Dietary rice bran protects against rotavirus diarrhea and promotes Th1 type immune responses to human rotavirus vaccine in gnotobiotic pigs

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Abstract

Rice bran (RB) contains a distinct stoichiometry of phytochemicals that can promote gut mucosal immune responses against enteric pathogens. Effects of RB on rotavirus diarrhea and immunogenicity of an attenuated human rotavirus (HRV) vaccine were evaluated in gnotobiotic pigs. Four treatment groups were included: RB plus vaccine, vaccine only, RB only, and mock. Pigs in the RB groups were fed with the amount of RB that replaces 10% of pigs’ total daily calories intake from milk starting from 5 days of age until euthanasia. Pigs in the vaccine groups were orally inoculated with two doses of the attenuated HRV vaccine. A subset of pigs from each group was orally challenged with the homologous virulent HRV on post-inoculation day 28. Diarrhea and virus shedding were monitored daily from post-challenge day 0 to 7. RB feeding significantly protected against diarrhea upon virulent HRV challenge and enhanced the protective rate of the vaccine against rotavirus diarrhea. Consistent with the protection, RB significantly increased IFN-γ producing CD4+ and CD8+ T-cell responses in intestinal and systemic lymphoid tissues. Furthermore, RB also increased the number of total IgM and IgA immunoglobulin secreting cells, total serum IgM, IgG and IgA titers and HRV-specific IgA titers in intestinal contents. RB reduced the numbers of intestinal and systemic HRV-specific IgA and IgG antibody secreting cells and reduced serum HRV-specific IgA and IgG antibody titers before challenge. These results demonstrated clear beneficial effects of RB in protection against rotavirus diarrhea and stimulation of non-specific and HRV-specific immune responses, as well as its biased Th1-type adjuvant effect for the vaccine.
Rice Bran (RB), a globally accessible, abundant and underutilized agricultural byproduct, has a distinct stoichiometry of bioactive compounds, phytochemicals, and minerals (1). It has been studied for bioactive functions such as the prevention and treatment of chronic diseases, growth of beneficial intestinal microbes, induction of mucosal and systemic immune responses, and protection against enteric pathogens (2-5). Thus, this agricultural byproduct represents a promising and practical dietary based solution for increasing innate resistance against enteric pathogens that cause diarrhea. In particular, its immune stimulatory functions can be potentially used as a vaccine adjuvant for enteric pathogen infections. Given that RB can support colonization of gut probiotics (e.g. Lactobacilli spp.), enhance mucosal IgA production (2), and significantly reduce the enteric burden of Salmonella infection in mice (5), continued investigation of dietary RB’s mechanisms for protection against viral pathogens that cause significant global morbidity and mortality (e.g. rotavirus) is warranted.

Previous studies have shown the immune-modulatory effects of RB on both innate and adaptive immunity in vitro and in vivo (4). RB oil enhanced T and B lymphocytes proliferation, production of Th1 cytokines (IL-2, IFN-γ and TNF-α) by lymphocytes, and reduced Th2 cytokines (both serum and lymphocyte derived IL-4) as well as the level of serum IgE and IgG1 (3). MGN-3, an arabinoxylan derived from rice bran, also increased levels of Th1 cytokines in human multiple myeloma patients (6). Importantly, γ-Oryzanol significantly promoted the development of antibody responses in rats stimulated with sheep red blood cells (4). Total local (feces) and systemic (serum) IgA levels, and IgA expression on Peyer’s patches B cells, was also
enhanced in mice fed 10% RB diet, suggesting RB promoted both mucosal and systemic B cell development (2). These studies demonstrated the immune-stimulatory effects of RB on multiple components of the immune system.

Given the RB-mediated protection against bacterial pathogens (7-9) and stimulatory effects on both the innate and adaptive immune systems, RB represents a promising natural food product for modulating mucosal immunity and protecting against diarrhea from major enteric pathogens, such as human rotavirus. The gnotobiotic (Gn) pig model of human rotavirus (HRV) infection and diarrhea has been extensively utilized to study HRV infection and vaccination (10-14). In this study, using the well-established neonatal Gn pig model, we aim to: 1) determine whether RB can reduce the susceptibility to infection and diarrhea upon virulent HRV challenge; 2) examine the ability of RB to promote the development of intestinal and systemic T and B cell immune responses and improve the protective efficacy of oral rotavirus vaccine compared to the control diet.

METHODS

Gn pig experimental groups and treatment

Neonatal Gn pigs were derived and maintained in sterile isolators as previously described (15). Four treatment groups were included: RB + AttHRV, AttHRV only, RB only, and mock control. Heat-stabilized, gamma radiated RB, (from the Neptune variety) provided by Anna McClung from the USDA-ARS Rice Research Center (Stuttgart, AK), was added to the Gn pigs’ milk diet (Ultra-high-temperature treated cow-milk), starting at 5 days of age (PPD 5) until the end of experiment. The amounts of RB were calculated to replace 10% of the pigs’ daily caloric intake.
from milk. AttHRV is produced from the 35th passage of Wa strain (G1P1A[8]) HRV in the MA104 cell culture (16). Two oral doses of the AttHRV vaccine were given at approximately 5 x 10^7 florescence forming units (FFU)/dose on PPD 5 and PPD 15. Mock group received neither RB nor AttHRV vaccine. A subset of pigs from each group were orally challenged with the virulent HRV (VirHRV) Wa strain (G1P1A[8]) at a dose of approximately 1 x 10^5 FFU on post first inoculation day (PID) 28. The 50% infectious dose (ID_{50}) of the virulent HRV in neonatal Gn pigs was determined as approximately 1 FFU (17). For measuring antibody titers, blood samples were collected weekly starting from PPD5 and upon euthanasia. Rectal swabs were taken daily for monitoring of virus shedding and diarrhea from post-challenge day (PCD) 0 to 7. Pigs were euthanized on PID 28 or PCD 7 and mononuclear cells (MNCs) from ileum, spleen, and blood were isolated for detection of T and B cell responses using flow cytometry and Enzyme-Linked ImmunoSpot (ELISPOT) assay. All animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University.

Detection of virus shedding by ELISA

Rectal swabs taken daily upon VirHRV challenge were processed by washing two swabs in 8ml Diluent #5 (MEM, 1% Penicillin and Streptomycin, 1% HEPES) and then centrifuged at 2100 rpm for 15 minutes at 4 °C. Supernatants were then aliquoted and stored at -20°C. Virus antigens in the fecal swabs were detected by enzyme-linked immunosorbent assay (ELISA) (18). Rectal swabs from mock treatment Gn pigs were used as negative controls.
Detection of total CD3+CD4+ (Th) cells, CD3+CD8+ (CTL) cells and IFN-γ producing T cell responses by flow cytometry

Frequencies of total and IFN-γ producing CD4+ and CD8+ T cells among CD3+ mononuclear cells in ileum, intraepithelial lymphocytes (IEL), spleen, and blood were determined by intracellular staining and flow cytometry according to a previous publication (19). Data were acquired using a BD FACSaria flow cytometer and data were analyzed using FlowJo 7.22 (Tree Star, Inc.)

Detection of total immunoglobulin secreting cells (IgSC), HRV-specific antibody-secreting cells (ASC), total and HRV-specific serum and intestinal antibody titers

The ELISPOT assay for rotavirus-specific ASC response and data reporting were performed as previously described (16, 20, 21). The ELISPOT assay for measuring the total IgSC response and data reporting followed a previously described protocol (16). To determine the HRV-specific IgA and IgG antibody titers in serum and intestinal contents, isotype-specific ELISA was performed according to the protocol previously described (22, 23). Total IgM, IgA and IgG antibody titers in serum and intestinal contents were assessed by following an established ELISA protocol (24).

Statistical analysis

Kruskal-Wallis rank sum test was used for comparisons of data on virus shedding and diarrhea, Th and CTL cells, IFN-γ+ CD4+ and CD8+ T lymphocytes, total IgSC antibody secreting cells
and HRV-specific ASCs. Fisher's exact test was used for comparisons on the percentages of virus shedding and diarrhea. Total and HRV-specific antibody titers in the serum were analyzed using the ANOVA-general linear model (GLM). Total and HRV-specific antibody titers in the large and small intestinal contents were analyzed using the ANOVA – Turkey test.

**RESULTS**

**RB reduced HRV diarrhea but not virus shedding**

The effects of RB on rotavirus infection and disease were determined by comparing the virus shedding and diarrhea parameters among the four treatment groups: RB + AttHRV; AttHRV only; RB only; and mock control. The results summarized in Table 1 showed that RB only group had significantly lower incidence (100% to 20%), shorter mean duration (5.6 to 0.2 days), and reduced severity of diarrhea (diarrhea score 14.4 to 4.4) compared to the mock control diet group. When compared to the AttHRV group, RB + AttHRV vaccine treated Gn pigs had significantly reduced incidence of diarrhea (67% to 0%), shorter mean duration (4.6 to 0 days), and reduced severity of diarrhea (diarrhea score 9.8 to 4.4). Importantly, RB only group had less diarrhea compared to the group with AttHRV vaccine alone, with reduced incidence (20 vs 67%) of diarrhea, significantly shorter mean duration (0.2 vs 4.6 days) and lower diarrhea scores (4.4 vs 9.8).

No significant difference in virus shedding was observed between RB only group and mock controls. Compared to the AttHRV vaccine group, RB + AttHRV group had increased virus shedding (50 vs 100%), significantly earlier onset (6.0 vs 2.0), and significantly longer mean duration of virus shedding (1.3 vs 3.2 days). In addition, the RB alone group had slightly longer
(not significantly) mean duration of virus shedding (6.2 vs 4.7 days) compared to the mock controls. These data suggest that RB protects against rotavirus induced diarrhea through mechanisms that are independent of affecting rotavirus replication.

Effect of RB on total Th and CTL development in intestinal and systemic lymphoid tissues

The frequency of total Th and CTL lymphocytes among lymphocytes in different tissues on PID 28 were determined. The results are shown in Figure 1. Compared to the Mock control group, RB only group had similar frequencies of total Th and CTL T cells among lymphocytes in both intestinal (ileum and IEL) and systemic (spleen and blood) lymphoid tissues. Similarly, there are no significant differences between AttHRV group and RB + AttHRV group, with the only exception of significantly down-regulated CTL response in IEL of the RB + AttHRV group. These results suggest that RB did not influence the development of total Th and CTL cells.

Rice bran enhanced IFN-γ+ CD4+ and CD8+ T cell responses

Effector T cell responses against rotavirus is an important protective mechanism against infection. The effects of RB on effector T cells was assessed by the frequency of IFN-γ producing CD4+ and CD8+ T cell populations among total CD3+ mononuclear cells in both intestinal tissues (ileum and IEL) and systemic lymphoid tissues (spleen and blood). The results are shown in Figure 2. Compared to the mock control group, RB only group had significantly increased frequencies of IFN-γ+CD4+ T cell populations in ileum, spleen and blood on PID 28.
Compared to the AttHRV vaccine group, RB + AttHRV group had significantly increased frequencies of both IFN-γ+CD4+ and IFN-γ+CD8+ T cell populations in ileum, spleen and blood on PID 28 and PCD 7, except for IFN-γ+CD4+ T cells in ileum and spleen on PCD 7. There were no significant differences in IEL at any time point. These data demonstrate that RB has strong stimulating effects that favor Th1 type immune responses.

**Rice bran promoted the development of intestinal and systemic IgSC**

Total immunoglobulins (Ig) in the intestinal and systemic tissues, particularly intestinal IgA, play a significant role in non-specific mucosal protection against viral infections. The number of IgM, IgA and IgG IgSCs in the ileum, spleen and blood were measured by ELISPOT and compared between RB only group and mock group (Figure 3). Rice bran group alone showed significantly increased numbers of IgM IgSC in ileum and spleen as well as numbers of IgA IgSC in spleen and blood at PID 28. The numbers of IgA IgSC in the ileum and numbers of IgG IgSC in all tissues did not differ significantly between RB only and mock control groups. These data indicated that dietary RB intake can promote the development of intestinal and systemic IgSCs (IgM in ileum and spleen, IgA in spleen and blood).

**Rice bran stimulated the production of total IgM, IgA and IgG in serum**

Total serum IgM, IgA and IgG antibody titers in Gn pigs fed with or without RB were determined using ELISA and the results are shown in Figure 4. On PID 21, RB only pigs had
significantly higher level of IgM and IgA titers compared to the controls. Additionally, RB only pigs had significantly higher levels of serum IgM, IgA and IgG antibody titers than the controls on PID 28. Post HRV challenge, RB only pigs had significant higher level of IgA titer than the controls. In addition, RB + AttHRV pigs had significantly higher levels of IgM and IgA titers on PID 28 and IgM titers on PCD 7 than the AttHRV pigs. These data demonstrate that RB promoted the production of total serum IgM, IgA and IgG antibody titer in both naïve and vaccinated Gn pigs.

Rice bran decreased the intestinal and systemic HRV-specific IgA and IgG ASC responses to AttHRV vaccination but not VirHRV challenge

HRV-specific serum IgA levels as well as numbers of intestinal IgA and IgG ASCs have been shown to be associated with the protection against rotavirus infection and diarrhea (16, 23). HRV-specific ASC responses are shown in Figure 5. Compared to the AttHRV alone group, the RB + AttHRV group has significantly lower numbers of both IgA and IgG ASC in the ileum, spleen and blood on PID 28. On PCD 7, compared to the non-vaccinated RB only and mock groups, both AttHRV and RB + AttHRV groups had significantly higher numbers of HRV-specific IgA and IgG ASC in the ileum. The two vaccinated groups also had significantly higher numbers of IgA ASC in the blood than the mock group. RB only group had significantly higher numbers of IgA ASC in the ileum and blood in comparison to the mock group. Together, these results demonstrated that RB down-regulated virus-specific IgA and IgG effector responses induced by the AttHRV vaccine at PID 28, but not memory B cell responses upon VirHRV challenge.
Rice bran reduced serum HRV-specific IgA and IgG antibody responses to AttHRV

To further confirm the results that RB down-regulated HRV-specific IgA and IgG ASC response at PID 28, HRV-specific serum IgA and IgG antibody titers were determined by ELISA (Figure 6). Consistent with HRV-specific IgA ASC data, serum IgA titer is significantly lower on both PID 21 and PID 28, but with no significant difference on PCD 7 in the RB + AttHRV group in comparison to the AttHRV vaccine group. For both RB + AttHRV and AttHRV groups, HRV-specific serum IgG antibody titers were not significantly different on PID 21, PID 28 and PCD 7, although RB + AttHRV group had significantly higher IgG antibody titer on PID 10. RB only group had significantly lower virus-specific IgG antibody titer than the mock group on PCD 7.

Rice bran increased HRV-specific IgA titer in the intestinal contents

Total immunoglobulins and HRV-specific antibody responses in the small intestinal contents (SIC) and large intestinal contents (LIC) were measured by ELISAs (Figure 7). RB did not significantly change the levels of total immunoglobulins (IgA, IgG and IgM) in the intestinal contents on PID 28 or PCD 7, except for the decreased total IgA titer in LIC on PID 28 compared to the control pigs on PID 28 (Fig. 7A). Important to note, HRV-specific IgA titers in both SIC and LIC of the RB + AttHRV pigs were higher at PID 28 and significantly higher at PCD 7 compared to the AttHRV pigs (Fig. 7B). These data demonstrated that RB can enhance the production of virus-specific IgA antibodies by intestinal memory B cells in the AttHRV-vaccinated pigs after VirHRV challenge, even though the numbers of virus-specific IgA ASC
DISCUSSION

In this study, we examined the effects of RB supplementation on rotavirus infection and diarrhea, the total and virus-specific T and B cell responses, and isotype-specific antibody responses induced by the AttHRV vaccine using neonatal Gn pigs as a model system. We observed that 10% dietary RB supplementation to milk significantly protected against rotavirus diarrhea, but did not reduce rotavirus replication. RB also strongly promoted the development of IFN-γ producing T cells, IgM and IgA producing IgSC, total serum IgM, IgA and IgG antibody and HRV-specific intestinal IgA production, but significantly reduced HRV-specific IgA and IgG ASC in intestinal and systemic lymphoid tissues as well as HRV-specific serum IgA production at PID 28.

Rice bran alone reduced rotavirus diarrhea incidence and severity without reducing rotavirus shedding. Surprisingly, while RB and AttHRV vaccine synergistically and completely protected against rotavirus diarrhea, the protection of AttHRV vaccine against rotavirus shedding was reduced by RB. These results strongly suggest that mechanisms by which RB protects against rotavirus diarrhea is independent of rotavirus infection. The underlying mechanisms for rotavirus-induced diarrhea are currently not completely understood. The pathogenesis of rotavirus induced diarrhea has been reviewed (25, 26). Four distinct but nonexclusive mechanisms have been implicated: 1) Malabsorption due to the destruction of absorptive enterocytes in the villus, caused by rotavirus infection and increased intracellular [Ca$^{2+}$]; 2)
NSP4 enterotoxin mediated increase in membrane permeability and tight junction disruption; 3)
Increased secretion from the crypt cells and intestinal motility via stimulation of enteric nervous
system by rotavirus or NSP4 enterotoxin; and 4) Villus ischemia caused by unidentified
vasoactive substances during rotavirus infection. RB could interfere with each of these four
mechanisms. In fact, extracts from RB have been shown to be effective in reducing diarrhea
through inhibition of the intestinal mucosal Cl⁻ ion secretion by intestinal epithelial cells (27, 28).
This mechanism is likely to have contributed to the protective effects of RB against rotavirus
induced diarrhea in the current study. It is also reported that zinc and enkephalinase inhibitors
attenuate rotavirus-induced diarrhea (26). Certain RB phytochemicals might have functioned as
such inhibitors. Further studies are underway to examine the effects of RB on the intestinal
barrier integrity and permeability during rotavirus infection.

Both effector T and B cell responses play important roles during rotavirus infection and are
associated with the protective efficacy of rotavirus vaccine against rotavirus infection and
diarrhea (19, 23, 29). The significantly increased frequencies of IFN-γ producing CD4⁺ and
CD8⁺ T cell responses in local (ileum) and systemic (spleen and blood) lymphoid tissues at both
PID 28 and PCD 7 suggest that RB promoted the development of effector T cell responses.
However, this effect was not due to the enhanced expansion of total Th and CTL cells, as RB did
not significantly increase their frequencies among lymphocytes in both intestinal and lymphoid
tissues.

Rice bran also significantly enhanced the development of total IgM IgSCs in ileum and spleen
and IgA IgSCs in spleen and blood, and levels of total serum IgM, IgA and IgG antibodies pre-
and postchallenge, as well as rotavirus-specific IgA antibody levels in intestinal contents after
challenge, indicating the stimulatory effect of RB on the development of total and specific B cell
responses to the AttHRV vaccine and VirHRV challenge. Similarly, a previous study showed that the number of peripheral blood lymphocytes was significantly increased in Wistar male rats fed a 10% hemicellulose extracted from RB fiber (RBF) diet for 2 weeks (30). However, the significantly reduced numbers of rotavirus-specific IgA and IgG ASCs in both local and systemic tissues as well as the correspondingly lower levels of rotavirus-specific serum IgA and IgG antibody titers on PID 28 suggest that the immune-stimulatory effect (adjuvanticity) of RB are biased towards Th1 T cell responses before challenge and this effect is antigen-specific. Thus RB functioned as Th1 type immune response “food adjuvant” for the AttHRV vaccine. This observation is consistent with previous studies showing that RB feeding in mice up-regulated Th1 cytokines and down-regulated Th2 and antibody responses. The reduction in rotavirus-specific B cell and serum antibody response at challenge may have contributed to the increased fecal rotavirus shedding in RB + AttHRV treatment group over the AttHRV only treatment group. However, RB did not negatively affect the rotavirus-specific memory B cell responses and enhanced rotavirus-specific intestinal IgA antibody responses at PCD 7, suggesting that RB increased priming of local virus-specific B cells even under the Th1 biased condition before challenge. The molecular mechanisms and kinetics by which RB modulates T and B cell responses warrant further studies.

In summary, results from the current study demonstrated that RB significantly reduced the susceptibility to rotavirus diarrhea without reducing rotavirus shedding upon virulent HRV challenge in Gn pigs compared to the control diet. Furthermore, RB promoted the development of intestinal and systemic IFN-γ producing CD4+ and CD8+ T cell responses, total IgM IgSC in ileum and spleen, total IgA IgSC in spleen and blood, as well as total serum IgM, IgA and IgG antibody production. Additionally, RB increased HRV-specific IgA titers in the intestinal
contents postchallenge. RB alone also significantly increased the virus-specific IgA ASC response postchallenge in ileum and blood. These results have significant clinical implications in the prevention and management of enteric pathogen-induced diarrhea using dietary RB in developing countries. Clinical trials should be conducted before RB is recommended for use alone and in combination with rotavirus vaccines (and other vaccines) to reduce diarrheal diseases and to improve human health.

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Figure Legends

Figure 1. Total Th and CTL responses in control or vaccinated Gn pigs fed with or without RB supplemented diet. MNCs from Gn pigs in each treatment group euthanized on PID 28 were analyzed by flow cytometry. The MNCs were stimulated with semi-purified AttHRV antigen for 17 hrs in vitro. Figure 1(A). Gating strategies for lymphocytes, CD3+CD4+ (Th) and CD3+CD8+ (CTL) cells. Figure 1 (B). Frequencies of CD3+CD4+ (Th) and CD3+CD8+ (CTL) cells among lymphocytes from each tissue were represented by frequencies of CD3+ CD4+ subset (left panel), or CD3+ CD8+ (right panel) T cell subset among lymphocytes. Numbers on the y-axis indicate the percentage of CD3+CD4+ or CD3+CD8+ T cells among lymphocytes in the respective tissues shown on the x-axis. Error bars indicate standard error of the mean. Different capital letters (A, B) indicate significant differences between groups, while shared letters indicate no significant difference (Kruskal-Wallis rank sum test, p < 0.05; n = 3-6).

Figure 2. IFN-γ producing CD4+ and CD8+ T cell responses in control or vaccinated Gn pigs fed with or without RB supplemented diet. MNCs from Gn pigs in each treatment group euthanized on PID 28 (left panel) or PCD 7 (right panel) were analyzed by flow cytometry after the MNCs were stimulated with semi-purified AttHRV antigen for 17 hrs. Frequencies of IFN-γ producing T cells among total CD3+ cells from each tissue were represented by frequencies of IFN-γ+ CD4+ subset (Top panel), or IFN-γ+ CD8+ (bottom panel) T cell subset among CD3+ cells. Numbers on the y-axis indicate the percentage of IFN-γ producing CD4+ or CD8+ T cells among CD3+ cells in the respective tissues shown on the x-axis. Error bars indicate standard error of the mean. Different capital letters (A, B, C) indicate significant differences between
Figure 3. Mean numbers of total IgSCs in Gn pigs fed with or without RB supplemented diet for 28 days. MNCs from Gn pigs in RB only and mock groups euthanized on PID 28 (without AttHRV vaccine and HRV challenge), were analyzed by total IgSC ELISPOT. Numbers on the y-axis indicate the number of total IgM-, IgA-, or IgG- immunoglobulin secreting cells per 5 x 10^5 MNCs in the respective tissues shown on the x-axis. Error bars indicate standard error of the mean. Different capital letters (A, B) indicate significant differences between groups (p < 0.05), while shared letters indicate no significant difference (Kruskal-Wallis rank sum test, p<0.05; n = 3-4).

Figure 4. Total serum IgM, IgA and IgG antibody responses in control or vaccinated Gn pigs fed with or without RB supplemented diet. Serum antibody titers were measured by ELISA. Data on PID 0, 9, 21, 28 and PCD 7 are shown. Different capital letters (A, B, C) indicate significant difference among different treatment groups for the same time point and same isotype while shared letters indicate no significant difference (ANOVA - general linear model [GLM], p<0.05; n = 10-18).

Figure 5. Mean numbers of HRV-specific ASCs in Gn pigs fed with or without RB supplemented diet at PID 28 and PCD 7. MNCs from Gn pigs in each treatment group euthanized on PID 28 (top figure) or PID 35/PCD 7 (bottom figure), were analyzed by HRV-specific ELISPOT. Numbers on the y-axis indicate the number of HRV-specific IgA or IgG
antibody secreting cells per $5 \times 10^5$ MNCs in the respective tissues shown on the x-axis. HRV-specific ASC responses were not detected in any tissue for both RB only and Mock groups on PID 28, therefore they are not presented. Error bars indicate standard error of the mean. Different capital letters (A, B, C) indicate significant differences between groups, while shared letters indicate no significant difference (Kruskal-Wallis rank sum test, $p < 0.05$; $n = 4-7$).

**Figure 6.** HRV-specific IgA and IgG antibody titers in serum of Gn pigs fed with or without RB supplemented diet. Antibody titers were measured by ELISA and are presented as geometric mean titers for each treatment group. Error bars indicate standard error of the mean. Different capital letters (A, B, C) indicate significant difference among different treatment groups for the same time point, while different lower case letters (a, b, c, d, e) indicate significant difference among different time points for the same treatment group. Shared uppercase or lowercase letters indicate no significant difference (ANOVA - general linear model [GLM], $p<0.05$; $n = 10-18$).

**Figure 7.** Total and HRV-specific IgA and IgG antibody titers in small intestinal contents (SIC) and large intestinal contents (LIC) of Gn pigs fed with or without RB supplemented diet. Antibody titers in intestinal contents were measured by ELISA and are presented as geometric mean titers for each treatment group. Error bars indicate standard error of the mean. Different capital letters (A, B) indicate significant difference among treatment groups for the same time point while shared letters indicate no significant difference (ANOVA – Turkey test, $p<0.05$; $n = 3-6$ for PID 28 and $n = 4-12$ for PCD 7).
Pigs with daily fecal scores of ≥2 were considered diarrheic. Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semiliquid; and 3, liquid.

Mean cumulative score calculation included all the pigs in each group. Standard error of the mean. In the groups where some but not all pigs had diarrhea or shedding, the onset of diarrhea or shedding for non-diarrheic/shed pigs were designated as 8 for calculating the mean days to onset.

For days of diarrhea and virus shedding, if no diarrhea or virus shedding until the euthanasia day (PCD7), the duration days were recorded as 0.

Fisher's exact test was used for comparisons. Different letters (A, B, C) indicate significant differences in protection rates among groups (p < 0.05), while shared letters indicate no significant difference.

Kruskal-Wallis rank sum test was used for comparisons. Different letters (A, B, C) indicate significant differences in protection rates among groups (p < 0.05), while shared letters indicate no significant difference.

**TABLE 1. Clinical signs and rotavirus fecal shedding in Gn pigs after VirHRV challenge**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>n</th>
<th>% with diarrhea\textsuperscript{a}</th>
<th>Mean days to onset\textsuperscript{**}</th>
<th>Mean duration (days)\textsuperscript{**}</th>
<th>Mean cumulative scores\textsuperscript{b}</th>
<th>% shedding virus\textsuperscript{b}</th>
<th>Mean days to onset\textsuperscript{**}</th>
<th>Mean duration (days)\textsuperscript{**}</th>
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<tbody>
<tr>
<td>RB+AttHRV</td>
<td>6</td>
<td>0\textsuperscript{c}</td>
<td>8 (0)\textsuperscript{A}</td>
<td>0 (0)\textsuperscript{c}</td>
<td>4.4 (1.2)\textsuperscript{C}</td>
<td>100\textsuperscript{AB}</td>
<td>2.0 (0.5)\textsuperscript{B}</td>
<td>3.2 (0.9)\textsuperscript{B}</td>
</tr>
<tr>
<td>AttHRV only</td>
<td>12</td>
<td>67\textsuperscript{AB}</td>
<td>4.4 (0.8)\textsuperscript{B}</td>
<td>4.6 (0.5)\textsuperscript{B}</td>
<td>9.8 (1.4)\textsuperscript{B}</td>
<td>50\textsuperscript{B}</td>
<td>6.0 (0.7)\textsuperscript{A}</td>
<td>1.3 (0.2)\textsuperscript{C}</td>
</tr>
<tr>
<td>RB only</td>
<td>5</td>
<td>20\textsuperscript{BC}</td>
<td>7.2 (0.8)\textsuperscript{AB}</td>
<td>0.2 (0.2)\textsuperscript{C}</td>
<td>4.4 (1.6)\textsuperscript{C}</td>
<td>100\textsuperscript{AB}</td>
<td>1.6 (0.2)\textsuperscript{B}</td>
<td>6.2 (0.2)\textsuperscript{A}</td>
</tr>
<tr>
<td>Mock</td>
<td>9</td>
<td>100\textsuperscript{A}</td>
<td>1.4 (0.2)\textsuperscript{C}</td>
<td>5.6 (0.3)\textsuperscript{A}</td>
<td>14.4 (1.0)\textsuperscript{A}</td>
<td>100\textsuperscript{A}</td>
<td>2.0 (0.3)\textsuperscript{B}</td>
<td>4.7 (0.7)\textsuperscript{AB}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Pigs with daily fecal scores of ≥2 were considered diarrheic. Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semiliquid; and 3, liquid.

\textsuperscript{b}Mean cumulative score calculation included all the pigs in each group.

\textsuperscript{c}Standard error of the mean. In the groups where some but not all pigs had diarrhea or shedding, the onset of diarrhea or shedding for non-diarrheic/shed pigs were designated as 8 for calculating the mean days to onset.

\textsuperscript{d}For days of diarrhea and virus shedding, if no diarrhea or virus shedding until the euthanasia day (PCD7), the duration days were recorded as 0.
Figure 1. Total Th and CTL responses in control or vaccinated Gm pigs fed with or without R8 supplemented diet.
Figure 2, IFN-γ producing CD4+ and CD8+ T cell responses in control or vaccinated 68 pig fed with or without RB supplemented diet.
Figure A. Mean numbers of total IgG1 in pigs fed with or without RB supplemented diet for 28 days.
Figure 4. Total serum IgM, IgA and IgG antibody responses in control or vaccinated Gn pigs fed with or without RB supplemented diet.
Figure 5. Mean numbers of HRV-specific ASCs in Gp pigs fed with or without RB supplemented diet at PID 28 and PCD 7.
Figure 6. HRV-specific IgG and IgA antibody titers in serum of pigs fed with or without RB supplemented diet.
Figure 7. Total and HRV-specific IgA and IgG antibody titers in small intestinal contents (SIC) and large intestinal contents (LIC) of Gn pigs fed with or without RB supplemented diet.