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Lactobacilli and Bifidobacteria enhance mucosal B cell responses and differentially modulate systemic antibody responses to an oral human rotavirus vaccine in a neonatal gnotobiotic pig disease model

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Keywords: B cell responses, human rotavirus, neonatal diarrhea, probiotics, vaccine

Abbreviations: APRIL, a proliferation-inducing ligand; ASC, antibody secreting cell; AttHRV, attenuated human rotavirus; AUC, area under the curve; Bb12, Bifidobacterium lactis Bb12; FFU, fluorescent foci forming unit; Gn, gnotobiotic; HRV, human rotavirus; LGG, Lactobacillus rhamnosus strain GG; MNCs, mononuclear cells; PCD, postchallenge day; PID, postinoculation day; Vac+Pro, vaccinated probiotic colonized group; RAM, rat anti-mouse; RV, rotavirus; Vac, 3XAttHRV Wa vaccinated only group; VirHRV, virulent human rotavirus; PBCD, post bacterial colonization day.

B cells play a key role in generation of protective immunity against rotavirus infection, a major cause of gastroenteritis in children. Current RV vaccines are less effective in developing countries compared to developed countries. Commensals/probiotics influence mucosal immunity, but the role of early gut colonizing bacteria in modulating intestinal B cell responses to RV vaccines is largely unknown. We co-colonized neonatal gnotobiotic pigs, the only animal model susceptible to HRV diarrhea, with 2 dominant bacterial species present in the gut of breastfed infants, *Lactobacillus rhamnosus* strain GG and *Bifidobacterium animalis lactis* Bb12 to evaluate their impact on B cell responses to an attenuated (Att) human rotavirus (HRV) Wa strain vaccine. Following HRV challenge, probiotic-colonized, AttHRV vaccinated piglets had significantly lower fecal scores and reduced HRV shedding titers compared to uncolonized, AttHRV vaccinated pigs. The reduction in HRV diarrhea was significantly correlated with higher intestinal IgA HRV antibody titers and intestinal HRV-specific IgA antibody secreting cell (ASC) numbers in probiotic-colonized, AttHRV vaccinated pigs compared to uncolonized, vaccinated pigs. The significantly higher small intestinal HRV IgA antibody responses coincided with higher IL-6, IL-10 and APRIL responses of ileal mononuclear cells (MNCs) and the immunomodulatory effects of probiotics genomic DNA on TGF-β and IL-10 responses. However, serum RV IgG antibody titers and total IgG titers were significantly lower in probiotic-colonized, AttHRV vaccinated pigs compared to uncolonized, vaccinated pigs, both pre- and post-challenge. In summary, LGG and Bb12 beneficially modulated intestinal B cell responses to HRV vaccine.

Introduction

The intestinal microbiota plays a significant role in maturation of mucosal immunity in neonates.[1] Recent studies have shown that composition of the microbiota also influences gut immune responses[2,3] and the immunomodulatory effects of commensals or probiotics are strain dependent.[4] Mice monocolonized with *L. johnsonii* produced higher antigen-specific IgA antibody responses as compared to animals colonized with *L. paracasei*.[5] Further, the colonization patterns of commensals also had significant effects on B cell responses in neonates.[6] In infants, early colonization with *Escherichia coli* and Bifidobacteria was associated with increased memory B cells, but colonization with *Staphylococcus aureus* was negatively associated with number of memory B cells.[7] Most of the previous studies mainly investigated the effects of specific commensals on total intestinal IgA responses.[8,9] The impact of individual or a combination of early colonizing commensals in modulating intestinal B cell responses to enteric pathogens such as rotavirus, as well as enteric vaccines, is largely unexplored.

Rotavirus (RV) is a leading cause of diarrhea in children worldwide. Rotavirus infection causes ~450,000 deaths in

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children <5 years of age annually worldwide. Approximately 85% of the rotavirus-associated deaths occur in developing countries.[10,11] The efficacy of currently available RV vaccines is >80% in developed countries,[12-14] but it is only ~50% in developing countries.[11] B cells play a significant role in generation of protective immunity against RV infection.[15] Thus modulating adaptive B cell responses using beneficial commensals may be a cost effective strategy to prevent RV infection. Probiotic intervention is a potential strategy to modulate adaptive immunity to enteric infections/vaccines. Indeed, the composition of commensals in the small intestine significantly influenced antibody responses to enteric vaccines in children.[16-20] *Bifidobacteria and Lactobacilli* are among the dominant members of the gut microbiota in breastfed infants[21,22] and among the most commonly used probiotic bacterial species. Each species possesses various immunomodulatory properties.[23-25] Supplementation of *Bifidobacterium lactis* Bb12 increased both total[26] and poliovirus-specific fecal IgA levels in children.[25] Interactions among probiotics affect their intestinal colonization patterns as well as their effects on gut immunity. An earlier study reported that initial *Lactobacillus rhamnosus* strain GG (LGG) colonization promotes subsequent colonization of *Bifidobacterium* in children.[27] However, the effects of co-colonization by *Bifidobacterium* and *Lactobacillus* combined, on intestinal B cell responses to an oral HRV vaccine and on resolution of virulent HRV (VirHRV) infection is largely unknown.

Neonatal gnotobiotic (Gn) piglets are susceptible to HRV induced diarrhea and also their immune responses, gut physiology and milk diet are similar to that of infants.[28] Thus Gn piglets are an important animal model to assess the impact of probiotics on a HRV vaccine or infection. In our earlier study, we demonstrated that dual colonization of *L. acidophilus* and *L. reuteri* had no impact on HRV specific immunity[29] which may have been the result of a very short interval (2 days) between LAB colonization and HRV challenge of Gn piglets.[29] In this study, we investigated the effect of co-colonization of *Lactobacillus rhamnosus* strain GG and *Bifidobacterium lactis* Bb12 on B cell responses to AttHRV Wa strain vaccine in a neonatal Gn pig model. We show that dual-colonization of Gn pigs with LGG and Bb12 significantly augmented small intestinal B cell responses including HRV specific IgA responses in vaccinated animals post-challenge. The enhanced intestinal HRV IgA antibody responses were inversely correlated with reduced fecal scores and also coincided with complete protection against virus shedding in probiotic colonized, vaccinated piglets post-challenge. In addition, LGG+Bb12 also enhanced intestinal as well as systemic total IgA responses.

Results

LGG and Bb12 colonized Gn piglets and the probiotics decreased fecal scores and virus shedding post-virulent HRV challenge

Co-colonization of LGG and Bb12 was confirmed by QPCR in rectal swab fluids from representative pigs from each group. Both LGG and Bb12 were recovered from fecal samples of all colonized Gn piglets throughout the study period,[30] and the uncolonized piglets remained bacteriologically sterile. In addition, recovery of the probiotic bacteria from duodenum, jejunum, ileum, cecum and colon tissues from representative Pro and Vac+Pro piglets at PID34/PCD7 confirmed colonization of these probiotics in the intestinal tract of both groups of gnotobiotic piglets.[30] Vac+Pro piglets had significantly ($P < 0.05$) lower mean cumulative fecal scores compared to Vac piglets.[31] Fecal virus shedding was undetectable in Vac+Pro piglets but Vac piglets had higher incidence of fecal virus shedding.[31] Further, a stronger significantly inverse correlation was observed between small intestinal HRV IgA titers and mean fecal scores in Vac+Pro piglets (**Fig. 1C**) compared with Vac piglets (**Fig. 1D**). This suggests that amelioration of RV severity in vaccinated probiotic colonized piglets may be partly mediated by probiotics-augmented intestinal B cell responses.

LGG+Bb12 probiotics significantly enhanced small intestinal HRV IgA antibody and total IgA responses in vaccinated piglets post-challenge

In the small intestine (**Fig. 1A**), Vac+Pro piglets had significantly ($P < 0.05$) higher HRV IgA antibody titers compared with Vac piglets post-challenge (PID34/PCD7). Thus LGG+Bb12 probiotics enhanced intestinal IgA antibody responses. Nonparametric Spearman correlation coefficient analyses revealed a significant negative correlation between small intestinal HRV IgA antibody titers and mean diarrhea scores that was higher in Vac+Pro (**Fig 1C**) compared to Vac piglets post-VirHRV challenge (**Fig 1D**). This finding suggests that the enhanced small intestinal HRV IgA antibody responses may play a role in ameliorating the severity of HRV infection in Vac + Pro compared to Vac piglets. Total IgA titers in the small intestine were also significantly higher in Vac+Pro piglets compared to Vac piglets post-challenge (PID34/PCD7) (**Fig. 1B**). These findings were further supported by the presence of higher total intestinal IgA IgSCs in Vac+Pro piglets compared to Vac piglets (**Fig 2C**). Thus LGG+Bb12 probiotics enhanced intestinal total IgA and HRV IgA antibody responses.

LGG+Bb12 probiotics significantly elevated duodenal HRV IgA ASC in vaccinated piglets post-challenge

HRV IgA and IgG ASC were determined in ileum and duodenum. Significantly ($P < 0.05$) higher duodenal HRV IgA ASC were observed in Vac+Pro piglets compared to Vac piglets post-challenge (**Fig. 2A**). A similar trend of higher HRV specific IgA ASC in Vac+Pro compared to Vac piglets was also observed in ileum (**Fig. 2A**). The enhanced small intestinal HRV IgA ASC (**Fig. 2A**) coincided with enhanced small intestinal HRV IgA antibody responses (**Fig. 1A**). However the Vac+Pro piglets had lower duodenal HRV IgG ASC (**Fig. 2B**) and HRV IgG antibody-responses (**Fig. 4A**) compared to Vac piglets post-challenge.

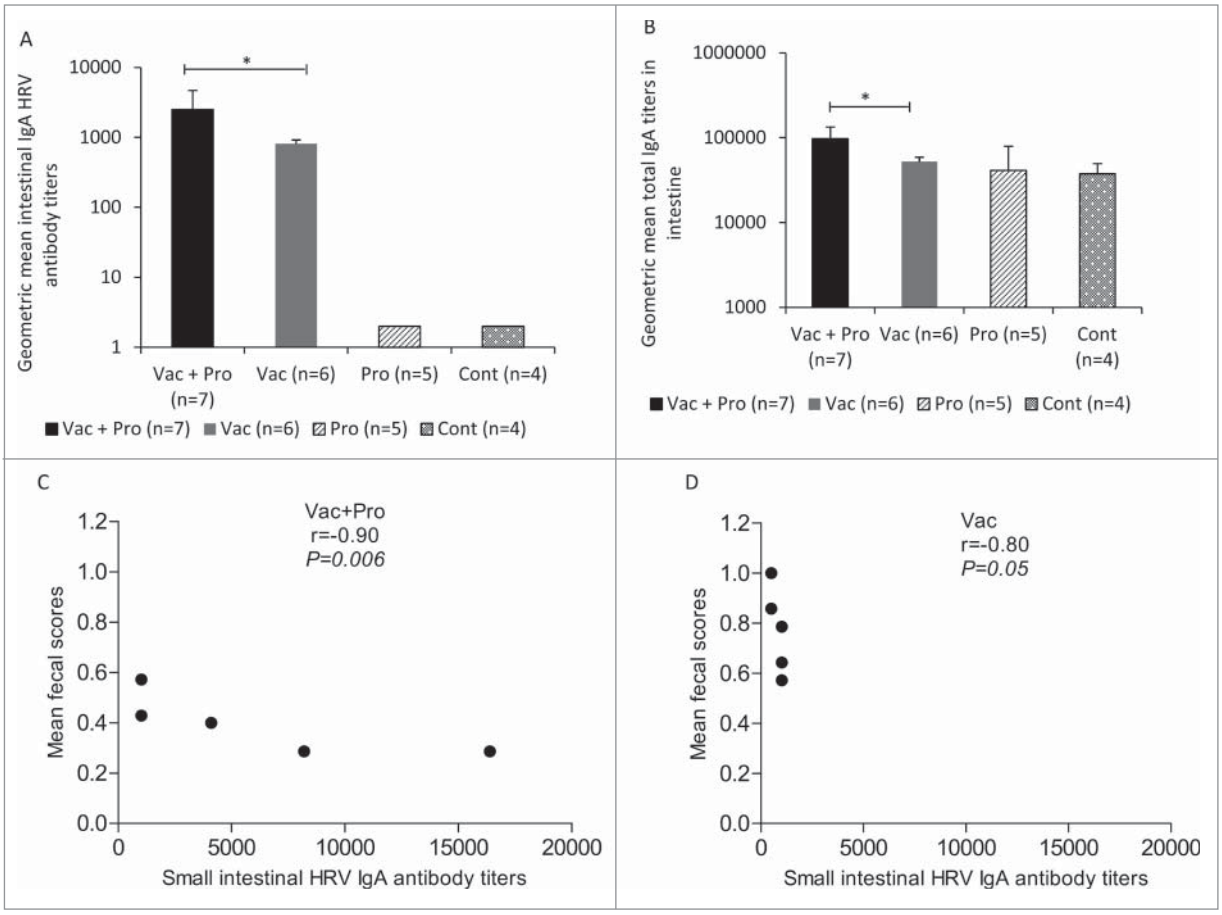

Figure 1. Intestinal HRV specific-IgA antibody and total IgA responses and correlation between small intestinal HRV IgA antibody titers and mean diarrhea scores at post-challenge (PID34/PCD7). Geometric mean titers (GMT) of HRV specific-IgA (**A**) and total IgA (**B**) antibody titers in intestinal contents of Gn pigs vaccinated with AttHRV with or without LGG + Bb12 probiotic colonization at post-HRV challenge (PID34/PCD7)(one-way ANOVA followed by Duncan's multiple range test on $\log_{10}$ transferred titers, $^*P < 0.05$). Correlations between SIC HRV IgA antibody titers and mean diarrhea scores (**C and D**) were determined using Spearman correlation coefficient. Error bars indicate SEM. PID-Post-inoculation day; PCD-Post-challenge day. SIC-Small intestinal contents; LIC-Large intestinal contents.

LGG+Bb12 probiotics enhanced the frequency of activated B cells as well as IgA$^+$ B cells in the small intestine

Vac+Pro piglets had significantly higher frequencies of IgA$^+$ B lymphocytes in spleen and ileum compared to that of Vac piglets. In addition, probiotic colonization alone resulted in significantly higher frequencies of IgA$^+$ B lymphocytes in blood and spleen in comparison to unvaccinated, non-colonized control piglets (**Fig. 3A**). Significantly higher frequencies of activated CD21$^+$CD2$^-$ B cells were observed in ileum and duodenum in Vac+Pro compared to Vac piglets (**Fig. 3C**) based on CD2 and CD21 expression (CD21$^+$CD2$^-$ B cells are defined as activated B cells in pigs).[32] The enhanced activation of B cells coincided with the higher frequencies of mature conventional dendritic cells (cDCs) as well as plasmacytoid DCs (pDC)[30] and higher intestinal IgA responses in Vac+Pro piglets compared to Vac piglets. Further, significantly higher frequencies of activated B cells were observed in unvaccinated piglets compared to vaccinated piglets in ileum, blood and spleen post-challenge (**Fig. 3C**). RV is known to activate B cells[33,34] and severe RV infection, as indicated by significantly higher RV shedding,[31] coincided with the increased frequency of activated B cells in the unvaccinated piglets compared to vaccinated piglets.

LGG+Bb12 probiotics decreased serum HRV IgG antibody responses

Serum HRV IgG antibody titers were significantly ($P < 0.05$) lower in Vac+Pro piglets compared to Vac piglets at pre- (PID14) and post- (PID34/PCD7) challenge (**Fig. 4A**). The differential systemic HRV IgG antibody responses were associated with significant differences in systemic IL-4 cytokine concentrations between Vac+Pro and Vac piglets.[31] However probiotics colonization had no effect on serum HRV IgA antibody responses in the vaccinated piglets (**Fig. 4C**). As expected, Vac piglets with or without probiotic colonization had significantly ($P < 0.05$) higher HRV IgG (**Fig 4A**) and IgA (**Fig 4C**) antibody titers compared to probiotic-colonized or control piglets at all PID or PCD time-points.

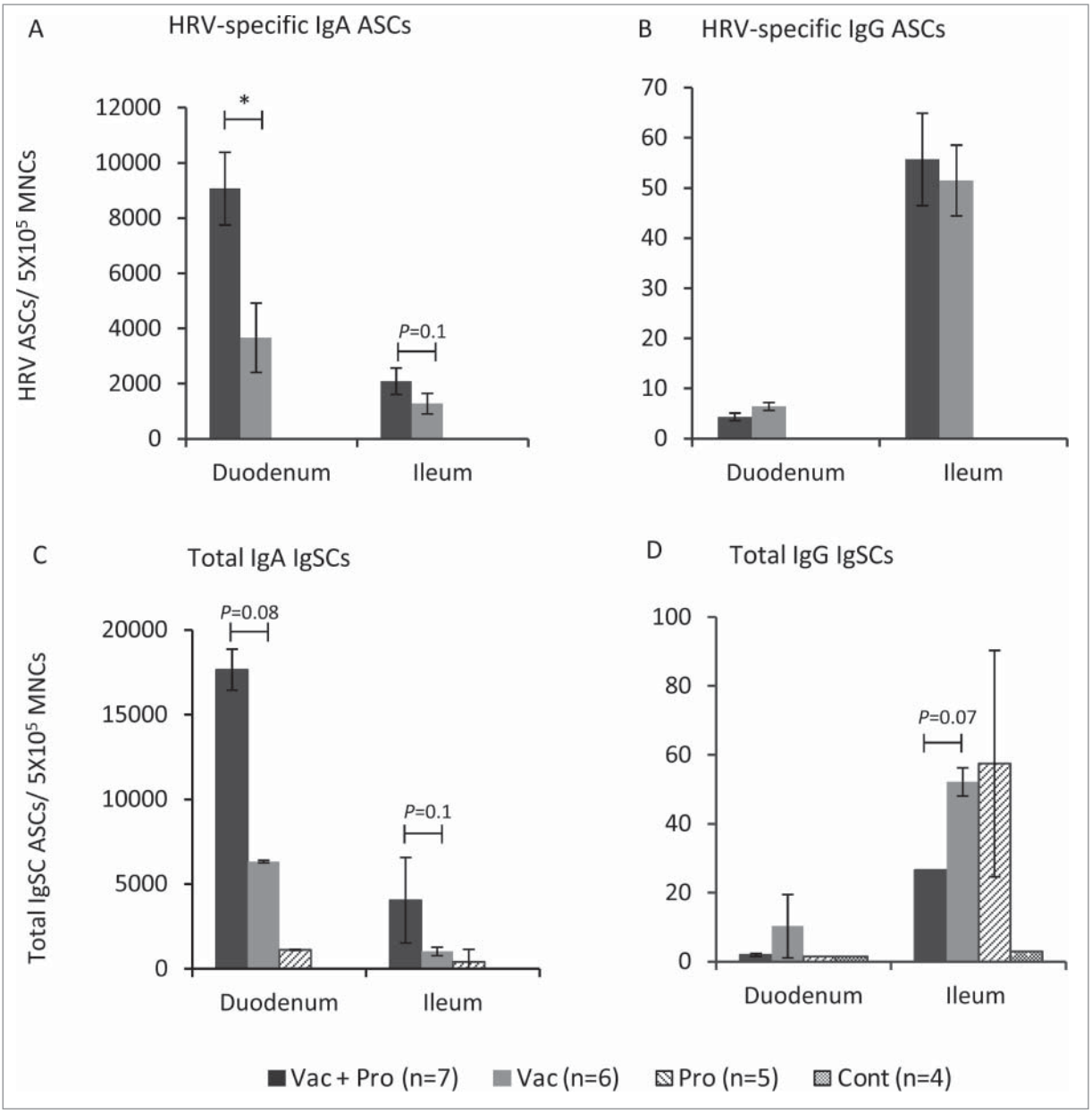

Figure 2. Small intestinal HRV IgA and IgG ASC and total IgA IgG IgSC responses at post-challenge (PID34/PCD7). HRV-specific ASC (**A and B**) and total IgSC (**C and D**) responses in duodenum and ileum of Gn pigs vaccinated with AttHRV with or without LGG + Bb12 probiotics colonization post-HRV challenge (PID34/PCD7). Data represent the mean numbers of HRV-specific ASC and total IgSC per 5×10^5 MNC, respectively (Kruskal–Wallis Test, $^*P < 0.05$). Error bars indicate SEM. PID-Post-inoculation day. PCD-Post-challenge day.

Systemic total Ig responses were modulated by LGG+Bb12 colonization

Total serum IgG titers also were consistently lower (**Fig. 4B**) post-probiotic colonization and were significantly lower at PID27/PCD0 (**Fig 4B**) in Vac+Pro piglets compared to Vac piglets. However, total serum IgA titers were significantly higher in Vac+Pro piglets compared to Vac piglets at PID34/PCD7 (**Fig. 4D**). In addition, the vaccinated piglets had significantly higher total serum IgA titers compared to unvaccinated piglets at PID14 irrespective of probiotic colonization (**Fig. 4D**). Pre-challenge (PID27/PCD0), the significantly higher (17-fold) total IgA titers in Pro piglets compared to Cont piglets (**Fig. 4D**) also indicates the marked effect of LGG+Bb12 probiotics on systemic total IgA antibody responses.

LGG+Bb12 probiotics increased IL-6 and IL-10 responses and decreased IL-8 and IL-17 responses in ileum

To assess immune responses at the induction site (ileum) of the small intestine,[35] cytokine responses were measured in culture supernatants from HRV antigen re-stimulated ileal MNCs isolated from Vac+Pro, Vac, Pro and Cont piglets (**Fig. 5**). Changes (Vac+Pro vs Vac and Pro vs Cont) in cytokine

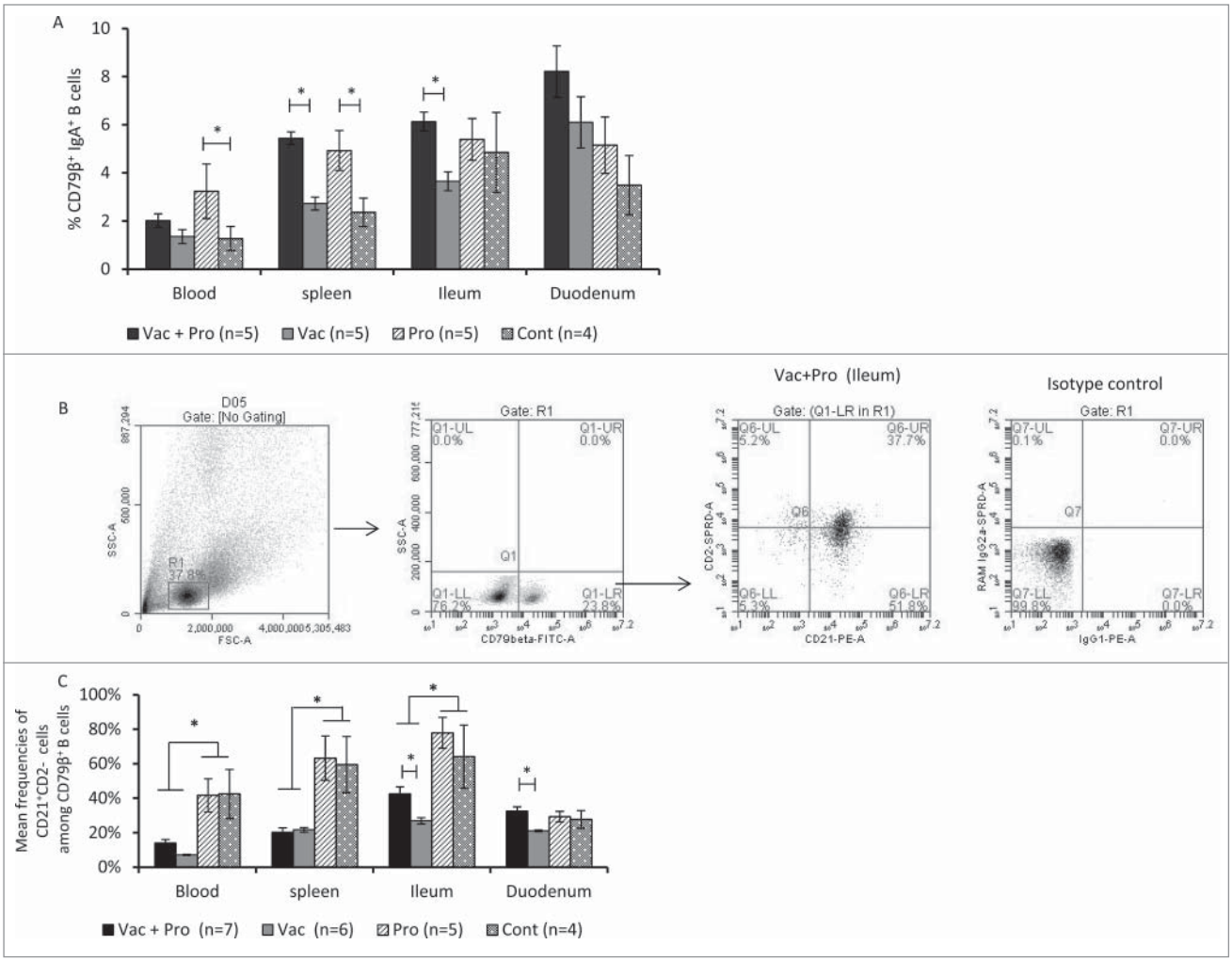

Figure 3. Probiotics colonization enhanced frequencies of intestinal activated B cells and IgA$^+$ B cells post-challenge (PID34/PCD7). Mean frequencies of CD79β$^+$IgA$^+$ B cells among lymphocytes (**A**), representative dot plot (**B**) and mean of frequencies (**C**) CD21$^+$CD2$^-$ B cells among CD79β$^+$ B cells in peripheral blood, spleen, ileum and duodenum of Gn pigs vaccinated with AttHRV with or without LGG + Bb12 probiotic colonization post-HRV challenge (PID34/PCD7). Data represent the mean frequencies of B cells ± SEM (Kruskal–Wallis Test, *$P < 0.05$). PID-Post-inoculation day. PCD-Post-challenge day.

responses were determined to assess effects of probiotics on induction of cytokines associated with IgA responses. Higher IL-6 responses were observed in probiotic colonized piglets compared to uncolonized piglets regardless of vaccination pre- and post-challenge (**Fig. 5A**). Pre-challenge, IL-10 levels were also higher in Vac+Pro piglets compared to Vac piglets (**Fig. 5B**). In contrast to IL-6 and IL-10 responses, a trend of lower IL-8 and IL-17 cytokine responses was observed in Vac+Pro piglets compared to Vac piglets pre-challenge (**Fig. 5C and D**). These results suggest that LGG+Bb12 colonization may enhance IL-6 and IL-10 cytokine responses which may in turn be involved in enhancing small intestinal RV specific IgA responses.

LGG+Bb12 probiotics bacterial DNA induces TGF-β, IL-10 and IL-12 cytokines in ileal MNC

Soluble factors or microbe associated molecular patterns of beneficial bacteria such as CpG DNA of probiotic bacterial genomic DNA might mediate some of the observed immunomodulatory and enhanced IgA effects. Both Bifidobacteria and LGG have high GC percentage in their genomic DNA.[36,37] Also, significantly higher small intestinal TLR9 expression was observed in vaccinated, probiotic colonized piglets compared to vaccinated, uncolonized piglets in our study.[30] TLR9 recognizes bacterial CpG motifs[38] and the higher expression of TLR9 of MNCs might have played a role in eliciting higher mucosal IgA responses in Vac+Pro piglets compared to Vac piglets. Treatment of ileal MNCs from naive Gn piglets (no prior exposure to any bacteria/virus) with probiotic bacterial genomic DNA (1:1 ratio of LGG and Bb12 genomic DNA) resulted in significantly higher levels of TGF-β (**Fig. 6A**), IL-10 (**Fig. 6B**) and IL-12 (**Fig. 6C**) cytokines under in vitro conditions. This indicates that probiotic bacterial genomic DNA may have induced a favorable microenvironment for enhanced IgA production in probiotic colonized, vaccinated piglets. In addition, pretreatment of MNCs

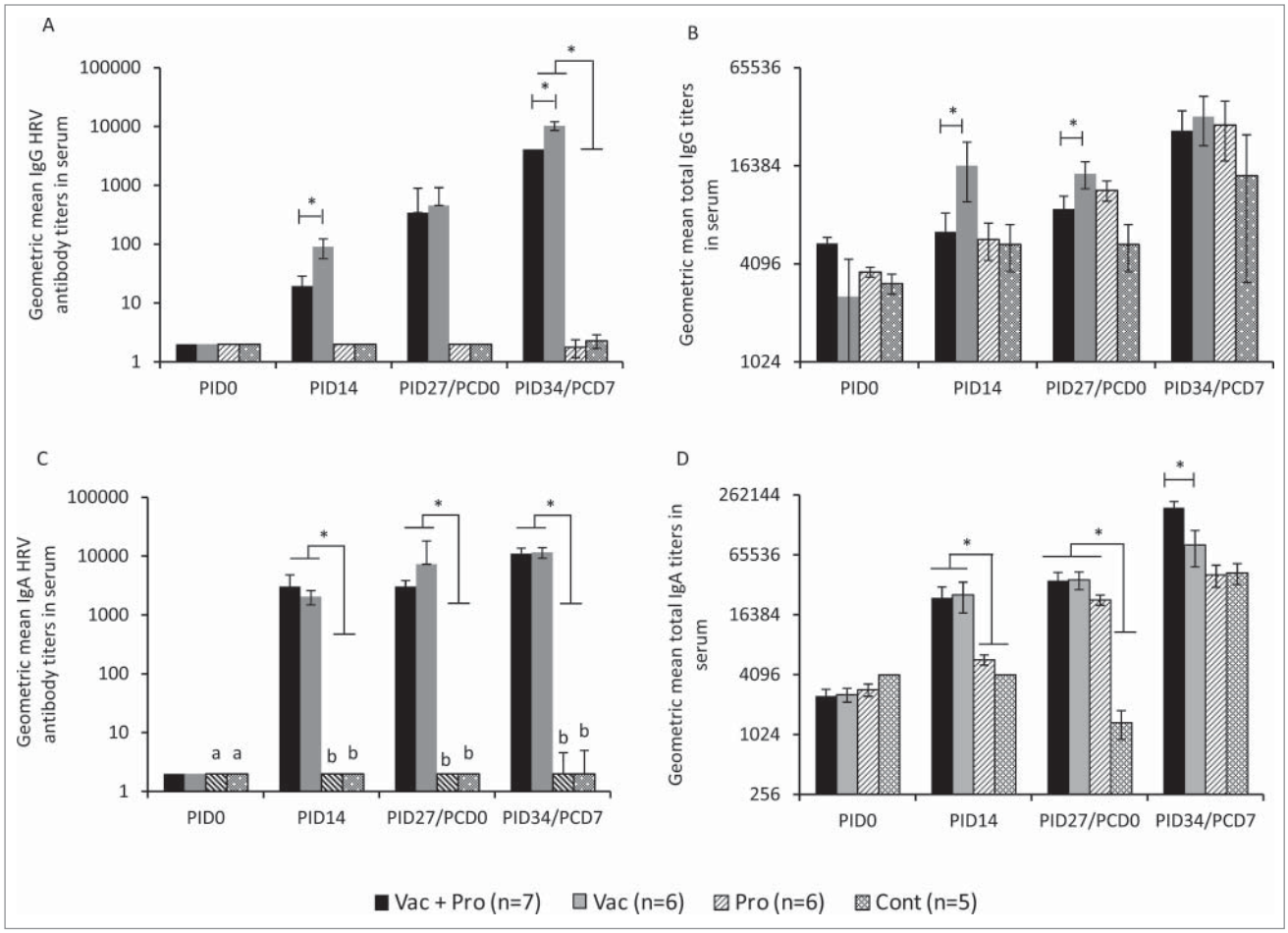

Figure 4. LGG and Bb12 probiotic colonization modulates serum HRV IgG antibody responses, and total serum IgA and IgG responses. Geometric mean titers (GMT) of IgG (**A**) and IgA (**C**) antibody to HRV, total IgG (**B**) and IgA (**D**) gemometric mean titers in serum of Gn pigs vaccinated with AttHRV with or without LGG + Bb12 probiotics colonization at indicated PID/PCD (one-way ANOVA followed by Duncan's multiple range test on $\log_{10}$ transferred titers, $^*P < 0.05$). PID-Post-inoculation day. PCD-Post-challenge day.

with chloroquine, a known inhibitor of TLR9, resulted in complete inhibition of probiotic bacterial DNA induced TGF-β and IL-12 cytokine responses, but had no effect on IL-10 production. These results suggest that bacterial genomic DNA may modulate production of factors associated with IgA responses through a TLR9 dependent manner.

The increased small intestinal IgA antibody responses in probiotic-colonized piglets may also be related to induction of T-cell independent IgA inducing factors.[39,40] To assess probiotics effect on T-cell independent IgA inducing factors such as APRIL and BAFF, ileal MNCS from control Gn piglets were treated with live LGG and Bb12 under in vitro condition. Probiotics treatment had no effect on BAFF expression in MNCs from naive piglets (data not shown). However, treatment of MNCs with live LGG and Bb12 probiotics or HRV antigen resulted in 15 (significant)- and 1.7-fold higher APRIL mRNA levels, respectively, compared to mock treatment (Supplementary F**ig. 1A**). Further, treatment of MNCs with a combination of the live probiotics and HRV antigen resulted in significantly higher APRIL mRNA (27-fold) compared to mock or LGG+Bb12 treatments (Supplementary **Fig. 1A**). This suggests a possible synergistic effect between LGG+Bb12 probiotics and HRV on induction of APRIL in ileum. Probiotic bacterial genomic DNA had no effect on APRIL and BAFF gene expression in MNCs under in vitro conditions (data not shown). Consistent with this in vitro finding, significantly higher levels of APRIL mRNA were also observed in ileal MNCs from Vac+Pro piglets compared to that of Vac piglets (Supplementary **Fig. 1B**). These findings suggest that APRIL may be involved in enhanced mucosal IgA production.

Discussion

Recent studies have shown a role of intestinal commensals in the development of mucosal immunity in neonates. In this study, we determined the specific impact of a combination of probiotics on B cell responses to RV vaccine post-VirHRV challenge. Our study demonstrates that co-colonization of LGG and Bb12 probiotics enhanced small intestinal HRV specific- and total

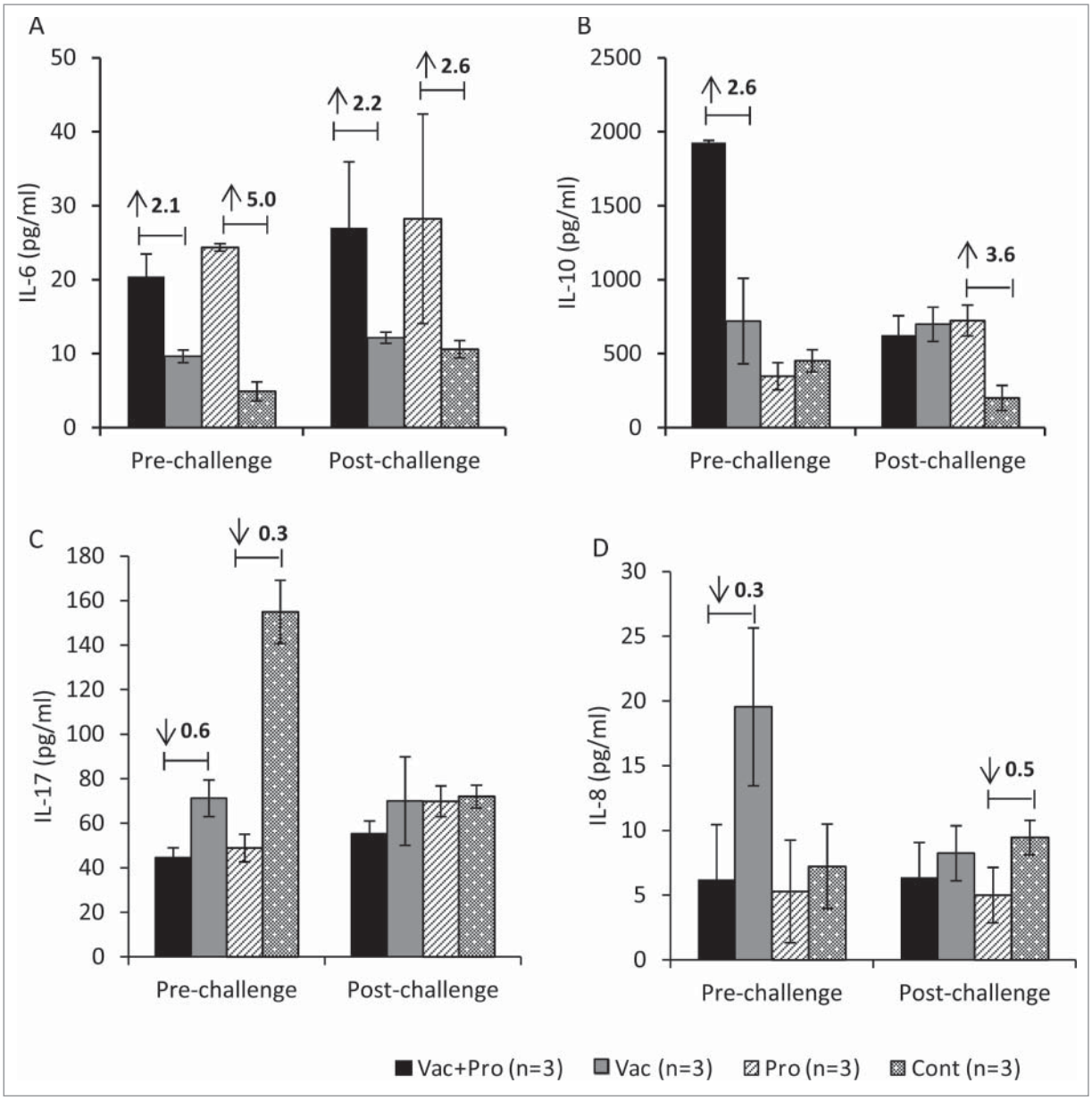

Figure 5. Ileal MNCs from Gn pigs vaccinated with AttHRV with or without LGG + Bb12 probiotics colonization pre- and post-challenge were co-cultured with HRV antigen under in vitro condition to determine cytokine responses. Ileal MNCs were treated with HRV antigen (12 μg/ml) for 48 h at 37°C and culture supernatants were collected to quantify concentrations of IL-6, IL-10, IL-17 and IL-8 (**A–D**) cytokines. Differences in cytokine levels between groups (Vac+Pro vs Vac and Pro vs Cont) were expressed as fold changes (Fold change with values greater than 1 representing increased levels and values less than 1representing decreased levels) as indicated by values at top of bar diagram.

IgA-responses and induced differential effects on systemic IgA- and IgG-antibody responses in Gn piglets.

Co-colonization of LGG+Bb12 probiotics enhanced both HRV specific- and total IgA responses in the small intestine. Although the immunomodulatory effects of either LGG or Bb12 on mucosal antibody responses were assessed in only a few previous studies,[24,25] it is unknown whether these probiotics exert their effects directly on host immunity or act through modulating the gut microbiota. Results from our Gn piglet model system (devoid of microflora) revealed that the observed effects of LGG+Bb12 probiotics on intestinal IgA responses could be mediated by direct modulation of host immune responses by the probiotics.

The potential mechanism(s) for the probiotic-enhanced intestinal IgA HRV antibody- and total IgA-responses or intestinal HRV IgA ASC may be through conditioning the intestinal microenvironment including modulating responses of dendritic cells to mediate increased IgA production. Surface MHC II expression on dendritic cells (DCs) is indicative of dentritic cell maturation[41] and in our study, significantly higher frequencies of

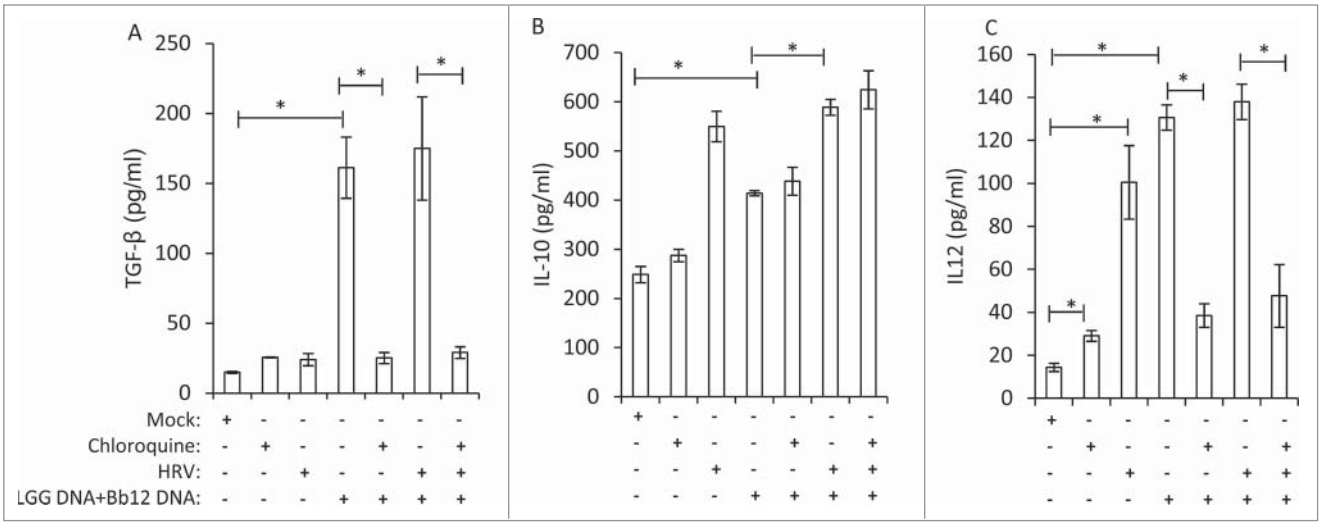

Figure 6. LGG and Bb12 probiotic bacterial DNA induces TGF-β, IL-10 and IL-12 cytokines in ileal MNC. Ileal MNCs from 28 day old control Gn piglets (no prior exposure to any bacteria/virus) were treated with a combination of LGG- (25 μg/ml) and Bb12-genomic bacterial DNA (25 μg/ml) in presence or absence of HRV antigen (12 μg/ml) for 48 h at 37°C, and supernatants were collected for TGF-β, IL-10 and IL-12 determination. When indicated, MNCs were pretreated with chloroquine (10 μM), a known inhibitor of TLR9, for 30 m prior to probiotic bacterial genomic DNA treatment. Results are mean ± SEM (n = 4, One-way ANOVA, $*P < 0.05$).

mature pDCs as well as cDCs were observed in ileum of LGG+Bb12 colonized, vaccinated piglets compared to uncolonized, vaccinated piglets.[30] Further, comparison of maturation status between pDCs and cDCs revealed that nearly all the pDCs have a mature phenotype, but only 10% of cDCs possess such a phenotype. These results suggest that the probiotics indeed enhanced maturation of intestinal DCs and the probiotic induced maturational changes in pDCs may have played a role in enhancing IgA antibody responses in probiotic colonized piglets compared to uncolonized piglets.[34,42]

LGG+Bb12 colonized, vaccinated piglets had significantly higher TLR9 expression and lower TLR4 and TLR2 expression levels in small intestinal MNCs compared to that of uncolonized, vaccinated piglets in small intestine.[30] Thus, probiotics-induced changes in expression of pattern recognition receptors may potentially modulate the IgA responses. Microbe associated molecular patterns such as bacterial nonmethylated CpG motifs can stimulate B cells and enhance antibody responses without BCR recognition.[43] Genomic DNA of both Bifidobacteria and LGG probiotics possess CpG motifs with immunomodulatory effects on B cells.[36,44] These motifs may have played a role in enhancing mucosal IgA production as observed in earlier studies.[45,46] Further, the ability of genomic DNA from LGG and Bb12 to induce cytokines that mediate IgA antibody responses such as TGF-β and IL-10[47] as shown in our study also suggest that these probiotics might possess immunomodulatory effects on IgA inducing factors in the small intestine. In addition, the complete inhibition of probiotic bacterial genomic DNA induced TGF-β response by chloroquine pretreatment in ileal MNCs also suggests possible involvement of TLR9 in induction of TGF-β. Further, colonization of various bifidobacterial strains from infants resulted in increased secretion of IL-10 and TGF-β cytokines in a Gn mouse model.[48] Collectively, these probiotic induced changes in MNCs may be involved in augmenting intestinal IgA antibody responses.

In addition, the increased IgA production may have occurred through either T-cell dependent or T-cell independent processes or a combination of both processes. In duodenum, significantly higher CD4 T cells as well as activated CD4 T cells were observed in Vac+Pro piglets as compared to Vac piglets pre-HRV challenge.[31] The higher frequency of activated CD4 T cells in LGG + Bb12 colonized, vaccinated piglets may act as a source of the cytokines that modulate IgA antibody responses. Others have shown that the cytokines TGF-β,[49] IL-10[47] and IL-6[50] are involved in stimulating intestinal IgA responses. Indeed, our finding of higher IL-6 and IL-10 cytokine responses of ileal MNCs from probiotic colonized, vaccinated piglets supports their potential role in mediating IgA induction in small intestine. The increased CD4 T cells along with higher IgA inducing cytokines such as IL-6 and IL-10 might have induced a favorable gut microenvironment for augmented intestinal IgA HRV antibody responses in vaccinated, probiotic colonized piglets.

T-cell independent IgA responses at mucosal sites also play a role in immunity against RV infection.[51] BAFF and APRIL are secreted mainly by monocytes, dendritic cells, and intestinal epithelial cells[52,53] and involved in generation of T-cell independent IgA responses.[54] The higher APRIL responses in MNCs of Vac+Pro piglets compared to Vac piglets also suggest the possible involvement of those factors in augmenting HRV specific intestinal IgA antibody responses in the probiotic-colonized, vaccinated piglets post-HRV challenge. These results were further confirmed by our in vitro experiment in which live LGG+Bb12

probiotic treatment of ileal MNCs from naive Gn piglets induced significantly higher APRIL responses which were synergistically enhanced by co-treatment with HRV antigen. These findings and our recent observation of translocation of LGG to the mesenteric lymph nodes in LGG monnocolonized gnotobiotic piglets (unpublished) suggest a potential role of the probiotics in enhancing expression of APRIL in intestinal tissues. In addition, a higher percentage of intestinal B cells were IgA^+ in probiotic-colonized piglets, which also indicates potential involvement of APRIL in augmenting intestinal IgA responses. These findings further coincided with the enhanced intestinal HRV IgA antibody responses.

Higher B cell activation in Vac+Pro compared to Vac piglets suggests that LGG+Bb12 probiotics played a role in activation of small intestinal B cells. Previous studies have shown that both genomic DNA of lactobacillus[36] or commensal bacteria[32] can activate B cells and the enhanced activation of B cells in Vac+Pro piglets may be induced by probiotic derived factors such as genomic DNA. Furthermore, a significantly higher frequency of activated B cells was observed post-challenge in the susceptible unvaccinated compared with the protected vaccinated piglets, regardless of probiotic colonization. The more severe rotavirus infection in unvaccinated piglets may have caused the increased activation of B cells as observed in a mouse model.[55] Indeed, the presence of significantly higher IFN-α,[30] a B cell activating factor that is induced during RV infection,[34] coincided with the higher B cell activation that we observed in unvaccinated piglets compared to vaccinated piglets.

Co-colonizaton of LGG and Bb12 probiotics had differential immunomodulatory effects on systemic HRV IgG antibody and total IgG responses compared to IgA responses in the Gn piglet model. Serum HRV IgG antibody titers were lower in the LGG+Bb12 colonized, vaccinated piglets compared to uncolonized, vaccinated piglets at all-time points post-bacterial colonization. Furthermore, more marked significant reductions in HRV specific IgG responses (five-fold lower) were observed at PID14 in comparison to differences in the IgG responses between Vac+Pro and Vac piglets during later time points. These observations are consistent with our earlier finding in which *Lactobacillus acidophilus* colonized, AttHRV vaccinated piglets had lower spleen HRV IgG ASC compared to uncolonized, vaccinated piglets.[56] These findings are also supported by an earlier study in which oral administration of Bifidobacteria to mice immunized with RV resulted in lower serum RV IgG antibody responses but enhanced serum RV specific IgA antibody responses as compared to immunized, non-probiotic colonized animals.[57] Consistent with these findings, a recent study also demonstrated that administration of a probiotic mixture including Lactobacillus and Bifidobacteria decreased antigen specific serum IgG antibody responses in a murine autoimmune disease model.[58] It appears that the Lactobacillus and Bifidobacterium probiotics may have an inherent capacity to induce systemic immune responses that favor systemic IgA- but not IgG-antibody responses. These results were further supported by presence of significantly higher probiotic specific IgA (supplementary **Fig. 2A**) - and lower probiotics specific IgG-responses (supplementary **Fig. 2B**) in serum of Vac+Pro piglets compared to Pro piglets at PID34/PCD7. One potential explanation is that the probiotic modulated systemic cytokine responses may be involved in the lower systemic IgG responses in the Vac+Pro piglets. In our study, serum IL-4 levels were significantly lower in Vac+Pro compared to Vac piglets post-challenge.[31] An earlier study[59] reported that IL-4 inhibits IL-21/CD40L mediated IgA isotype switching but enhances the IgG isotype switching process. Thus, lower systemic IL-4 levels in Vac+Pro piglets compared to Vac piglets might have caused the lower systemic IgG antibody responses. Further, dose dependent effects of IL-4 at lower concentrations on IgG responses (supplementary **Fig. 3A and B**) of RV antigen stimulated splenic B cells from AttHRV vaccinated pigs also suggest the potential role of IL-4 in differential systemic IgG antibody responses. Total IgG responses also followed a similar pattern in which LGG+Bb12 colonized, vaccinated piglets had lower total IgG titers as compared to uncolonized, vaccinated piglets. Lower serum HRV specific- and probiotic specific-IgG antibodies might have contributed to the lower serum total IgG responses in probiotic colonized, vaccinated piglets. Vac+Pro and Vac piglets had comparable serum total IgA titers pre-challenge (PID27/PCD0), but Vac+Pro piglets had higher serum total IgA titers at PID34/PCD7 indicating a potential synergistic interaction between the immunomodulatory effects of probiotics and VirHRV infection.

Experimental evidence in animal models has shown that B cell responses play a critical role in development of long-lasting protective immunity against RV infection.[15,60] Our results suggest that LGG and Bb12 probiotics, which effectively colonized the Gn piglets, beneficially modulated B cell responses to HRV vaccine. One caveat of this study is that gnotobiotic piglets without other confounding microbiota were used to determine the specific beneficial effects of only LGG+Bb12 on rotavirus induced immunity. Whether similar effects will be observed using the selected probiotics in the presence of a complex gut microbiota remains to be determined. Colonization of beneficial probiotics prior to RV vaccination in children may be achieved directly through treatment of children or through maternal supplementation of probiotics for indirect treatment of infants. Schultz and co-workers,[61] reported that maternal supplementation of LGG probiotics resulted in colonization of the LGG in infants for at least 12 months. In addition, maternal supplementation of LGG also promoted a bifidobacteria profile in infants without affecting gut microbiota diversity.[62]

Materials and Methods

Probiotic bacterial strains

The selected probiotic strains, *Lactobacilli rhamnosus GG* strain ATCC 53103 (ATCC, Manassas, VA) and *Bifidobacterium animalis* subsp. *lactis* Bb12 (Christian Hansen Ltd., Horsholm, Denmark) were used to colonize Gn piglets. Growth and enumeration of colony forming units (CFU) in bacterial cultures prior to feeding the probiotics to the piglets was done as described previously.[29]

Experimental design

All studies were approved by The Ohio State University Institutional Animal Care and Use Committee. Cesarean-derived Gn piglets from near-term sows were maintained in sterile isolators as described previously.[63] The Gn piglets were assigned to one of the following 4 groups: Probiotic-colonized and 3XAttHRV Wa vaccinated (Vac+Pro, n = 7); 3XAttHRV Wa vaccinated and uncolonized (Vac, n = 6), unvaccinated and probiotic-colonized (Pro, n = 5), and unvaccinated and uncolonized negative controls (Cont, n = 4). For probiotic colonization, pigs were first inoculated at 3 days of age (post bacterial colonization day, PBCD0) with Bb12 at a dose of 10^5 CFU; subsequently, the Bb12 colonized piglets were inoculated at 5 days of age (PBCD2) with both LGG and Bb12 at a 1:1 ratio, and at a dose of 10^5 CFU of each bacteria per pig. For vaccination, cell culture adapted AttHRV Wa strain (G1P1A[8]) vaccine was given orally at a dose of 5×10^7 fluorescent-forming units (FFU) at 6- (Post inoculation day, PID0), 15- (PID9) and 26- (PID20) days of age. Serum samples were collected to assess HRV antibody responses at PID0, 14, 27 and 34. For VirHRV challenge, piglets were challenged with 10^5FFU VirHRV Wa strain at PID27 (Pre-challenge) and subsets euthanized at PID27/post-challenge day 0 (PCD0) and PID34/PCD7. To measure immune responses in intestinal and systemic lymphoid tissues, mononuclear cells (MNCs) were isolated from blood, spleen, duodenum, and ileum as previously described.[31]

Fecal probiotic counts, clinical signs and virus shedding

The total LGG and Bb12 colonies in rectal swab fluids and tissues and the specific colonization patterns of the probiotic bacteria were determined as described previously.[29,31] VirHRV challenged piglets were examined daily from PCD0 to PCD7 to assess fecal consistency, duration of diarrhea[60] and fecal virus shedding, all of which were determined as described previously.[60]

ELISA, ELISPOT and flow cytometry assays

HRV specific IgA and IgG antibody responses and total IgA and IgG antibody responses were measured by ELISA as described previously.[60,64] LGG and Bb12 specific-IgA and -IgG responses were measured by ELISA as described previously.[29] Enumeration of both isotype-specific HRV antibody secreting cells (ASC) and total immunoglobulin secreting cells (IgSC) were performed by enzyme-linked immunosorbent spot (ELISPOT) assay as previously described.[60] Frequencies of IgA^+ or IgG^+ B lymphocytes were determined by identifying $CD79\beta^+IgA^+$ and $CD79\beta^+IgG^+$ B cell, respectively. Briefly, 1×10^6 cells were stained with monoclonal anti-porcine IgA (Clone K61 1B4, Serotec) or biotinylated anti-porcine IgG (Clone F007–1241, BD Biosciences) antibody for 15 min at 4°C. Subsequently, cells were washed and incubated with anti-mouse IgG1-Allophycocyanin (BD Biosciences, CA) or streptavidin PE-Cy7 (BD Biosciences) secondary antibodies. After washing, stained cells were permeabilized and then stained with porcine cross- reactive anti-mouse CD79β-FITC antibody (Clone AT107-2, Serotec, NC). To determine activated B cells ($CD21^+CD2^-$), MNCs were stained with anti-porcine CD21-PE (Clone BB6–11C9.6, SouthernBiotech, AL) and anti-porcine-CD2 (Clone MSA4, VMRD, WA), followed by anti-mouse IgG2a-SPRD (Cat#1080–13, SouthernBiotech, AL). Subsequently, cells were stained with CD79β-FITC as described previously. Appropriate isotype matched control antibodies were included. Subsequently, 100,000 events were acquired per sample using BD Accuri C6 flow cytometer (BD Biosciences, CA) and data were analyzed using C6 flow sampler software.

In vitro stimulation with probiotics, probiotic bacterial genomic DNA and HRV antigen

Effects of probiotics or probiotics genomic DNA on induction of cytokine- as well as T-cell independent IgA inducing factor-responses were assessed in freshly isolated ileal MNCs from naive Gn piglets (no prior exposure to any bacteria/viruses). Isolated Ileal MNCs (2.5×10^5 cells/well in 48 well cell culture plate) from 28 day old naive Gn piglets were treated with live LGG and Bb12 bacteria at ratio of 10:1 (bacteria : MNCs) in the presence or absence of purified inactivated HRV antigen (12 μg/ml) for 24 h in RPMI containing 8% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 mM HEPES, and antibiotics (E-RPMI). Subsequently, total RNA was isolated from the cells using RNeasy mini kit (Qiagen, MD) to determine changes in expression of B cell activation factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL). Genomic DNA from LGG and Bb12 probiotics was isolated using GenElute Bacterial Genomic DNA Kit (Sigma, MO) to determine probiotic bacterial genomic DNA-induced cytokine responses. Ileal MNCs from control Gn piglets were co-cultured with a combination of LGG (25 μg/ml) - and Bb12 (25 μg/ml)-genomic DNA with or without HRV antigen (12 μg/ml). When indicated, cells were pretreated with chloroquine (10 μM) (InvivoGen, CA), a known inhibitor of TLR9, for 30 min. Culture supernatants were collected for determination of TGF-β, IL-10 and IL-12 cytokines by ELISA as previously described.[31,65]

Cytokine responses of ileal MNCs from Vac+Pro, Vac, Pro and Cont piglets stimulated with HRV antigen under in vitro condition were also determined. Briefly, freshly isolated MNCs were treated with inactivated purified HRV antigen (12 μg/ml) for 48 h at 37°C in E-RPMI. Subsequently, culture supernatants were collected to quantify IL-6, IL-10, IL-17 and IL-8 cytokine concentrations by ELISA as previously described.[31,65]

Quantitative real-time RT-PCR (QPCR)

The MNCs from the ileum of pigs vaccinated with AttHRV with or without LGG+Bb12 probiotic colonization were extracted from euthanized pigs on PID34/PCD7 (Post-HRV challenge). Subsequently, total RNA was isolated from the isolated MNCs using RNeasy Mini kit for QPCR. QPCR was performed for BAFF, APRIL, and β-actin genes using gene-specific primers (**Table 1**). The QPCR was performed using a Quanti-Tect SYBR Green RT-PCR Kit (Qiagen, USA) as instructed by

Table 1. Details of primer sequences used for QPCR experiments

Gene	Forward and reverse primer sequences (5′→3′)	Amplicon length (bp)	Accession number
β-actin	5′- CAGGTCATCACCA TCGGCAACG -3′ 5′- GACAGCACCGTGTTGGCGTAGAGGT -3′	164	DQ845171
APRIL	5′- CAGCCTCATCTCCTTCCTTG -3′ 5′- TTTGCAGCTCTGTTTGTTGG -3′	162	NM_001112690
BAFF	5′- CAGCTCCATTCAAAGCAACA -3′ 5′- CCGTTTCTTTGACCACGATT -3′	203	NM_001097498

the manufacturer. QPCR data were analyzed by the $2^{-\Delta\Delta CT}$ method.[66]

Statistical analysis

Log_{10} transformed isotype-specific ELISA antibody titers were analyzed using one-way ANOVA followed by Duncan's multiple range test. The HRV-specific ASC, total IgSC and frequencies of $CD79\beta^{+}IgA^{+}$ B cells were compared among groups using the Kruskal–Wallis rank sum test. All statistical analyses were performed using SAS program (SAS Institute, NC) or GraphPad Prism version 5 (San Diego, CA). Differences were considered significant at $P < 0.05$. Error bars indicate the standard error of the mean (SEM).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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