Thus, microbiome characteristics associated with outcome in this model should be multifactorial and broadly applicable.

To date, we have completed two studies evaluating the microbiome associations with outcome following co-infection with PRRSV and PCV2. In the first study (Niederwerder et al., 2016), we evaluated the fecal microbiome differences between pigs with the best and worst clinical outcomes after co-infection. An experimental population of 95 nursery pigs was challenged with PRRSV and PCV2 at approximately 6 weeks of age and followed for 70 days post-infection (dpi). At the conclusion of the trial, the experimental population could be divided into two groups based on growth performance and clinical disease. Ten of the best clinical outcome pigs were selected due to having the highest average daily gain and a complete lack of overt clinical disease; ten of the worst clinical outcome pigs were selected due to having the lowest average daily gain and 10 or more days of moderate to severe clinical disease. Complete necropsies were performed and fecal samples were collected at 70 dpi for microbial analysis. At the level of the fecal microbiome, best clinical outcome pigs had two significant characteristics, including 1) increased microbiome diversity and 2) increased prevalence of *Escherichia coli*. Best clinical outcome pigs had higher absolute weekly weights on each of the 10 weeks post-infection, a significant reduction in severity of interstitial pneumonia, decreased gross necropsy lesions, reduced overall virus replication, and decreased bacterial presence in serum (Niederwerder et al., 2016).

In the second study (Niederwerder et al., unpublished data), we investigated the microbiome characteristics that may predispose or predict outcome following co-infection with PRRSV and PCV2. An experimental population of 50 nursery pigs were challenged with PRRSV and PCV2 at approximately 8 weeks of age and followed for 42 dpi. At the conclusion of the trial, 20 representative pigs were selected due to having high or low growth rates post-infection. Fecal samples collected on 0 dpi were analyzed from all 20 pigs representing the high and low growth rate groups. Preliminary analyses have shown that pigs with high growth rates following co-infection have significantly greater microbiome diversity prior to challenge (data not shown) (Niederwerder et al., unpublished data).

In both studies performed by our group, microbiome diversity was associated with enhanced growth performance and/or reduced clinical disease after co-infection with PRRSV and PCV2. Increased microbial density or diversity have also been associated with improved response to enteric diarrheal pathogens in swine (Costa et al., 2014; Messori et al., 2013) and improved response to respiratory pathogens in swine and mice (Table 1). As previously discussed, increased fecal microbiome diversity was also beneficial in the response of pigs to challenge with *Mycoplasma hyopneumoniae* (Schachtschneider et al., 2013). Taken together, these studies provide evidence that microbiome diversity may be

Table 2
Associations between the nasal microbiome and outcome in infectious respiratory disease.

Pathogen or disease	Species	Beneficial microbiome characteristic(s)	Outcome	Reference
Haemophilus parasuis	Pig	Increased microbial species richness and diversity	No clinical respiratory disease on farm	Correa-Fiz et al. (2016)
Bovine respiratory disease	Cattle	Increased microbial species richness and diversity	Not treated for clinical respiratory disease at feedlot	Holman et al. (2015)
Influenza virus	Mice	*Lactobacillus rhamnosus*[a]	Increased survival, reduced viral titers in lung	Youn et al. (2012)
Bordetella pertussis	Mice	Endogenous microbiota, *Staphylococcus* and *Klebsiella* species[a]	Decreased susceptibility to infection, higher dose required for colonization	Weyrich et al. (2014)

[a] Microorganism(s) administered to animal prior to pathogen challenge.

broadly beneficial for both viral and bacterial causes of pneumonia in swine.

In the first study performed by us, members of the Proteobacteria phylum, including *Escherichia coli*, *Erwinia amylovora*, *Campylobacter lari*, *Dechlorosoma suillum*, and *Mannheimia haemolytica*, were only detected in pigs with the best clinical outcome after co-infection with PRRSV and PCV2. Specifically, nonpathogenic *E. coli* was detected at a significantly higher rate in the best clinical outcome pigs compared to the worst clinical outcome group ($p = 0.03$, Fisher's exact test) (Niederwerder et al., 2016). The beneficial effects of gastrointestinal *Escherichia coli* in response to pathogenic challenge have been recognized for decades. In 1979, Moxon and Anderson reported that the administration of commensal *E. coli* prior to *Haemophilus influenza* type b challenge in mice reduced pathogen dissemination, decreased meningitis, and enhanced antibody response (Moxon and Anderson, 1979). More recently, investigations have discovered associations between *Escherichia coli* and weight gain or reduced wasting. For example, *E. coli* has been associated with increasing weight gain in pregnant women (Santacruz et al., 2010), promoting growth in swine (Looft et al., 2012), increasing body weight and fat deposition in rats (Karlsson et al., 2011), and preventing cachexia in mice after infection with respiratory and gastrointestinal pathogens (Schieber et al., 2015). Members of this phylum may contribute to improved clinical outcome and weight gain in pigs under viral challenge conditions.

6. Conclusions

Understanding the complex interactions and relationship between the gastrointestinal microbiome with health and disease of humans and animals is in its early stages. Although the last 5 years have unveiled numerous associations between the microbiome and pulmonary infections, much more work is needed to understand the causative relationship between microbiome composition and outcome following exposure to pathogens. This is particularly true in livestock as alternative approaches to managing infectious disease become essential to maintaining healthy populations.

PRRSV emerged at a time when pig production was transitioning to primarily large specialized operations where pigs are housed indoors and segregated by age. Considering the factors supporting the "hygiene hypothesis" in humans, in which the apparent increase in susceptibility to immune-mediated conditions is due to reduced exposure to microorganisms in early life, interesting parallels can be drawn with modern swine production in developed countries. These factors include high antibiotic use, low incidence of gastrointestinal parasitism, and housing with strict sanitation (Stiemsma et al., 2015; Wills-Karp et al., 2001). As we continue to understand how the immune response contributes to PRRS-associated pathology (Cino-Ozuna et al., 2015, 2014; Gomez-Laguna et al., 2013), it will be increasingly important to consider how reduced microbial exposure and the clean nature of modern pig production shapes immunity and affects susceptibility to PRRS.

As shown by several studies looking at PRRSV and other respiratory pathogens, increased gastrointestinal microbiome diversity is associated with improved outcome, supporting the hypothesis that exposure to diverse microorganisms improves immunity. Similar findings have been reported with regards to the swine nasal microbiome (Correa-Fiz et al., 2016); however, the opposite is true in regards to the microbiomes of the tonsil and serum (Jaing et al., 2015; MacInnes et al., 2014; Niederwerder et al., 2015b). It is clear that additional research is needed to understand how the microbiome plays a role in both the exacerbation and resolution of disease. Future areas of research should focus on elucidating specific microorganisms that are beneficial or detrimental to outcome following PRRSV exposure, investigating intervention strategies such as microbiome modulation that may be applied to a production setting, and understanding the molecular mechanisms, such as immune or metabolic signaling by gastrointestinal microorganisms, which may contribute to continued growth and reduced clinical disease in the presence of PRRSV.

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