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Probiotics and colostrum/milk differentially affect neonatal humoral immune responses to oral rotavirus vaccine

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Abstract

Breast milk (colostrum [col]/milk) components and gut commensals play important roles in neonatal immune maturation, establishment of gut homeostasis and immune responses to enteric pathogens and oral vaccines. We investigated the impact of colonization by probiotics, *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium lactis* Bb12 (Bb12) with/without col/milk (mimicking breast/formula fed infants) on B lymphocyte responses to an attenuated (Att) human rotavirus (HRV) Wa strain vaccine in a neonatal gnotobiotic pig model. Col/milk did not affect probiotic colonization in AttHRV vaccinated pigs. However, unvaccinated pigs fed col/milk shed higher numbers of probiotic bacteria in feces than non-col/milk fed colonized controls. In AttHRV vaccinated pigs, col/milk feeding with probiotic treatment resulted in higher mean serum IgA HRV antibody titers and intestinal IgA antibody secreting cell (ASC) numbers compared to col/milk fed, non-colonized vaccinated pigs. In vaccinated pigs without col/milk, probiotic colonization did not affect IgA HRV antibody titers, but serum IgG HRV antibody titers and gut IgG ASC numbers were lower, suggesting that certain probiotics differentially impact HRV vaccine responses. Our findings suggest that col/milk components (soluble mediators) affect initial probiotic colonization, and together, they modulate neonatal antibody responses to oral AttHRV vaccine in complex ways.

Keywords

Probiotics (LGG+Bb12); Colonization; Antibody responses; Rotavirus vaccine; Colostrum/milk; TGFβ

Introduction

Acquisition of commensal microbiota after birth promotes maturation and regulation of the neonatal immune system, and establishment of gut homeostasis [1-3]. This critical stage is

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decisive for imprinting of neonatal immune responses. Initial microbial colonization in infants depends on the mode of delivery and the type of feeding. *Lactobacilli* and *Bifidobacteria* species are common in breast fed infants, in contrast to more diverse flora belonging to *Bacteriodes*, *Atopobium*, *Clostridium*, and *Enterococci* in formula fed infants [4-6]. The lower rate of gastrointestinal infections in breastfed infants compared to formula-fed infants may be attributed, not only to breast milk antibodies, but also to differences in gut microbiota. Breast milk or colostrum/milk (col/milk) promotes colonization by commensals and provides maternal antibodies and various biological soluble mediators such as CD14 (sCD14), cytokines, growth factors, lactoferrin, etc. [7-10]. Recently, we reported that sow col/milk contains large amounts of TGF β (T regulatory) and IL-4 (T helper 2) cytokines, and sCD14, similar to that in human breast milk. Besides acting locally in the gut, these soluble mediators were also transferred to the serum of suckling neonatal pigs [9,10], suggesting that they may influence commensal colonization and immune responses to vaccines and infections. The impact of breast milk and its components on generation of the microenvironment to promote colonization by selected commensals (*Bifidobacteria* and *Lactobacilli*), and their combined effect on subsequent immunologic maturation of the neonatal gut and oral vaccines, is largely undefined.

Rotavirus (RV) is a leading cause of viral diarrhea in infants and children. Currently available RV vaccines are effective against severe RV gastroenteritis in developed countries (>80% efficacy), but for unknown reasons, they show reduced efficacy (~50%) in impoverished countries [11]. *Lactobacilli* and *Bifidobacteria* spp are reported to reduce the severity of RV diarrhea and RV shedding in children, although mechanisms are undefined [12,13]. Colonization by certain probiotics, which were selected based on their ability to reduce infectious diarrhea may also act as adjuvants to enhance the efficacy of HRV vaccines [14].

Piglets resemble human infants in gastrointestinal physiology, anatomy and development of mucosal immune responses [15,16]. The gnotobiotic (Gn) piglets, devoid of microflora and sow col/milk, are a unique animal model to investigate initial interactions between col/milk components and the probiotics that commonly colonize breast fed neonates. These initial interactions imprint neonatal immunity, which may also affect immune responses to oral AttHRV vaccines. For this study, our major objectives were: a) to investigate whether col/milk influences dual *Lactobacilli rhamnosus* GG (LGG) and *Bifidobacterium animalis* subsp. *lactis* (Bb12) colonization, persistence and distribution in the gut; and b) to determine if LGG+Bb12, without sow col/milk (mimick formula fed infants) or in association with col/milk (mimick breastfed infants) enhance antibody responses to an oral AttHRV Wa strain (G1P1A[8]) vaccine that is genotypically similar to the current HRV vaccine (RotaRix, G1P[8]). In addition, this study also highlights the role of probiotics in modulating antibody responses in the presence of passive HRV-specific col/milk antibodies.

Material and methods

Probiotic Strains

The probiotics LGG strain ATCC 53103 (ATCC, Manassas, VA, USA) and Bb12 (Christian Hansen Ltd., Horsholm, Denmark) were used to colonize the Gn pigs. The LGG and Bb12 were propagated overnight at 37° C in anaerobic conditions in Man-Rogosa-Sharpe broth with and without 0.05% cysteine hydrochloride, respectively. The CFU¹ were enumerated as previously described [17].

¹colony forming units

Sow colostrum and milk

Colostrum and milk were collected from RV-field exposed seropositive, non HRV-vaccinated lactating sows and were pooled and centrifuged (1,850g, 30 minutes) to remove fat and cellular fractions. The whey fraction was collected for further use and will be referred to as col/milk supplement for this study. The sow colostrum and milk whey were sterilized by treating with 0.05% β -propiolactone (BPL, Sigma) for 1 h and then agitated at 37 °C for 2 h to break up BPL and make it safe for *in vivo* use. The pooled, treated col/milk samples were retested to verify sterility by culturing in non-selective media under aerobic and anaerobic conditions.

Experimental design

All experimental procedures were approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC protocol number: 2010A0088). Gn piglets were surgically derived as previously described [18] and were divided into two major groups: one group was initially fed (n=16) sterile sow col/milk for the first 6 days of life and the other was fed (n=20) ultra high temperature processed commercial cow milk (Parmalat) at derivation and throughout the study. Sow col/milk fed pigs received sow colostrum for the first two days of life and subsequently sow milk for 4 days, followed by parmalat for the duration of the experiment (Fig 1A). Piglets from each major group were assigned randomly to one of the following four groups: 3XAttHRV vaccinated and probiotic colonized (Vac+Pro, n=5; Vac+Pro+Col/milk, n=4); 3XAttHRV vaccinated only (Vac, n=5; Vac+Col/milk, n=4); probiotic colonized only (Pro, n=5; Pro+Col/milk, n=4); and negative controls (Cont, n=5, Col/milk, n=4). Cell culture adapted AttHRV Wa (propagated in a rhesus monkey kidney cell line, MA 104) was diluted in antibiotic free minimum essential medium (Sigma) and used as vaccine at a dose of 5×10^7 FFU² [19]. Vaccinated pigs were orally inoculated with the first dose at 6 days of age and then received two additional doses at 10 day intervals (post-inoculation day [PID] 10 and PID20). All pigs received 5 ml of 100mM NaHCO₃ before each vaccine/mock dose to reduce gastric acidity. Probiotic groups were sequentially colonized orally at day 3 of age with Bb12 and at day 5 of age with LGG+Bb12 (1:1) at a dosage of 10⁵ CFU/pig/time-point in 0.1% peptone water (Fig 1A.). Pigs were euthanized at PID27 and intestinal tissues (ileum and duodenum), spleen and blood were collected aseptically as previously described [19].

Probiotic shedding/colonization and enumeration

Rectal swabs were collected for measuring probiotic colonization and shedding on post-bacterial colonization days (PBCD) 3, 6, 11, 14, 19 and 25. To assess bacterial colonization of the gastrointestinal tract, sections of small intestinal and large intestinal tissues were collected at euthanasia (PID27) and were rinsed and homogenized. Enumeration of probiotics in rectal swab fluids and gut tissues was performed as previously described [17]. The total LGG and Bb12 colonies in rectal swab fluids and tissues were enumerated collectively as total CFU. However, the specific colonization of LGG and Bb12 was determined by real time qPCR on DNA extracted from representative rectal swab fluids and tissues using species specific primers and probes (LGG; courtesy of Dr. Gloria Solano-Aguilar, USDA, and Bb12 [20]). Probiotic counts in the gut tissues included bacteria in the mucus layer, epithelial surface and in the tissue and will be referred to as mucosa associated bacteria.

²fluorescent focus-forming units

Isolation of mononuclear cells (MNCs) and assessment of B cell responses

The MNCs were isolated from blood, spleen, duodenum and ileum at PID27 as previously described [21,22]. The HRV specific and total immunoglobulin (Ig) A and IgG in serum and intestinal contents were detected as previously described [17,22]. The HRV specific IgA and IgG antibody secreting cells (ASC) were measured as previously described [17,21]. The frequency of B cells was measured by detecting CD21⁺CD3⁻ cells. Briefly, 1×10^6 MNCs were stained with anti-porcine CD3-SpectralRed and CD21-Phycoerythrin antibodies (Southern Biotech, Birmingham, AL) for 15 minutes at 4 °C. Stained cells were washed and samples were acquired and analyzed using Accuri C6 flow cytometer and C6 flow sampler software, respectively.

TGFβ (TGFβ1) and IL4 cytokine ELISA

TGFβ and IL4 cytokine concentrations in BPL treated and untreated colostrum and milk and in the serum of piglets at early time-points were measured by using porcine TGFβ and IL4 specific coating and detection antibody pairs (Biosource) as previously described [23].

Statistical Analysis

Statistical analysis was done by SAS version 9.3 (SAS Institute, Cary, NC USA). Mean probiotic fecal shedding, probiotic tissue colonization, total Ig and RV specific antibodies were log transformed and compared using one-way analysis of variance (ANOVA-general linear model), followed by Duncans multiple range test. Frequencies of CD21⁺CD3⁻lymphocytes, RV specific ASC, and serum TGFβ concentrations among and within groups were compared using Kruskal-Wallis rank sum (non-parametric) test.

Results

Col/milk positively impact fecal shedding of probiotics in unvaccinated pigs

Bacteria colonized Gn pigs effectively. We confirmed colonization of both LGG and Bb12 by real-time qPCR in rectal swab fluids and tissues from representative pigs from each group. Since both species were detected in similar numbers (data not shown), we enumerated only the total CFUs from all samples. Col/milk feeding did not result in immediate beneficial effects on probiotic colonization at early time-points (PBCD3, PBCD6). The bacteria shedding was higher in Pro+Col/milk group at PBCD11, PBCD14 and was significantly higher at PBCD19 and PBCD25 compared to Pro group, suggesting the role of col/milk components in enhancing probiotic colonization at later time-points (fig 1B). However, increased fecal probiotic shedding was not observed in Vac+Pro+Col/milk group compared to the Vac+Pro group at any time throughout the study (fig 1C).

The probiotics colonized the large intestine at higher levels, but the counts were reduced in col/milk fed pigs

At PBCD30/PID27, the probiotic counts ranged from 10^9 - 10^{11} CFU/g of tissue in cecum and colon, 10^6 - 10^8 CFU/g in ileum and jejunum and 10^3 - 10^5 CFU/g in duodenum (fig 2). This pattern of colonization was similar to that observed in human intestines, with higher bacterial load in the large intestine [24]. No significant differences in probiotic counts were observed in any of the three small intestine segments between different groups (fig 2A). Non-col/milk fed pigs had higher bacterial counts in the cecum and colon as compared to col/milk fed pigs at PBCD30/PID27 irrespective of vaccination (fig 2B, 2C). Fecal probiotic shedding did not correlate with intestinal tissue colonization (data not shown).

Col/milk fed pigs had higher serum TGF β concentrations irrespective of probiotic colonization (pre-AttHRV vaccination)

β -propiolactone treated sow col/milk was sterile. The BPL treated colostrum and milk had 1.5-3 fold lower TGF β and undetectable levels of IL4 as compared to untreated colostrum (TGF β 2201 pg/ml, IL4 149 pg/ml) and milk (TGF β 615 pg/ml, IL4 67pg/ml). However, col/milk fed pigs had significantly higher serum TGF β concentrations compared to non-col/milk supplemented pigs before bacterial colonization (PBCD-1/PID-4) and immediately after the second dose of probiotics (PBCD3/PID0) suggesting successful transfer of TGF β from colostrum and milk to pigs (Fig 3). Probiotic colonization did not affect serum TGF β levels in col/milk fed and non-fed pigs (data not shown) at PBCD3/PID0.

Probiotics helped to overcome maternal antibody suppressive effects on IgA HRV antibodies in col/milk fed vaccinated pigs after the 3rd vaccine dose

The BPL treated colostrum and milk had 2-4 fold lower IgA and IgG HRV antibody titers compared to untreated colostrum and milk (data not shown). Nevertheless col/milk fed pigs had significantly higher serum IgA and IgG HRV antibody titers compared to non-col/milk fed pigs at PID0 suggesting effective passive transfer of Ig into serum (fig 4).

i) Antibody responses in unvaccinated control groups—The non-col/milk fed and unvaccinated pigs had undetectable IgA and IgG HRV antibody titers. The HRV IgA antibody titers in col/milk fed unvaccinated pigs decreased with age and were undetectable at PID27 (Fig 4A), whereas HRV IgG antibodies decreased, but persisted at PID27 (fig 4C), confirming a longer half-life for serum IgG compared to IgA HRV antibodies.

ii) Antibody responses in vaccinated groups—The Vac+Pro+Col/milk and Vac+Col/milk groups had 12 fold and 3 fold lower serum IgA HRV antibody titers at PID14 compared to Vac+Pro and Vac groups, respectively, suggesting early inhibitory influences of maternal antibodies through two vaccine doses (fig 4B). However, after three vaccine doses (PID27), probiotic colonization in Vac+Pro+Col/milk pigs resulted in significantly higher mean IgA HRV antibody titers (2.5 fold) as compared to Vac+Col/milk pigs and antibody titers were similar to those of the Vac and Vac+Pro groups (fig 4B). Probiotic colonization did not affect IgA HRV antibody titers in non-col/milk fed vaccinated pigs at PID14/PID27.

Interestingly, probiotic colonized, non-col/milk fed vaccinated pigs had significantly lower serum IgG HRV antibody titers at PID14 (12 fold) and lower at PID27 (4 fold) as compared to non-probiotic colonized, non-col/milk fed vaccinated pigs (fig 4D). Probiotic colonization did not alter serum IgG HRV antibody titers in the sow col/milk fed vaccinated pigs at PID14 or PID27 (fig. 4D), contrary to the increase observed in IgA HRV antibody titers at PID27 in this group (fig 4B).

iii) Intestinal antibody responses—Mean intestinal IgA HRV antibody titers as proportion of total intestinal IgA titers were higher in col/milk fed pigs compared to non-col/milk fed pigs at PID27, irrespective of probiotic colonization, suggesting beneficial effects of col/milk feeding (Table 1). Pro+Col/milk and Col/milk pigs had significantly higher intestinal IgA HRV antibody titers compared to undetectable levels in Pro and Cont pigs at PID27, respectively, reflecting the passive persisting maternal HRV IgA antibodies (Table 1).

Probiotics + col/milk increased intestinal IgA HRV ASC

IgA and IgG HRV ASC were detected in ileum (induction/ effector site) and duodenum (effector site) at PID27. Vac+Pro+Col/milk group had significantly higher numbers of IgA

HRV ASC in ileum (2.5 fold) and higher numbers in duodenum (2 fold) compared to Vac +Col/milk group, suggesting immune enhancing effects of probiotics with col/milk components (fig 5A), which coincided with higher IgA HRV antibodies observed in the former group (fig 4B). Probiotic colonization in vaccinated pigs without col/milk feeding did not enhance intestinal IgA HRV ASC numbers.

Overall, col/milk fed vaccinated pigs $\pm$ probiotics, had lower duodenal and ileal IgG ASC compared to counterpart non-col/milk fed vaccinated pigs, suggesting suppressive effects of maternal antibodies on intestinal B cells (fig 5B). Frequencies of ileal IgG HRV ASC were significantly lower in Vac+Pro group vs Vac group, which coincided with the lower serum IgG HRV antibody titers (fig 4D) in the former group.

Discussion

Few studies have investigated the impact of selected beneficial probiotics on responses to oral vaccines in neonates, especially in the context of col/milk feeding. We examined how LGG+Bb12 colonization alone or with col/milk (to mimic breast fed infants), can affect development of B cell responses to an oral AttHRV vaccine in a relevant Gn pig model.

Supplementation of col/milk and AttHRV vaccination affected probiotic colonization. Supplementation of col/milk (containing TGF β) increased fecal probiotic shedding from PBCD11 through the study duration suggesting that milk containing regulatory cytokines and other soluble factors (glycans etc.) can promote establishment and extended colonization by probiotics (LGG+Bb12) [25-27]. Breast milk is a major source of TGF β for neonates when intrinsic production is limited [25]. TGF β promotes intestinal immune responses including class-switch to IgA isotype, induction of regulatory T lymphocytes, attenuation of pro-inflammatory responses and it reduces immune mediated and allergic conditions [25,28,29]. The high TGF β levels like we observed may allow early maturation of regulatory immune responses and suppression of anti-microbial responses, contributing to higher probiotic colonization. Although not examined, porcine col/milk like human milk contains oligosaccharide lacto-N-neotetraose, which promotes the growth of *Bifidobacterium* [30] and may contribute to our observed higher fecal bacteria counts. Unexpectedly, an increase in probiotic fecal shedding was not observed in col/milk fed vaccinated pigs, possibly due to AttHRV vaccine related reduction in serum TGF β and increase in pro-inflammatory and Th1 cytokines (IL6 and IL12), resulting in conditions less favorable for commensal colonization [23].

Interestingly, lower counts of probiotics were detected in cecum/colon of col/milk fed pigs, irrespective of vaccination, suggesting a differential impact of col/milk on fecal bacterial shedding vs mucosal adherence. Maternal antibodies in sow col/milk to bacterial components including peptidoglycan (detected by ELISA, data not shown) may prevent mucosal adhesion of probiotics and result in lower mucosal associated counts. Similarly, previous studies of suckling Gn mice have suggested sequestration of commensals in the lumen by maternal secretory antibodies [31,32].

We observed that supplementation of col/milk and probiotics affected antibody responses to AttHRV in complex interactions. Serum and intestinal IgA HRV antibodies and ASC are correlates of protection against HRV diarrhea in pigs and humans [16,33]. Combined probiotic colonization and col/milk supplementation in vaccinated pigs enhanced serum IgA HRV antibody titers and intestinal IgA HRV ASC, which was not observed Vac+Pro pigs suggesting a complex interaction between probiotics and col/milk components. Col/milk containing HRV antibodies transiently suppressed serum IgA antibody responses after two vaccine doses irrespective of probiotic colonization, but this effect was ameliorated in Vac

+Pro+Col/milk pigs after the three dose vaccine regimen. Thus colonization with LGG+Bb12 in breast fed vaccinated infants (with preexisting col/milk HRV antibodies) may overcome at least the suppressive effects of maternal antibodies on IgA antibody responses. Although, TGF β in colostrum was positively correlated with serum IgA antibody responses in infants [28,34], this effect may have been masked in our studies due to the antigen-specific suppressive effect of maternal antibodies to HRV. We speculate that the interaction of LGG+Bb12 and col/milk components (TGF β , lactadherin, lactoferrin, etc) may have resulted in higher frequency and activation of antigen presenting cells resulting in higher T cell help for IgA responses (Saif et al, unpublished) [35,36]. Similar to our study, Isolauri et al. [37] showed enhanced HRV IgA responses in LGG fed infants of unknown milk feeding status after oral immunization with live oral rotavirus vaccine. In contrast, supplementation of *Bifidobacterium breve strain Yakult* (BBG-01) in breast fed children resulted in no significant difference in vibriocidal antibody responders following oral cholera vaccination [38]. Moreover, cholera toxin B subunit specific IgA antibody responders were lower in the BBG-01 group and this effect was more pronounced in young compared to older children. The quantity of maternal antibodies received in utero (humans) or through colostrum (pigs) and the duration of breast milk feeding are critical factors in determining active antibody responses of neonates to oral vaccines. A recent study of breastfed infants showed higher enhancing effects of supplementation of *Lactobacillus paracasei strain F19* on anti-diphtheria antibodies in infants breastfed for <6 months as compared to those breastfed >6 months [39], suggesting complex interactions between probiotics, col/milk and host immune responses.

Probiotics alone did not enhance HRV IgA antibody responses in non-col/milk supplemented pigs, suggesting lack of adjuvancy of LGG+Bb12 for primary IgA responses under the conditions tested. Similarly Perez et al. [40] have shown a lack of adjuvant effect of probiotics in enhancement of tetanus and pneumococcal IgA and IgG antibodies in children after parenteral immunizations. In contrast, use of *Bifidobacterium spp* in formula fed infants have shown enhancement of poliovirus and rotavirus IgA antibodies [14,41]. Probiotic effects vary with strain, dose, vaccine type and route, level of maternal antibodies, duration of breast feeding, age and other factors [37-42], and further studies are needed to elucidate reasons for inconsistent results.

Probiotic colonization resulted in significantly lower serum HRV IgG antibody titers and IgG HRV ASC in non-col/milk fed vaccinated pigs. This suggests that LGG+Bb12 may enhance gut barrier integrity in multiple ways, similar to other probiotics [12,42]. This would reduce systemic translocation and exposure to AttHRV and reduce IgG HRV ASC and antibody responses. However, this hypothesis has not been investigated directly in this study. Feeding LGG+Bb12 in formula fed infants may be still advantageous, not only by directly enhancing IgA HRV antibodies, but also by reducing systemic exposure.

β -propiolactone treatment of col/milk reduced levels of soluble biological mediators (antibodies, cytokines etc.), potentially decreasing overall effects on immune responses in piglets. Also, lower cross-reactive/protective HRV Wa specific antibodies in normal sow col/milk and restricted col/milk feeding of piglets may have reduced the impact of the suppressive effects of maternal antibodies in our study. In future studies, we will investigate whether the adjuvant effects of probiotics observed in our study are protective against virulent HRV challenge in col/milk fed pigs and affect memory B cell responses to HRV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Col/milk supplementation enhanced probiotic fecal shedding in unvaccinated animals.
- Col/milk fed pigs had suppressed IgA HRV antibody responses after 2nd vaccine dose.
- Probiotics ameliorated IgA HRV responses in Pro+Vac+Col/milk pigs after 3rd vaccine dose.
- Probiotic colonization in Vac+Pro pigs reduced IgG HRV antibody responses.

Fig. 1. Schematic for experimental design and fecal shedding of probiotics in different groups
 (A) Schematic for probiotic and col/milk feeding, administration of vaccines and collection of blood samples. (B) and (C) Mean number of probiotic (LGG+Bb12) CFU/ml in feces of pigs vaccinated with AttHRV (Vac) with or without LGG+Bb12 (Pro) and/or colostrum/milk (Col/milk) at various time-points. Data shows mean values $\pm$ standard error of the mean. *: significant difference between Pro+col/milk vs Pro groups was determined by one-way ANOVA followed by Duncans multiple range test on $\log_{10}$ transformed data. Arrows indicate vaccine time-points (Fig. B)

Fig. 2. Probiotic colonization in the small and large intestines

Mean number of probiotic (LGG+Bb12) CFU/g in (A) small intestine, (B) cecum and (C) Colon of pigs vaccinated with AttHRV (Vac) with or without LGG+Bb12 (Pro) and/or colostrum/milk (Col/milk) at PBCD30/PID27. A): Data shows mean values $\pm$ standard error of the mean. B) and C): Bars represent mean values with each dot indicating individual animals. Different letters indicate statistically significant difference (one-way ANOVA followed by Duncan's multiple range test on $\log_{10}$ transformed data) among the 4 groups.

Fig. 3. Serum TGF β concentrations at early time-points

Serum TGF β concentrations (pg/ml) in sow col/milk fed and non-fed pigs as determined by ELISA at PID-4/PBCD-1 and PID0/PBCD3. Bars indicate means $\pm$ standard error of the mean and each dot represents an individual animal. The p value is derived by non-parametric Kruskal-wallis rank sum test.

Fig. 4. Serum IgA and IgG HRV antibody titers in different groups

Geometric mean titers of serum IgA (A,B) and IgG (C,D) HRV antibodies in pigs vaccinated with AttHRV with or without LGG+Bb12 (Pro) and/or colostrum/milk (col/milk) as determined by ELISA at indicated time-points. The numbers of animals for each treatment group were 4-5. Data shows mean values $\pm$ standard error of the mean. *: significant difference between indicated groups (one-way ANOVA followed by Duncan's multiple range t test on log₁₀ transformed titers). Arrows indicate vaccine time-points.

Fig. 5. HRV specific IgA and IgG ASC in ileum and duodenum

HRV specific (A) IgA and (B) IgG ASC/5x10⁵ MNCs in pigs vaccinated with AttHRV (Vac) with or without LGG+Bb12 (Pro) and/or colostrum/milk (Col/milk) in ileum and duodenum as determined by ELISPOT at PID 27. The numbers of animals for each treatment group were 4-5. Data shows mean values + standard error of the mean. Significant differences between groups are indicated with p values determined by non-parametric Kruskal-wallis rank sum test.

Table 1

Geometric mean titers (GMT) of HRV IgA antibodies and intestinal total IgA titers and their ratios in the vaccinated and control groups at PID27.

Groups	Number of pigs	IgA HRV antibody GMT $\pm$ SEM ^a	Total IgA GMT $\pm$ SEM ^a	Ratio (HRV IgA titers/total IgA titers) $\pm$ SEM ^a
Vac+Pro+Col/milk	4	512 $\pm$ 221	6,888 $\pm$ 2896	0.08 $\pm$ 0.02
Vac+Col/milk	4	861 $\pm$ 367	11,585 $\pm$ 3072	0.11 $\pm$ 0.05
Vac+Pro	5	256 $\pm$ 190	9,410 $\pm$ 3009	0.03 $\pm$ 0.01
Vac	5	512 $\pm$ 188	16,384 $\pm$ 0	0.04 $\pm$ 0.01
Pro+Col/milk	4	8 $\pm$ 2 [*]	4,096 $\pm$ 3405	-
Col/milk	4	11.3 $\pm$ 6 [*]	4,096 $\pm$ 886	-
Pro	5	2 ^b	4,096 $\pm$ 2718	-
Cont	5	2 ^a	1,176 $\pm$ 647 [#]	-

^aSEM: Standard error of the means

Statistical significant difference between groups derived by one-way ANOVA followed by Duncan's multiple range test on log₁₀ transformed titers

^{*} significant difference for intestinal IgA HRV antibodies compared to negative control and probiotic treated only groups.

[#] significant difference for intestinal total IgA compared to all vaccinated groups irrespective of probiotic colonization and/or col/milk feeding.

^b actual titers <4 but for statistical purpose titer value of 2 was used for all the pigs in Pro and Cont groups