Chapter 3
Pathogenesis, Immunity and the Role of Microbiome/Probiotics in Enteric Virus Infections in Humans and Animal Models

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Abstract The gut microbiota has a profound impact on the resistance, pathogenesis, and immunity of enteric viral pathogens. Commensal microbes may prevent the host from infection or enhance infection by altering virus stability, attachment or cellular entry. Additionally, microbiota members can stimulate or suppress host immune responses to the viral infection. In most cases, the gut microbiota plays a role in host resistance against invading enteric viral pathogens; hence, germ-free animals are more susceptible to infection of various enteric pathogens. However, increasing evidence has demonstrated that certain commensal bacteria can enhance enteric viral infection. Exact mechanisms by which specific bacteria carry out these effects are not clearly understood in most instances. In this chapter, human norovirus (HuNoV) and human rotavirus (HRV), the two most important viral pathogens causing gastroenteritis, are chosen for the discussion of the impacts and mechanisms of microbiome–host interactions on viral gastroenteritis. The pathogenesis and immunity of HuNoV and HRV in humans and in germ-free animal models, particularly gnotobiotic (Gn) mice and pigs, and Gn pigs transplanted with human gut microbiota are reviewed. Findings from studies on host–microbiome interactions on the pathogenesis and immunity of the two viruses, and mechanisms of probiotics/prebiotics in ameliorating their infection and diseases, are summarized. Unraveling the role of microbiome and specific probiotics in the infectivity, pathogenesis, and immunity of HuNoV and HRV facilitates the development of strategies for manipulating the microbiome against viral infections. Further studies are needed to improve our understanding of mechanisms underlying host–microbiome interactions in the pathophysiology of enteric viral diseases.

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List of Abbreviations

ASC	Antibody-secreting cells
AttHRV	Attenuated human rotavirus
Dpi	Days post-inoculation
EcN	*Escherichia coli* Nissle 1917
Gn	Gnotobiotic
HBGA	Histo-blood group antigen
HHGM	Healthy human gut microbiota
HRV	Human rotavirus
HuNoV	Human norovirus
LGG	*Lactobacillus rhamnosus* GG
MNCs	Mononuclear cells
NHPs	Nonhuman primates
UHGM	Unhealthy human gut microbiota
VirHRV	Virulent human rotavirus

3.1 HuNoV Pathogenesis and Immunity in Humans and in Animal Models

3.1.1 HuNoV Gastroenteritis, Pathogenesis, and Cell Tropism in Humans

Human noroviruses (HuNoVs) are positive-sense, single-stranded, non-enveloped RNA viruses that belong to the genus *Norovirus* in the family Caliciviridae (Zheng et al. 2006). Since the introduction of rotavirus vaccines (RotaTeq in 2006 and Rotarix in 2008), HuNoVs have become the predominant cause of viral epidemic acute gastroenteritis across the globe (Pringle et al. 2015; Hemming et al. 2013; Payne et al. 2013). Viral transmission occurs via the fecal–oral route by contaminated food or water and person-to-person spread (Patel et al. 2009). HuNoV gastroenteritis is generally self-limiting, with a duration of 2–3 days and consists of moderate to severe acute diarrhea episodes, sudden onset of vomiting, and mild or no fever (O'Ryan et al. 2010), but the diseases can become more severe and prolonged in infants, the elderly, and individuals with impaired immunity (Karst 2010). Despite its importance in public health, no virus-specific therapeutics or vaccines are currently available to treat or prevent HuNoV gastroenteritis (Kocher and Yuan 2015), mainly because HuNoV research has been hampered by the long absence of a robust cell culture system and small-animal model. HuNoV biology has been explored most frequently by viral challenge studies in human volunteers (Karst 2010), chimpanzees (Bok et al. 2011), gnotobiotic (Gn) calves and pigs (Souza et al. 2008; Bui et al. 2013; Cheetham et al. 2006), and immunodeficient mice (Taube et al. 2013) (Table 3.1).

Table 3.1 Features of animal models for human norovirus (HuNoV) infection

Host	Gem-free condition	Viral strain	Route of infection	Viral antigen location	Fecal virus shedding	Viremia	Diseases	Pathological changes
Immunocompetent humans	–	Multiple	Oral	N/A	+	±	Vomiting and severe diarrhea	Yes (Agus et al. 1973; Schreiber et al. 1973, 1974; Dolin et al. 1975; Karst et al. 2014)
Immunocompromised humans	–	Multiple	Oral	IECs	+	N/A	Vomiting and severe diarrhea	Yes (Karandikar et al. 2016; Bok and Green 2012; Green 2014)
Chimpanzees	–	GI.1	Oral (Wyatt et al. 1978) intravenous (Bok et al. 2011)	Intestinal DC and B cells	+	–	Asymptomatic	No (Bok et al. 2011)
Balb/c RAG/$\gamma c^{-/-}$ mice	–	GII mix	Intraperitoneally	Mφ-like cells in liver and spleen	–	N/A	Asymptomatic	No (Taube et al. 2013)
Gnotobiotic pigs	+	GII.4	Oral	IECs	+	+	Mild diarrhea	Yes (Bui et al. 2013; Cheetham et al. 2006)
Gnotobiotic calves	+	GII.4	Oral	IECs and intestinal Mφ-like cells	+	+	Mild diarrhea	Yes (Souza et al. 2008)

IECs intestinal epithelial cells, *Mφ* macrophage, *N/A* not available

Challenging HuNoV in immunocompetent volunteers resulted in acute gastroenteritis, and biopsy specimens from the individuals who acquired clinical gastroenteritis displayed histological changes in the small intestine, including mucosal inflammation, villus blunting, microvillus shortening, and abnormal organelles such as endoplasmic reticulum and mitochondria (Agus et al. 1973; Schreiber et al. 1973, 1974; Dolin et al. 1975). Although intestinal epithelial cells (IECs) are the target for most enteric pathogens, the presence of HuNoV virions or antigen have not been reported in these biopsies from immunocompetent humans, and the cellular tropism of HuNoV has long been elusive (Agus et al. 1973; Schreiber et al. 1973, 1974; Dolin et al. 1975; Karst et al. 2014). Chronic HuNoV infection occurs in immunocompromised transplant patients. A recent study using intestinal biopsies from a patient cohort showed that HuNoV infection was observed in duodenal and jejunal enterocytes, and HuNoV-associated histopathological changes were present as the flattening of epithelial cells and the severe loss of villin in enterocytes (Karandikar et al. 2016). In addition, stem cell-derived and nontransformed human intestinal enteroids have been recently established as a reproducible cultivation system for multiple HuNoV strains, confirming enterocytes as target cell types for HuNoV infection in vitro and in vivo (Ettayebi et al. 2016). B cells were suggested to be a permissive cell type for HuNoV replication in vitro, which is a novel HuNoV cultivation system in the BJAB cell line supplemented with free histo-blood group antigen (HBGA) or HBGA-expressing inactivated enteric bacteria (Jones et al. 2014). However, this cell culture system produced inconsistent results in other laboratories (Jones et al. 2015; Lei et al. 2016c), and HuNoV infection was observed in B cell-deficient patients and Gn pigs (Brown et al. 2016; Lei et al. 2016b), along with the low virus yields in such an in vitro cell system compared with high-level virus shedding in patients (Bok and Green 2012), suggesting that B cells might not be the primary target cell of HuNoV.

3.1.2 HuNoV Infection and Pathophysiology in Conventional Animal Models

Nonhuman primates (NHPs), particularly chimpanzee (99%) (Kehrer-Sawatzki and Cooper 2007), share the greatest genome similarities with humans, which makes them desirable models for studies on several fastidious viral pathogens, such as human immunodeficiency virus and hepatitis viruses (O'Neil et al. 2000; Pfaender et al. 2014; Purcell and Emerson 2001). The chimpanzee was presented as a viable animal model for subclinical GI.1 HuNoV infection, characterized by intravenous inoculation, asymptomatic fecal virus shedding, and viral associated serum antibody responses (Bok et al. 2011). Biopsies from the jejunum and duodenum showed no histological changes after HuNoV infection, although the viral genome was detectable up to 21 days post-inoculation. Interestingly, viral capsid antigen was only observed in cells of the duodenal and jejunal lamina propria, and further

investigations indicated that viral antigen-positive cells were dendritic cells and B lymphocytes (Bok et al. 2011). However, the chimpanzee is not available for biomedical research any longer owing to ethical concerns.

Another animal model of subclinical HuNoV infection is the Balb/c mouse deficient in recombination activation gene (RAG) and common gamma chain (γc or IL2RG), which lacks T cells, B cells, and natural killer cells. In this mouse model, a HuNoV GII mix was inoculated intraperitoneally (Taube et al. 2013). Although virus shedding and gastrointestinal diseases were not observed in those Balb/c RAG/$\gamma c^{-/-}$ mice, viral genome was detected in the intestinal and systemic sites, with increased levels over the input virus 1–2 days post-inoculation. Viral structural and nonstructural proteins were observed in cells morphologically resembling macrophages in the liver and spleen, validating HuNoV propagation (Taube et al. 2013). Moreover, Balb/c RAG/$\gamma c^{-/-}$ mice can be used for the evaluation of anti-HuNoV drugs such as the nucleoside analog 2′-C-methylcytidine, which inhibited HuNoV replication in vivo (Kolawole et al. 2016).

3.1.3 HuNoV Infection and Pathophysiology in Gnotobiotic Large Animal Models

The neonatal Gn pig model is well suited for the evaluation of HuNoV pathogenesis and vaccine efficacy, and it reflects HuNoV biology in terms of supporting the natural oral route of infection, resulting in diarrhea, transient viremia, and virus shedding in feces (Cheetham et al. 2006; Bui et al. 2013; Kocher et al. 2014; Souza et al. 2007a, b). Viral structural and nonstructural proteins were detected in enterocytes in wild-type Gn pigs experimentally infected with HuNoV genotype GII.4 (Bui et al. 2013; Cheetham et al. 2006; Lei et al. 2016c), indicating viral infection and replication in Gn pigs. HuNoV-induced diarrhea in Gn pigs was associated with mild villus atrophy and cytopathological changes in the small intestine, manifested as blunting and shortening of microvilli and necrosis and apoptosis of enterocytes (Bui et al. 2013; Cheetham et al. 2006), which recapitulate the hallmark pathological features in humans.

Twenty-four units of the P domain of HuNoV capsid protein can form P particle, which efficiently induces innate, humoral, and cellular immune responses in mice (Fang et al. 2013). Together with its easy and economical preparation in *E. coli*, P particle has gained recognition as a promising vaccine candidate against HuNoV infection (Kocher and Yuan 2015). In the study of P particle vaccination in Gn pigs, P particle exhibited 47% cross-variant protection against HuNoV diarrhea, and the protection correlated positively with T cell expansion in the ileum and spleen, while correlating inversely with T cell expansion in the duodenum (Kocher et al. 2014). Persistent HuNoV infection in immunocompromised patients can lead to increasingly debilitating and life-threatening gastroenteritis with prolonged virus shedding (Bok and Green 2012; Green 2014). Similarly, in RAG2/IL2RG-deficient Gn pigs,

HuNoV infection was severe and prolonged owing to the severe combined immunodeficiency of the host, and enterocytes of the duodenum and jejunum were sites of HuNoV infection (Lei et al. 2016b).

Neonatal Gn calves serve as another large animal model that supports GII.4 HuNoV infection; viral capsid protein was detected in enterocytes of the jejunum and ileum, and in cells morphologically resembling macrophages in the lamina propria (Souza et al. 2008). Similar to the findings in Gn pigs, HuNoV challenge in Gn calves resulted in diarrhea along with intestinal lesions and mild villous atrophy, fecal virus shedding, transient viremia, and intestinal and systemic immune responses (Souza et al. 2008).

Notably, pigs are natural hosts of noroviruses GII (genotypes 11, 18, and 19); however, all porcine noroviruses were detected from conventional pigs without clinical signs (Knowles and Reuter 2012). Porcine norovirus has been detected in many countries and geographical distribution indicates the worldwide occurrence of porcine noroviruses among pigs on farms. The QW101/2003/US (GII.18) isolate from a healthy adult pig was genetically and antigenically related to HuNoVs and replicated in Gn pigs with fecal shedding coincident with mild diarrhea (Wang et al. 2005). Seroprevalence of norovirus GII in pigs was reported to be 97% in the USA. Attempts have been made, but failed to infect conventional Göttingen miniature pigs (Marshall BioResources, North Rose, NY, USA) with HuNoV (Tin et al. 2017). The miniature pigs shed neither virus nor seroconvert after oral and intravenous HuNoV inoculation. The difference in the susceptibility to norovirus infection and lack of disease in conventional pigs suggest that the gut microbiota or maternal antibodies might be protective. Effects of the gut microbiota on the resistance and immunity to norovirus infection are currently under investigation.

3.2 Effects of the Microbiome on Norovirus Infection, Immunity, and Disease

The notion that commensal bacteria can enhance enteric viral infection was demonstrated by two landmark studies published in 2011 using poliovirus, reovirus, and mouse mammary tumor virus (Kuss et al. 2011; Kane et al. 2011). When intestinal bacteria were depleted by administering a cocktail of antibiotics to mice, poliovirus infection was dramatically attenuated in comparison with normal mice with gut microbiota, as characterized by the reduced fecal virus shedding and mortality (Kuss et al. 2011). In addition, the reduced poliovirus infection was reversed by fecal transplantation to reconstitute intestinal microbes, and the status of the intestinal microbiota did not affect viral infectivity when poliovirus was inoculated intraperitoneally (Kuss et al. 2011), indicating the role of intestinal bacteria in enhancing enteric viral infection. Poliovirus was shown to directly bind to the bacterial outer-membrane component lipopolysaccharide, resulting in virion

thermo-stabilization and attachment to host cells (Kuss et al. 2011; Wilks et al. 2015). As a result, the interactions between host-microbiome and enteric viruses have been gaining intense attention. However, the understanding of effects of the intestinal microbiota on HuNoV has been impeded by the absence of a suitable cell culture system and animal model. Limited studies analyzing stool samples from human patients showed that HuNoV infection could alter microbial composition (Nelson et al. 2012).

Murine norovirus (MNV) was first identified in 2002 from the brain of an immunocompromised mouse, RAG/STAT1$^{-/-}$ strain, because of its lethal infection (Karst et al. 2003). Since then, MNV has been used widely as a surrogate to explore HuNoV biology regarding viral pathogenesis, host immunity, and interplays with gut microbiota. Antibiotic treatment reduced the acute MNV infection, and lower virus titers in the distal ileum, mesenteric lymph nodes, and colon were observed compared with control mice (Jones et al. 2014). Antibiotics also prevented persistent MNV infection in mice, but persistent infection could be restored by microbial colonization (Baldridge et al. 2015), indicating the stimulatory role of microbiota in MNV infectivity. However, major disruptions of the microbiome were not observed following acute or persistent MNV infection in mice (Nelson et al. 2013). MNV infection is asymptomatic in wild-type mice, but mucosal inflammation was observed in IL-10$^{-/-}$ mice maintained in a specific pathogen-free environment, and MNV-induced pathological changes such as reduced tight junction proteins and inflammatory lesions were lacking in germ-free IL-10$^{-/-}$ mice, suggesting that MNV-triggered intestinal diseases might be induced via bacterial microbiota (Basic et al. 2014).

3.3 Mechanisms of Probiotics/Prebiotics in Ameliorating Norovirus Infection and Disease

Probiotics have been increasingly recognized as vaccine adjuvants and therapeutic agents to ameliorate pediatric acute gastroenteritis (Schnadower et al. 2015; Licciardi and Tang 2011). The underlying mechanisms of their beneficial health effects include modulating gut microbiota composition, enhancing intestinal barrier function, and promoting mucosal immunity (Wen et al. 2009). Notably, *Lactobacillus* spp. exhibit promising properties against viral infection and diseases in human clinical trials (Guandalini et al. 2000; Sindhu et al. 2014; Szajewska et al. 2014), and their binding capacity with viral P particles holds great promise for reducing HuNoV infectivity and disease in vivo (Rubio-del-Campo et al. 2014). Evaluation of the effects of consuming *Lactobacillus casei* strain Shirota fermented milk on HuNoV gastroenteritis during an outbreak in Japan demonstrated that the elderly HuNoV-infected patients (about 84 years old) who continuously consumed the milk experienced a shorter duration of fever than the nontreated patients

(1.5 vs. 2.9 days), although the incidence of HuNoV gastroenteritis did not differ between the two groups (Nagata et al. 2011).

Probiotic bacteria can bind HuNoV P particles on their surface in vitro, and the presence of *L. casei* BL23 and *Escherichia coli* Nissle 1917 (EcN) may inhibit P particle attachment to epithelial cells (Rubio-del-Campo et al. 2014). *Lactobacillus rhamnosus* GG (LGG) is another probiotic strain with HuNoV-binding capacity, and a recent study showed that the binding between HuNoV and LGG/EcN was associated with their inhibitory role of HuNoV shedding in Gn pigs (Lei et al. 2016a). In addition, daily supplement of prebiotic rice bran in LGG/EcN colonized Gn pigs was highly protective against HuNoV diarrhea and shedding. The mechanism involves enhancement of IFN-γ-producing T cell responses, increased production of total intestinal IgA and IgG antibodies, and maintenance of longer villi compared with the non-rice bran-fed and non-probiotic-colonized control group (Lei et al. 2016a). Norovirus infection leads to epithelial barrier dysfunction and an increase in epithelial apoptosis, which results in a reduction in villus height (Troeger et al. 2009). The antiviral effects of IFN-γ and mucosal antibodies induced by rice bran can attenuate the damage to the intestinal epithelia by HuNoV infection to reduce diarrhea and maintain longer villi. In another study, the presence of *Bifidobacterium adolescentis* inhibited the attachment of HuNoV GI.1 virus-like particle to epithelial cells in vitro (Li et al. 2016), indicating the inhibitory role of probiotics on the initial viral infection stage. However, instead of affecting the viral attachment, *B. adolescentis* decreased the replication of MNV in RAW 264.7 cells (Li et al. 2016). Vitamin A was shown to inhibit MNV replication in mice by upregulating lactobacilli in gut microbiota, and anti-MNV effects of lactobacilli were confirmed in RAW264.7 cells (Lee and Ko 2016). Given the natural source and commercial accessibility, probiotic and prebiotic treatments may constitute a novel, safe, and effective measure in clinical practice against HuNoV infection and disease.

3.4 Rotavirus Pathogenesis and Immunity in Humans and in Animal Models

3.4.1 Rotavirus Pathogenesis and Immunity

Rotaviruses are double-stranded, segmented, non-enveloped, RNA viruses belonging to the Reoviridae family. Worldwide, rotaviruses are a major cause of acute gastroenteritis in infants and young children, which is characterized by diarrhea, vomiting, and dehydration. They were responsible for approximately 500,000 deaths a year, mostly in low-middle income countries before the two commercial vaccines (Rotarix and RotaTaq) became available (Desselberger 2014; Desselberger and Huppertz 2011). Diarrhea is caused by viral damage to enterocytes, villus ischemia,

action of the enterotoxin NSP4, and activation of the enteric nervous system (Desselberger 2014; Desselberger and Huppertz 2011).

Rotaviruses replicate in mature, nondividing enterocytes near the tips of the villi. The pathological changes due to rotavirus infection are mostly limited to the small intestine (Ramig 2004). Systemic rotavirus infections have been documented in humans and animals and systemic disease does occur in rare cases (Ramig 2007). In humans, after primary symptomatic or asymptomatic rotavirus infection, the patient is typically protected from subsequent severe disease (Desselberger and Huppertz 2011). Correlates of protection include rotavirus-specific serum IgA and fecal IgA (Desselberger 2014; Desselberger and Huppertz 2011). In some studies, there is a lack of correlation between neutralizing antibody titers and protection (Desselberger 2014). Rotavirus-specific T cells help to eliminate virus after infection and memory B cells provide long-term protection (Desselberger 2014). In humans, newborns are provided with additional protection through transplacental and breast milk antibodies (Desselberger and Huppertz 2011).

3.4.2 Animal Models of Rotavirus Infection and Disease

In addition to humans, many animals are susceptible to rotavirus infection and disease, and can be used as models (i.e., pigs, calves, lambs, rats, rabbits, mice, and NHPs) to study rotavirus pathogenesis and immunity. These models have been reviewed in extensive detail elsewhere (Yuan and Wen 2017). The Gn pig model has many benefits over other animal models. Pigs and humans share high genomic and protein sequence homologies, omnivorous diet, similar gastrointestinal physiology, and similar immune systems (Wang and Donovan 2015; Saif et al. 1996). Additionally, there is no transfer of maternal antibodies across the porcine placenta and Gn pigs are deprived of sow colostrum/milk, which prevents maternal antibodies from interfering with studies. Gn pigs are susceptible to genotype 1 (G1) and genotype 3 (G3) human rotavirus (HRV) infections. Inoculation of Gn pigs, up to at least 8 weeks of age with Wa strain (G1P1A[8]) HRV results in diarrhea (Yuan et al. 1998). Based on duodenal biopsies from children with acute rotavirus infection, the histopathological changes are similar to those found in piglets (Barnes and Townley 1973; Davidson and Barnes 1979; Ward et al. 1996a). Extensive work has been done with Gn pigs and Wa strain HRV. The virulent Wa human rotavirus strain (VirHRV) allows assessment of host response to natural infection, whereas the attenuated human rotavirus (AttHRV) can be used to study vaccination (Yuan and Saif 2002; Saif et al. 1997).

After oral inoculation with VirHRV, Gn pigs develop diarrhea, shed virus, and develop nearly complete protection against subsequent clinical disease and viral shedding when challenged with VirHRV after recovery (Yuan et al. 1996; Ward et al. 1996b; Iosef et al. 2002). Diarrhea develops approximately 13 h after inoculation and is associated with viral antigen in enterocytes at villus tips; villus atrophy develops 24 h post-infection and correlates with peak fecal viral titers

(Ward et al. 1996a). Gn pigs orally inoculated with AttHRV seroconvert, but have little to no virus shedding and no clinical signs, and protection from diarrhea and viral shedding after challenge is less efficacious than what is seen in pigs receiving primary VirHRV oral inoculation (Yuan et al. 1996; Ward et al. 1996b; Iosef et al. 2002).

Gnotobiotic calves have also been used to study rotavirus; however, reports are not as numerous as those in Gn pigs. Gn calves can be infected with some HRV strains, but clinical illness does not always develop (Tzipori et al. 1980). In a study in which calves successfully developed diarrhea after administration of an HRV strain, they had histological lesions consistent with rotavirus infection (Mebus et al. 1977). In addition to the fact that Gn calves are not as consistent as Gn pigs as a model of HRV infection and disease, ruminant microbiota is very different from that of humans; therefore, calves are not a proper model for the study of the role of microbiota in HRV infection and immunity.

Despite the close genetic relationship between NHPs and humans, they are not a superior rotavirus animal model compared with Gn pigs. Often, HRV strains are naturally attenuated in NHPs (McNeal et al. 2005). There have been reports of oral inoculation of simian (SA11) or human (Wa) rotavirus into different NHPs with development of diarrhea; however, it is usually during the first week of life, after which disease is not observed, and older animals may not shed virus or even seroconvert (McNeal et al. 2005). Even in a study evaluating a wild-type macaque rotavirus in 14- to 42-day-old macaques, they remained clinically normal, despite shedding large amounts of virus (McNeal et al. 2005).

Mice are attractive animal models because of their size, ease of maintenance compared with Gn pigs, and availability of numerous strains and genetic knockouts. The major downside of the murine rotavirus model is that mice are only susceptible to disease for approximately 15 days after birth (Ward et al. 1990). Adult mice can be used to study rotavirus infection; however, infections are subclinical and often do not predict protective efficacy of interventions against clinical infection (Ward et al. 1990; Yuan and Saif 2002).

3.5 Effects of Microbiome on Rotavirus Infection, Immunity, and Disease

3.5.1 Studies Comparing Conventional and Germ-Free Mice

A French research group pioneered the study on the impact of the microbiota on rotavirus pathogenesis nearly 30 years ago (Heyman et al. 1987). They compared intestinal absorption of macromolecules during murine rotavirus infection in conventional versus germ-free newborn mice derived from seronegative dams. The study showed that rotavirus infection caused a transient increase in gut permeability to undegraded proteins; this increase occurred earlier after infection in conventional

pups and later in germ-free pups. Furthermore, the length of virus excretion was different in conventional and germ-free mice; rotavirus titers in intestinal homogenates were still high at 8 days post-inoculation (dpi) in conventional mice, whereas they become very low in germ-free mice. However, there was no correlation between virus excretion and diarrhea in mice, as diarrhea was observed from 2 to 8 dpi in both conventional and germ-free mice, and no differences were detected on diarrhea kinetics. When the intestinal microbiota was absent, clinical and physiological disturbance were more severe, i.e., greater weight loss after rotavirus infection, and a more marked and long-lasting augmentation in intestinal permeability to intact proteins. This study indicates that intestinal microbiota has a significant impact on both rotavirus replication and pathogenesis, as supported by the timing, magnitude, and duration of increased epithelial permeability and virus excretion (Heyman et al. 1987).

A recent study showed that rotavirus infection was reduced by 42% and diarrhea was decreased in incidence and duration in germ-free mice (via ablation of microbiota by antibiotics) compared with mice with conventional microbiota (Uchiyama et al. 2014). Based on the non-altered ratio of positive to negative sense rotavirus RNA strands, the authors suggested that the antibiotics used to ablate the microbiota affected entry of the virus into host cells (Uchiyama et al. 2014). These antibiotic-treated mice had more durable mucosal and systemic humoral immune response and the durability correlated with small intestinal rotavirus-specific IgA antibody-secreting cells (ASCs) (Uchiyama et al. 2014). Mice treated with low levels of dextran sodium sulfate to increase exposure of immune cells to the microbiota had decreased rotavirus-specific antibodies. Further studies are needed to understand how the microbiota and antibiotics interact to induce the immunological differences between the mouse groups. The contradictory findings between the two studies on the role of microbiota in rotavirus infection and diarrhea are most likely due to the difference between using true germ-free newborn mice (Heyman et al. 1987) versus using mice ablated of the microbiota with antibiotics (Uchiyama et al. 2014). In addition to killing bacteria, antibiotics have many effects on the physiology and immune cell development of the host, which need to be taken into consideration and should be properly controlled in these types of studies.

3.5.2 Studies in Gn Pigs and Human Gut Microbiota-Transplanted Gn Pigs

To identify the influence of microbiota in the response of the Gn pig to HRV and to more closely mimic human infants with the model, Gn pigs transplanted with newborn human gut microbiota (HGM) and infected with HRV have been evaluated (Zhang et al. 2014). HGM successfully colonized the Gn pig intestine after three oral inoculations. Sequencing of the V4 region of 16S rRNA genes

demonstrated that the pigs carried a microbiome similar to that of the cesarean-section-delivered human infant donor (Zhang et al. 2014). This model was used to test the effects of probiotics on the gut microbiome structure during a VirHRV infection and the development of AttHRV vaccine-induced immune responses were compared between the HGM- and non-HGM-transplanted Gn pigs (Wen et al. 2014). The AttHRV vaccine conferred overall similar protection against rotavirus diarrhea and virus shedding in Gn pigs and HGM-transplanted Gn pigs. HGM promoted the development of the neonatal immune system, significantly enhancing IFN-γ-producing T cell responses and reducing Treg cell responses in the AttHRV-vaccinated pigs (Wen et al. 2014).

Furthermore, a Gn pig model of enteric dysbiosis and rotavirus immunity has been developed (Twitchell et al. 2016). Using this model, it has been shown that after vaccination with AttHRV, pigs colonized by gut microbiota from children who had a good immune response to oral rotavirus vaccination and low enteropathy scores (healthy human gut microbiota, HHGM) had more rotavirus-specific IFN-γ T cells in the ileum, spleen, and blood than pigs colonized by microbiota from children who did not respond well to the oral rotavirus vaccine and showed evidence for enteropathy (unhealthy human gut microbiota, UHGM) (Twitchell et al. 2016). UHGM pigs had higher viral shedding titers and more severe clinical signs than HHGM pigs after challenge with VirHRV (Twitchell et al. 2016). There was a significantly positive correlation between *Collinsella* and significantly negative correlations between *Clostridium* spp. or *Anaerococcus* and frequencies of IFN-γ T cells at the time of challenge. HHGM pigs had an increased mean relative abundance of *Bacteroides* after VirHRV challenge (Twitchell et al. 2016). As the only variable that differed between these groups was microbiota composition, this study clearly demonstrated that the differences in immune responses and clinical disease are due to the influence of the different microbiota.

It has been shown that human intestinal cells incubated with soluble factors from *Bacteroides thetaiotaomicron* and *L. casei* were protected from rotavirus infection (Varyukhina et al. 2012). The protection was attributed to increased cell surface galactose induced by the bacterial factors, which blocked rotavirus infection. This mechanism is significant in rotavirus infection because these viruses use glycan recognition to attach to enterocytes (Varyukhina et al. 2012). Perhaps a similar mechanism was at play in the Gn pig enteric dysbiosis study and may partially explain why HHGM pigs had decreased viral shedding compared with UHGM pigs (Twitchell et al. 2016).

3.5.3 *Studies of the Microbiome in Rotavirus Infection and Vaccination in Humans*

The abundance of *Bacteroides* species in rotavirus infected and uninfected children was different. *B. fragilis* was increased whereas *B. vulgatus* and *B. stercoris* were

decreased in the intestines of infected children (Zhang et al. 2009). A rotavirus vaccine study in rural Ghana evaluated pre-vaccination fecal microbiome of vaccine responders and non-responders and then compared them with age-matched healthy Dutch infants (Harris et al. 2016). The Ghanaian vaccine responder microbiome was more like the healthy Dutch infant microbiome than the Ghanaian nonresponders. Vaccine response correlated with an increased abundance of *Streptococcus bovis* and decreased Bacteroidetes phylum (Harris et al. 2016). The significance of these findings needs to be further elucidated. These studies suggest that certain bacterial components of microbiome might play a modulatory role in the development of rotavirus infection and immunity. Although the underlying mechanisms of specific host–bacteria and virus–bacteria interactions that lead to the different outcomes in enteric viral diseases and immunity have not been identified, studies of probiotics shed some light on the mechanisms.

3.6 Mechanisms of Probiotics/Prebiotics in Ameliorating Rotavirus Infection and Disease

Prebiotics and probiotics are being developed as a nonpharmacological means of preventing or ameliorating gastroenteritis caused by enteropathogens, and as vaccine adjuvants. Mechanisms by which prebiotics and probiotics affect infection, disease, and immunity are an active area of study. Effects vary based on strain, dose, and frequency of administration.

3.6.1 Mechanisms for Reducing Rotavirus Diarrhea Using Probiotics

Among all commercially available probiotics, LGG has been most extensively studied in rotavirus infection, disease, and immunity. LGG has been shown to protect the intestinal barrier in studies using conventional pigs and Gn pigs. When supplemented with LGG and then challenged with rotavirus, conventional pigs had increased villus-to-crypt ratios, villus height, sIgA, IL-4, mucin1 and mucin2 concentrations, and ZO-1, occludin, and Bcl-2 mRNA in jejunal mucosa, and decreased Bax mRNA and NSP4 in the jejunum compared with controls (Mao et al. 2016). Gn pigs supplemented with LGG were partially protected from HRV-induced increases in adherens junction proteins α-catenin and β-catenin, tight junction proteins occludin, claudin-3 and claudin-4, and leakage of protein claudin-2 compared with controls (Liu et al. 2013). In both studies, LGG-supplemented pigs had reduced diarrhea compared with controls after rotavirus challenge (Mao et al. 2016; Liu et al. 2013). One mechanism by which LGG may reduce diarrhea is by protecting small intestinal barrier function. A recent

study showed that metabolites of *L. casei* and *B. adolescentis* significantly reduced NSP4 production and $Ca2^{++}$ liberation in vitro, suggesting activity against rotavirus infection (Olaya Galan et al. 2016).

LGG can improve innate immunity. It has been shown that LGG increases mRNA levels of TLR3 when incubated with intestinal organoids (Aoki-Yoshida et al. 2016). In vivo, single and a 7-day course of LGG increased TLR3 mRNA levels in the small intestine of C57/BL6N mice (Aoki-Yoshida et al. 2016). Co-colonization of Gn pigs with LGG and *Bifidobacterium lactis* Bb12 (Bb12) induced upregulation of TLR3 after VirHRV challenge and downregulation of TLR 2 and TLR4 expressing mononuclear cells (MNCs) after AttHRV vaccination (Vlasova et al. 2013). *L. ruminis* SPM02111, *Bifidobacterium longum* SPM1205 and SPM1206 were able to inhibit rotavirus replication in neonatal mice, inhibit Wa HRV infection of Caco-2 cells, increase IFN-α and IFN-β, and increase gene expression of IFN-inducible antiviral effectors when compared to controls (Kang et al. 2015). *Lactobacillus reuteri* and *Lactobacillus acidophilus* with HRV infection have an additive effect on TLR2 and TLR9 expressing antigen presenting cell responses in Gn pigs (Wen et al. 2009). This same study demonstrated increased IFN-γ and IL-4 responses in serum of the probiotic colonized pigs while serum IFN-α response to HRV were reduced (Wen et al. 2009).

Lactobacillus rhamnosus GG, in combination with other probiotics, has been shown to influence T cell and humoral responses. Nonvaccinated Gn pigs colonized with LGG and Bb12 challenged with VirHRV had less diarrhea and viral shedding than nonvaccinated, noncolonized pigs, and the increased protection was associated with higher T regulatory cells before and after challenge; higher serum TGF-β; and lower proinflammatory cytokines after viral challenge (Chattha et al. 2013). AttHRV-vaccinated pigs colonized with these two probiotics had enhanced serum IFN-α, splenic and blood IFN-γ-producing T cells, and serum Th1 cytokines compared with noncolonized vaccinated pigs (Chattha et al. 2013). Gn pigs colonized with LGG and *Bifidobacterium animalis lactis* Bb12 had lower diarrhea scores and viral shedding after AttHRV vaccination and VirHRV challenge than noncolonized vaccinated pigs (Kandasamy et al. 2014). The decreased clinical signs in the colonized pigs correlated with higher intestinal rotavirus-specific IgA titers, and rotavirus-specific IgA ASC (Kandasamy et al. 2014).

Modulation of microbiome by probiotics has been studied in Gn pig models. AttHRV-vaccinated Gn pigs colonized with infant gut microbiota showed that LGG prevented changes in the microbiome structure caused by VirHRV challenge that were seen in non-LGG-supplemented groups (Zhang et al. 2014).

Bifidobacterium spp. are commonly studied probiotics. *B. thermophilum* RBL67 is thought to inhibit rotavirus infection by competing for adherence on cells, as demonstrated in vitro with Caco-2 and HT-29 cells (Gagnon et al. 2016). When incubated before rotavirus infection of cells to assess exclusion and incubated with rotavirus to assess competition, there was decreased viral attachment in the *B. thermophilum*-treated cells; however, the probiotic did not appear to displace virus already adhered (Gagnon et al. 2016). *B. longum* subsp. infantis CECT 7210 can inhibit rotavirus replication in vitro via an 11-aminoacid peptide (11-mer

peptide) released into supernatant along with a protease that releases the 11-mer peptide (Chenoll et al. 2016). In vivo studies have shown the effectiveness of *B. thermophilum* during rotavirus infection. Administration of *B. thermophilum* RBL67 to CD-1 suckling mice before challenge with simian rotavirus SA-11 decreased the duration of diarrhea, viral replication, recovery time, and histological lesions, and stimulated rotavirus-specific IgG and IgM (Gagnon et al. 2016).

The combination of EcN and LGG have been evaluated in vivo and in vitro. Gn pigs colonized by EcN had lower mean peak viral shedding titers and mean cumulative fecal scores compared with LGG or noncolonized pigs (Kandasamy et al. 2016). Total IgA levels after challenge in the intestine and before challenge in serum were higher in EcN than LGG-colonized pigs (Kandasamy et al. 2016). EcN but not LGG induced IL-6, IL-10, and IgA in MNCs treated with EcN or LGG in vitro (Kandasamy et al. 2016). EcN colonization was associated with better protection against HRV and induced higher frequencies of plasmacytoid dendritic cells (pDCs), increased NK-cell function, and decreased frequencies of apoptotic and TLR4+MNC compared with LGG-colonized pigs (Vlasova et al. 2016). EcN-treated splenic or intestinal MNC produced higher levels of IFN-α, IL-12, and IL-10, compared with MNC treated with LGG (Vlasova et al. 2016). These studies demonstrate that different probiotic strains do not have the same immunomodulatory functions and that strain selection should be based on the effect desired.

Bacteria are not the only microorganisms used as probiotics. When the yeast *Saccharomyces boulardii* was given to children with acute rotavirus diarrhea, the mean duration of diarrhea and hospitalization were shorter than in controls; however, there was no difference between the groups in the number of children requiring parenteral rehydration or who had diarrhea lasting beyond 7 days (Das et al. 2016). It is believed that *S. boulardii* decreases diarrhea by preventing rotavirus-induced oxidative stress and thus activation of NSP4 and subsequent chloride secretion based on results obtained in Caco-2 cells and human intestinal organ culture (Buccigrossi et al. 2014).

3.6.2 Dose Effects of Probiotics in Modulating Rotavirus Vaccine-Induced Immune Responses

Differences in the dosing schedule of the probiotics influence host immune response. A Gn pig study looking at the influence of LGG on protection provided by AttHRV vaccination showed that rotavirus-specific intestinal memory B cell responses and virus-specific intestinal IgA ASCs were enhanced by a five-dose regimen of LGG, but not nine-dose regimen, although both doses enhanced the rotavirus-specific serum IgA response and rotavirus-specific IFN-γ producing effector/memory T cell responses, with the nine-dose regimen having a stronger effect (Wen et al. 2015). This study demonstrated how the dosing regimen can affect the immune response; in this case, the five-dose regimen favored a mucosal

IgA response, whereas the higher dosing schedule favored the T cell response (Wen et al. 2015). Another AttHRV vaccine study in Gn pigs showed that pigs receiving 14 doses of LGG had increased large intestinal LGG titers and a shifted microbiota structure, which correlated with increased rotavirus-specific IFN-γ-producing T cells, suggesting a Th1 adjuvant effect (Wang et al. 2016). However, pigs in the same study receiving nine doses of LGG had enhanced TLR9 signaling, which may suggest that this dosing regimen might have enhanced innate immunity (Wang et al. 2016). A third study also demonstrated a differential effect from LGG dosing schedules. In this study, using HGM=transplanted Gn pigs, it was shown that a 14-dose regimen of LGG enhanced rotavirus-specific, IFN-γ-producing T cell response to AttHRV vaccination, whereas a nine-dose regimen was ineffective (Wen et al. 2014). The effects of dosing schedules are seen with other probiotics in addition to LGG. Gn pigs colonized with the *L. acidophilus* NCFM, vaccinated with AttHRV, and challenged with VirHRV demonstrated that a nine-dose regimen of *L. acidophilus* but not a 14- or five-dose regimen improved protection provided by the vaccine and this was associated with enhanced rotavirus-specific antibody, ASC, and memory B cell responses to the vaccination (Liu et al. 2014). Neither the high-dose (14) nor the low-dose (5) regimen enhanced antibody or ASC responses, and thus did not improve vaccine efficacy (Liu et al. 2014). The differential modulating effects of different doses of probiotics are intriguing. The underlying mechanisms require further investigation. It has been reported that the effect of low-dose microbe-associated molecular patterns (MAMPs), such as lipopolysaccharide, was strikingly different than that of high-dose MAMPs on macrophage cell functions: low-dose lipopolysaccharide induced a strong inflammatory response in macrophages (Maitra et al. 2011). It is plausible that a similar interaction occurs between the MAMPs from probiotics and immune cells in the gut. Future studies are needed to identify the molecular mechanisms of the dose responses of different MAMPs.

3.6.3 Mechanisms for Reducing Rotavirus Diarrhea Using Prebiotics

Prebiotics are another nonpharmacological category of agents being investigated for treatment or prevention of diarrhea with or without concurrent probiotic administration. Rice bran contains phytochemicals that can promote intestinal mucosal immunity to enteropathogens (Yang et al. 2014). Gn pigs fed rice bran were protected from diarrhea after VirHRV challenge and AttHRV was more protective in these pigs than in nonrice bran-fed pigs (Yang et al. 2014). IFN-γ-producing CD4+ and CD8+ T cells were increased in intestinal and systemic lymphoid tissues, IgM ASC, IgA ASC, total serum IgM, IgG, IgA, and rotavirus-specific IgA intestinal titers were increased in rice bran-fed pigs compared with nonrice bran-fed pigs (Yang et al. 2014). Results support rice bran as a stimulator of nonspecific and

rotavirus-specific immune responses (Yang et al. 2014). Gn pigs colonized with LGG and EcN were fed a diet supplemented with rice brain daily (Yang et al. 2015). Rice bran completely prevented rotavirus diarrhea in the colonized pigs after VirHRV challenge and promoted growth of both probiotic strains LGG and EcN compared with nonrice bran-fed pigs (Yang et al. 2015). In addition, after VirHRV challenge, the rice bran-fed pigs had increased intestinal IFN-γ and total IgA levels, and fewer histological changes in the ileum, compared with the nonrice bran-fed group (Yang et al. 2015).

Prebiotics are often evaluated with probiotics as they can have synergistic effects on each other. A study evaluating *B. lactis* B94 and inulin in children with acute diarrhea showed that the duration and amount of diarrhea was reduced in the group receiving the prebiotics and probiotics (Islek et al. 2014). The clinical effects were most pronounced in cases of rotavirus diarrhea (Islek et al. 2014).

3.7 Concluding Remarks

Germ-free animal models provide an indispensable tool for the study of the consequences of bacterial colonization and mechanisms underlying host–microbiome interactions in enteric virus infection and gastroenteritis. The Gn pig model, with its distinct advantages, has greatly contributed to studies on the effects and mechanisms of gut microbiota and probiotics on enteric virus infections and vaccines. However, the drawback of using pig models is the decreased availability of species-specific molecular reagents and gene knockout pigs compared with mouse models, which hinders in-depth mechanistic studies. Further optimization of the pig models, including genetic modification using CRISPR/Cas9 technology, humanization of the immune system through stem cell transfer, and transplantation with HGM from donors representing different health, disease, and immune statuses will further improve the usefulness and reliability of pig models for mimicking HuNoV and HRV infection and disease in humans. Unraveling the role of the microbiome and specific probiotics in the infectivity, pathogenesis, and immunity of HuNoV and HRV will facilitate the development of strategies for manipulating the microbiome against viral infections.

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