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Bifidobacterium animalis subspecies lactis modulates the local immune response and glucose uptake in the small intestine of juvenile pigs infected with the parasitic nematode *Ascaris suum*

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ABSTRACT

An evaluation of a localized intestinal allergic type-2 response concomitant with consumption of probiotic bacteria is not well documented. This study investigated the effect of feeding probiotic *Bifidobacterium animalis subspecies lactis (Bb12)* or a placebo in weaned pigs that were also inoculated with *Ascaris suum (A. suum)* eggs to induce a strong Th2-dependent allergic type 2 immune response. Sections of jejunal mucosa were mounted in Ussing chambers to determine changes in permeability and glucose absorption, intestine and liver samples were collected for analysis of type-2 related gene expression, jejunum examined histologically, and sera and intestinal fluid were assayed for parasite antigen specific antibody. The prototypical parasite-induced secretory response to histamine and reduced absorption of glucose in the jejunum were attenuated by feeding *Bb12* without a change in mucosal resistance. Parasite antigen-specific IgA response in the serum and IgG1 and IgG2 response in the ileal fluid were significantly increased in *A. suum*-infected pigs treated with *Bb12* compared to infected pigs given the placebo. *Ascaris suum*-induced eosinophilia in the small intestinal mucosa was inhibited by *Bb12* treatment without affecting the normal expulsion of *A. suum* 4th stage larvae (L4) or the morphometry of the intestine. Expression of genes associated with Th1/Th2 cells, Treg cells, mast cells, and physiological function in the intestine were modulated in *A. suum* infected-pigs treated with *Bb12*. These results suggested that *Bb12* can alter local immune responses and improve intestinal function during a nematode infection by reducing components of a strong allergenic type-2 response in the pig without compromising normal parasite expulsion.

Introduction

Two major functions of the intestinal epithelium are to provide a selective barrier to luminal contents and to transport water, nutrients, and electrolytes.[1-3] The intestinal epithelium is also the interface between luminal contents and the mucosal immune system. It functions not only to reduce responses to food antigens and commensal bacteria, but to activate host defenses against pathogens.[4] Alterations in epithelial barrier function, therefore, can play a significant role in regulating gastrointestinal disease.[5]

Infection with gastrointestinal parasites affects the physiological and immunological responses of the host. Helminth infection induces a hyper-contractility of intestinal smooth muscle, inhibition of intestinal glucose absorption, and changes in intestinal secretion that are IL-4 receptor-dependent and STAT6-inducible.[6] There is growing interest in the use of probiotics to ameliorate the negative effects of infection and allergic disease.[7] Probiotics have been used to treat a variety of gastrointestinal (GI) ailments including inflammatory bowel disease (IBD), irritable bowel syndrome, viral enteritis, and antibiotic-associated diarrhea.[8-11] Although little is known definitively about their mechanism of action, probiotics seem to have protective and anti-inflammatory effects on the intestinal mucosa. Proposed mechanisms include the production of ammonia, hydrogen peroxide, and bacteriocins that inhibit the growth of pathogenic bacteria,

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competition for bacterial adhesion sites on intestinal epithelia, alterations of composition and function of the gut microbiome, and an adjuvant-type stimulation of the immune system against pathogenic organisms.[12-16] In the present study, we investigated the effect of *Bifidobacterium animalis subspecies lactis (strain Bb12)*, a human-derived probiotic used extensively in dairy products,[17,18] on immune activation and intestinal function of young pigs exposed to migrating *A. suum* larvae.[19] Our results showed that *Bb12* modulated the liver and intestinal immune response and intestinal physiology without compromising the normal expulsion of the worms. The parasite-induced reduction in intestinal glucose absorption was ameliorated, the tissue eosinophilic response was reduced, and serum and intestinal antibody responses to the worm were enhanced by feeding *Bb12*.

Results

Quantitative detection of Bifidobacterium animalis subspecies lactis (Bb12) in the intestine

Bb12-treated pigs born to *Bb12*-treated sows (T/T) had significantly higher copies per gram (cpg) of the *Bifidobacterium animalis subspecies lactis tuf* gene[20] in proximal colon contents of *Ascaris suum (A. suum)*-infected T/T ($1.8 \pm 0.9 \times 10^5$ cpg) compared to non-

infected control placebo-treated pigs born to placebo-treated sows (C/C) ($0.07 \pm 0.07 \times 10^4$ cpg; $p < 0.05$) or *A. suum*-infected C/C ($0.006 \pm 0.004 \times 10^4$ cpg; $p<0.05$) with no difference when compared to non-infected T/T pigs ($1.2 \pm 0.9 \times 10^5$ cpg) (Figure 1A).

In the second experiment, T/T pigs had higher cpg in fecal samples collected before *A. suum* infection at six weeks of age ($0.5 \pm 0.1 \times 10^6$ versus $0.2 \pm 0.01 \times 10^6$ cpg; $p<0.05$) and at 17 days post-inoculation with *A. suum* eggs at nine weeks of age ($2.1 \pm 0.5 \times 10^6$ versus $0.2 \pm 0.01 \times 10^6$ cpg; $p<0.05$) with a non significant change in cpg when weaned at 3 week of age ($2.56 \pm 1.5 \times 10^6$ cpg vs $0.2 \pm 0.01 \times 10^6$ cpg) (Figure 1B).

In the second study, there was also an increase in *Bifidobacterium animalis subspecies lactis tuf* gene cpg in the proximal colon contents of *A. suum* infected *Bb12*-treated pigs (T/T) (7.3×10^5 cpg) compared to *A. suum*-infected placebo-treated pigs (C/C)) ($1.4 \pm 0.1 \times 10^5$ cpg) ($p<0.05$) at the time of necropsy (Figure 1C).

Parasite recovery

Ascaris suum L4 are resident in the proximal small intestine at 10 days post-inoculation (p.i.), and are normally expelled distally from day 17 through day 21
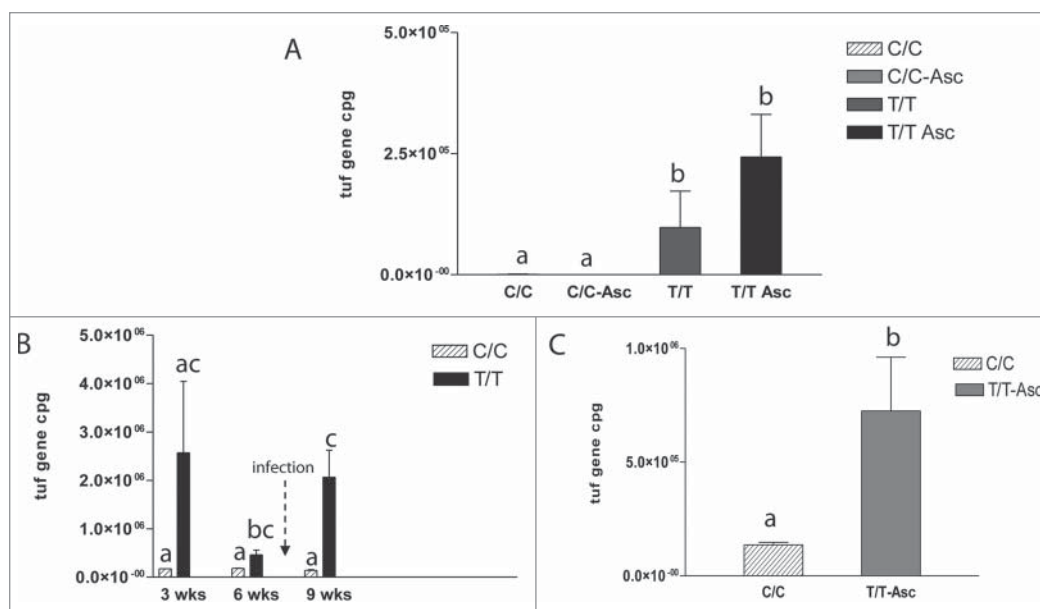

Figure 1. Abundance of *Bifidobacterium lactis* in feces and proximal colon contents. Bacterial abundance was determined by detection of the *Bifidobacterium animalis subspecies lactis* species-specific *tuf*-gene by real-time PCR. Data is expressed as mean ± SEM (n = 6-8 per treatment) of *tuf* gene copies per gram (cpg) in feces or intestinal contents. Proximal colon samples from first experiment were collected 21 days p.i. at 2.5 months of age (A). Fecal samples from a second experiment were collected at weaning (3 weeks), pre-infection (6 weeks) and 3 weeks post-inoculation with *Ascaris suum* (9 weeks) (B). Proximal colon contents from the second experiment were collected at 9 weeks (C). Different letters denote differences among treatments after ANOVA (*p* < 0.05).
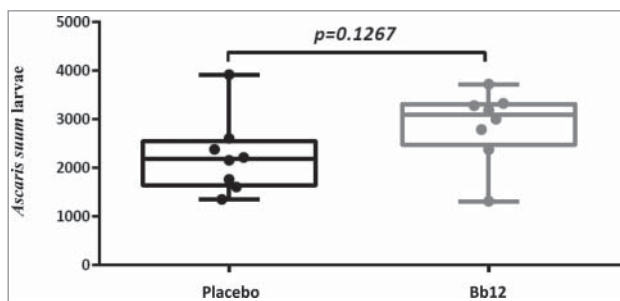
Figure 2. Detection of *Ascaris suum* fourth-stage larvae (L4). *Ascaris suum* L4 were counted 17 days post inoculation (p.i.) with 2×10^4 infective eggs in the small intestine. Bar represent mean average counts per treatment group from the entire intestine (n = 7 per treatment).

p.i., as part of the normal protective response to infection. This pattern was consistent among all *A. suum*-infected pigs regardless of treatment with *Bb12* or placebo. In the first experiment, three of eight pigs from the T/T and three of six pigs from the C/C treatment groups had <30 L4 in the entire small intestine, and the remaining pigs had the predominant larval burden shifted to the distal two sections of the intestine; the
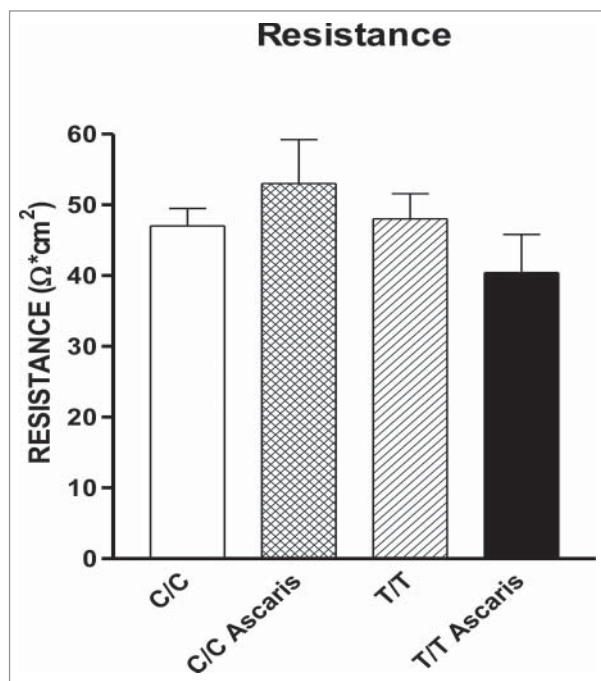

Figure 3. Ussing chamber measurement of intestinal permeability. Segments of muscle-free jejunum mucosa were mounted in Ussing chambers to measure changes in tissue resistance (an index of epithelial permeability) in *A. suum*-infected pigs after continuous treatment with probiotic bacteria *Bb12*. Values are means ± SE for resistance measures expressed as change in Ω/cm2 which is inversely correlated to intestinal permeability. Bars represent average resistance values ± SE (n = 6 per treatment group).

total number of L4 were not significantly different between the two groups (data not shown). In the second experiment, there was no significant difference in the number of L4 detected in the entire small intestine of T/T (2875 ± 746) versus C/C (2249 ± 793) treated pigs at 17 days p.i. (Figure 2).

Intestinal function

There was no significant change in resistance in muscle-free mucosa isolated from the jejunum of pigs from any treatment group (Figure 3) in both experiments, which suggested that mucosal resistance was not impaired by parasite infection or feeding *Bb12*. Sections of muscle-free mucosa from the jejunum of pigs infected with *A. suum*, however, showed an increased basal I_{sc} representing the net Cl^- ion flux in response to secretagogues like histamine ($p<0.05$) (Figure 4A), but not to PGE_2 (Figure 4B). The prototypical *A. suum*-induced pro-secretory response to histamine was attenuated significantly ($P<0.05$) by treatment with *Bb12* (Figure 4A), while the responses to PGE_2 remained unchanged (Figure 4B). Infection with *A. suum* elevated mucosal responses to 5-HT that persisted in pigs fed either placebo or *Bb12* (Figure 4C). Jejunal mucosa from pigs infected with *A. suum* had a characteristic decrease in the absorption of glucose that was attenuated in pigs also fed *Bb12* but not placebo (Figure 5).

Interaction between Bb12 and A. suum at the mRNA level in the jejunum

A selected gene array was evaluated to test the hypothesis that the *Bb12*-modulated genes that regulate a local type-2 response and glucose absorption in the jejunum.. Forty-three genes were selected to represent categories of relevant cytokines, cytokine receptors, transcription factors, chemokines, mast cell and goblet cell markers, intestinal tight junction, and physiological markers. The jejunum of pigs treated with *Bb12* showed no significant change in gene expression compared to placebo-treated pigs except for a <2-fold decrease in expression of *IL4* (Figure 6). Infection with *A. suum* significantly increased ($P<0.05$) expression of the high affinity Fc receptor of IgE (*FCER1A*), Resistin-like molecule *β* (*RETNLB*), and increased expression of *IL-9, IL-13*, and mast cell tryptase (*TPSAB1*) ($P<0.1$). There was a significantly decreased expression of eosinophil chemotactic
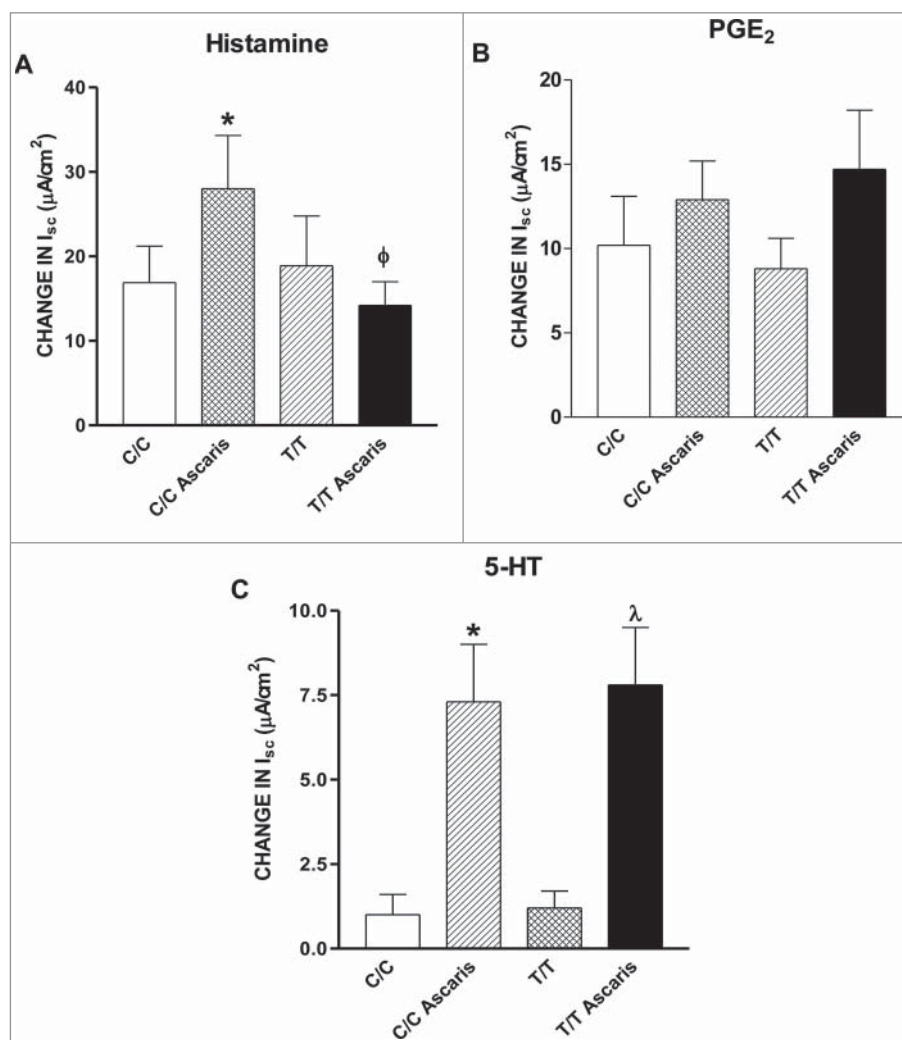
Figure 4. Mucosal epithelial response to secretagoges. Segments of muscle-free jejunum mucosa were mounted in Ussing chambers to measure concentration-dependent changes in I_{sc} in response to Histamine (A) PGE2 (B) or 5-hydroxytryptamine 5-HT (C) in pigs infected with *A. suum* after continuous daily feeding with *Bb12*. Values are means ± SE for repeated experiments, *, $P < 0.05$ vs C/C control pigs; Φ, $P < 0.05$ vs C/C –*A. suum*-infected pigs; λ, $P < 0.05$ vs T/T *Bb12*-treated pigs.

protein *CCL11*, and the inducible gene for cyclooxygenase-2, Cox-2 (*PTGS2*) (P<0.1) compared to control non-infected pigs (Figure 6). The interaction between *Bb12* treatment and infection with *A. suum* showed a significant (P <0.05) up-regulation in *RETNLB, IL-13, FCER1A, IGHE,* and *IL25*(*IL-17E*).There was a significant (P<0.05) decrease in expression of the signature Th1-associated gene *IFNG*, as well as *IL13RA1, IL10, MUC5AC, TLR9, PAR-1* and Treg-derived *SOCS3* (Figure 6). Mast cell-associated markers *CMA1* and *IL3*, and the gene for the tight junction protein, *TJP1*, and *PTGS2*, also were significantly (P<0.05) down-regulated in *A. suum*-infected pigs fed *Bb12* (Figure 6).

Interaction between probiotic and A. suum at the mRNA level in the colon, mesenteric lymph nodes (MLN) and liver

Gene expression was measured in the proximal colon mucosa where high numbers of *Bb12* were detected. Significant down-regulation of gene expression in the proximal colon mucosa was only detected for Forkhead box P3 (*FOXP3*) in pigs infected with *A. suum*, and in *RETNLB* and *IL3* in pigs treated with *Bb12* (data not shown). The interaction between infection and *Bb12* significantly decreased (P<0.05) the expression of *TBX21, FOXP3, CMA, IL3, SOCS3, IL25,* and *TJP1* while only *IGHE* expression was significantly increased (data not shown) Changes in gene
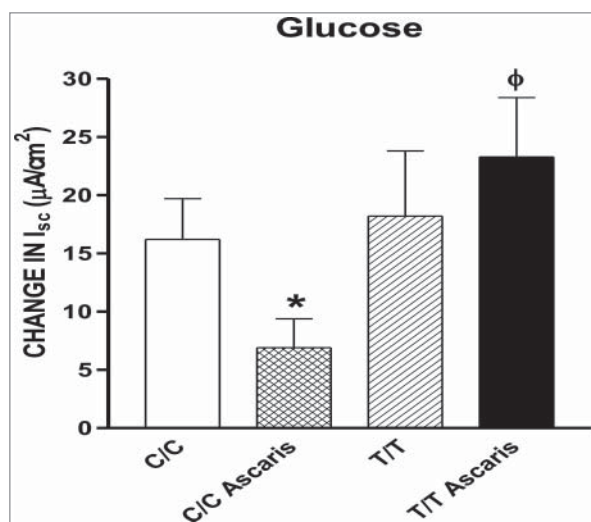
Figure 5. Mucosal epithelial response to glucose. Segments of muscle-free jejunal mucosa were mounted in Ussing chambers to measure concentration-dependent changes in I_{sc} in response to glucose in *A. suum*-infected pigs after continuous daily feeding with *Bb12*. Values are means $\pm$ SE for repeated experiments, *, $P < 0.05$ vs control pigs; Φ, $P < 0.05$ vs C/C-*A. suum*-infected pigs.

expression in liver between *Bb12* and placebo-treated pigs were compared at 17 days p.i. (Table 1). There was a 2.9-fold reduction in *MRC1*, a pattern recognition receptor, a 5.7-fold reduction in *IL25*, a 9.6-fold reduction in *STAT6*, a 1.8-fold reduction in *IL4*, and a 2.2-fold reduction in *IL1B*. No major change in gene expression was detected in the MLN of pigs from the various treatment groups (data not shown).

Antibody titers to A. suum and Bb12 antigens

Parasite antigen-specific antibody responses in the serum and intestinal ileal fluid of *A. suum*-infected and *Bb12*-treated pigs were measured 21 days p.i. to determine changes in acquired immunity. Infection with *A. suum* significantly (P>0.05) increased serum IgG1, and IgM anti-*Ascaris* L3/L4 antibodies compared to non-infected controls in both *Bb12* and placebo treated pigs (p<0.05), while the levels of IgA anti-*Ascaris* L3/L4 antibodies were increased in *A. suum*-infected pigs treated with *Bb12* (T/T infected) compared to *A. suum*-infected pigs given placebo (C/C-infected) (Figure 7). In addition, there was a significant increase in IgG1 and IgG2 anti-*Ascaris* L3/L4 antibodies (P<0.05) in the ileum fluid of *A. suum*-infected pigs treated with *Bb12* (T/T-infected) compared to the other groups. (Figure 8). IgM and IgA anti-*Ascaris* L3/L4 antibodies in the ileum fluid were not significantly affected by feeding *Bb12* (Figure 8).

Histological and morphometric analysis

Morphometric measurements were taken from sections of the jejunum of pigs 21 days p.i. to evaluate inflammation and cellular infiltration. There was no significant difference in the average villus height and crypt depth from five representative fields among the treatment groups (data not shown). There was a significant increase, however, in the number of eosinophils in *A. suum*-infected pigs fed the placebo (6.2 $\pm$ 1.6) compared to non-infected control pigs fed the placebo (1.6 $\pm$ 0.3) that was inhibited in infected pigs fed *Bb12* (2.5 $\pm$ 0.3; p<0.05) (Table 2). Goblet cell numbers were not significantly changed, and mast cells were not detected.

4. Discussion

Clinical studies suggest that some probiotic bacteria can promote health and prevent disease;[1,8,21,22] however the mechanisms underlying these beneficial effects are not well understood. In this study, the *in vivo* effect of *Bifidobacterium animalis subspecies lactis* (*Bb12*) on intestinal function and immunity against a parasitic nematode was evaluated. *Ascaris suum* is a persistent and common problem in swine raised worldwide, and the related species in humans, *A. lumbricoides* (large roundworm) infects an estimated 804 million people.[23] Although infections are common in countries with poor sanitation, ascariasis exhibit a cosmopolitan distribution with sporadic cases also described in developed countries[24] where pigs can be a source of infection.[25] Examination of the immunological and physiological responses to intestinal helminths also has been used to study local and systemic allergic disease.[26,27]

The analysis of muscle-free segments of jejunum mucosa from *Bb12*-treated pigs showed no change in mucosal resistance or in net Cl$^-$ ion flux in response to PGE2, histamine, 5-hydroxytryptamine (5-HT), acetylcholine, and glucose compared to placebo-treated pigs indicating that the intestinal epithelium is functionally quiescent in pigs fed *Bb12*. Likewise, mucosal resistance, and secretion in response to PGE$_2$, remained unchanged in *A. suum*-infected pigs at 21 days p.i., but the secretory response to histamine and 5-HT were significantly increased. The enhanced mucosal responsiveness to these secretagogues is consistent with the mucosal mast cell hyperplasia that follows infection with *A. suum in vivo*,[28] and the
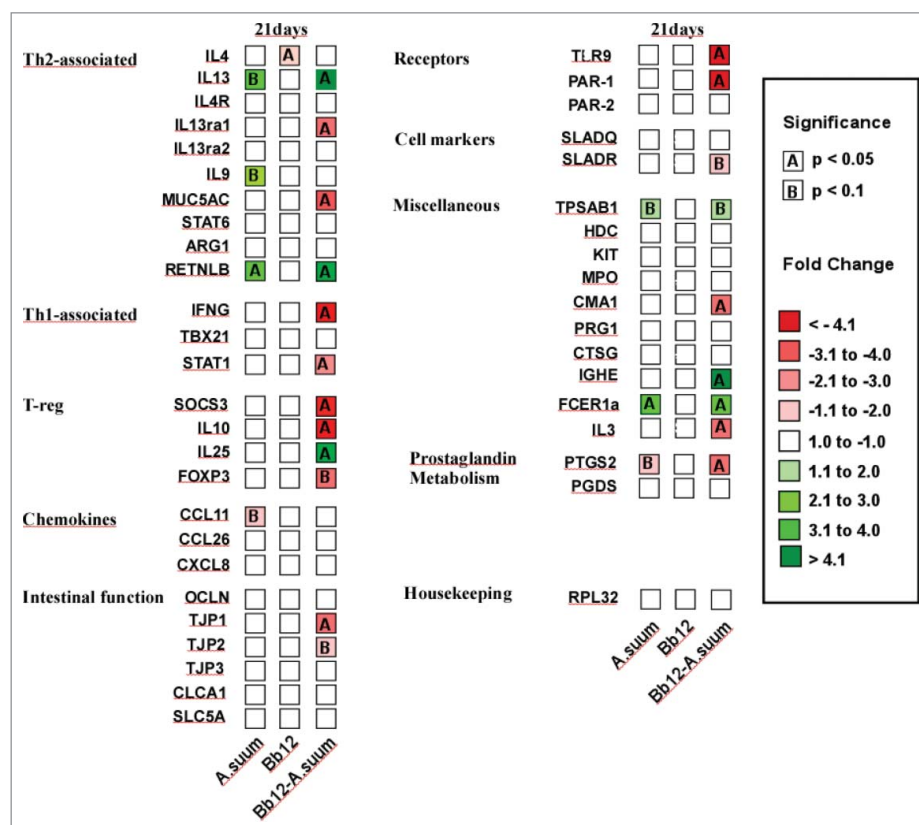
Figure 6. Gene expression in jejunum. Sections of jejunum from the small intestine of *A. suum*-infected pigs were collected 21 days p.i. All samples taken were processed and stored at $-70°$C. RNA and cDNA were prepared from each sample and expression of the indicated genes was analyzed using quantitative real-time PCR. Fluorescence signals measured during amplification were processed after amplification and were considered positive if the fluorescence intensity was 20-fold greater that the standard deviation of the baseline fluorescence. This level was the C_t value. Gene expression was normalized to a constant amount of RNA and cDNA amplified relative to the housekeeping gene LBP32. A statistical analysis was performed by using Statview 5.0 for Macintosh. Color-coded squares denote fold changes in gene expression; columns represent from left to right *A. suum*-infected pigs vs control pigs; *Bb12*-treated pigs vs control pigs; and *A. suum*-infected and *Bb12*-treated pigs vs control pigs, respectively. Statistical significance is indicated by A ($P \leq 0.05$) and B ($P < 0.1$).

functional changes and parasite antigen-induced release of histamine from mast cells isolated from the intestine of *A. suum*-infected pigs.[29] In the current study, however, a significant increase in mast cell numbers was not found by histological examination of the jejunum of *A. suum*-infected pigs. Jejunal tissues were fixed in buffered-formalin with the specific objective to evaluate potential morphometric changes due to exposure to *Bb12* or the interaction with *A. suum* infection. Metachromatic staining for pig mast cells, however, is facilitated by acidic fixation in Carnoy's and low pH staining with toluidine blue to preserve intracytoplasmic granule structure, which was not used in this study. In addition, young confinement-reared pigs have few detectable mast cells in the intestines compared to pigs raised on dirt or after chronic exposure to parasitic infection.[28]

Nevertheless, the increase in basal I_{sc} in intestinal mucosa from *A. suum*-infected pigs in response to histamine *in vitro* is comparable to mast cell-dependent increases in fluid in the intestinal lumen that facilitate

Table 1. Type2-associated gene expression in liver of *A. suum*-infected pigs.

	Placebo	Bb12	Fold change relative to Placebo treatment
MRC1	6.21 ± 0.34^{1a}	7.72 ± 0.37^b	−2.9
GATA3	8.70 ± 0.18	8.96 ± 0.18	0.8
IL25	11.29 ± 0.36^a	13.81 ± 0.48^b	−5.7
FCER1A	6.67 ± 0.24	6.69 ± 0.24	0.9
IL13	13.36 ± 0.54	13.02 ± 0.37	1.3
STAT6	4.97 ± 0.32^a	8.23 ± 0.80^b	−9.6
CCL26	4.08 ± 0.88	2.77 ± 0.53	2.5
IL4	12.89 ± 0.24^a	13.75 ± 0.22^b	−0.5
IL1B	10.23 ± 0.30^a	11.38 ± 0.23^b	−2.2

[1]Data represent the mean $\pm$ SE of Δ Ct for associated Th2 signature genes. Means with different superscript letters are different with $p < 0.05$.
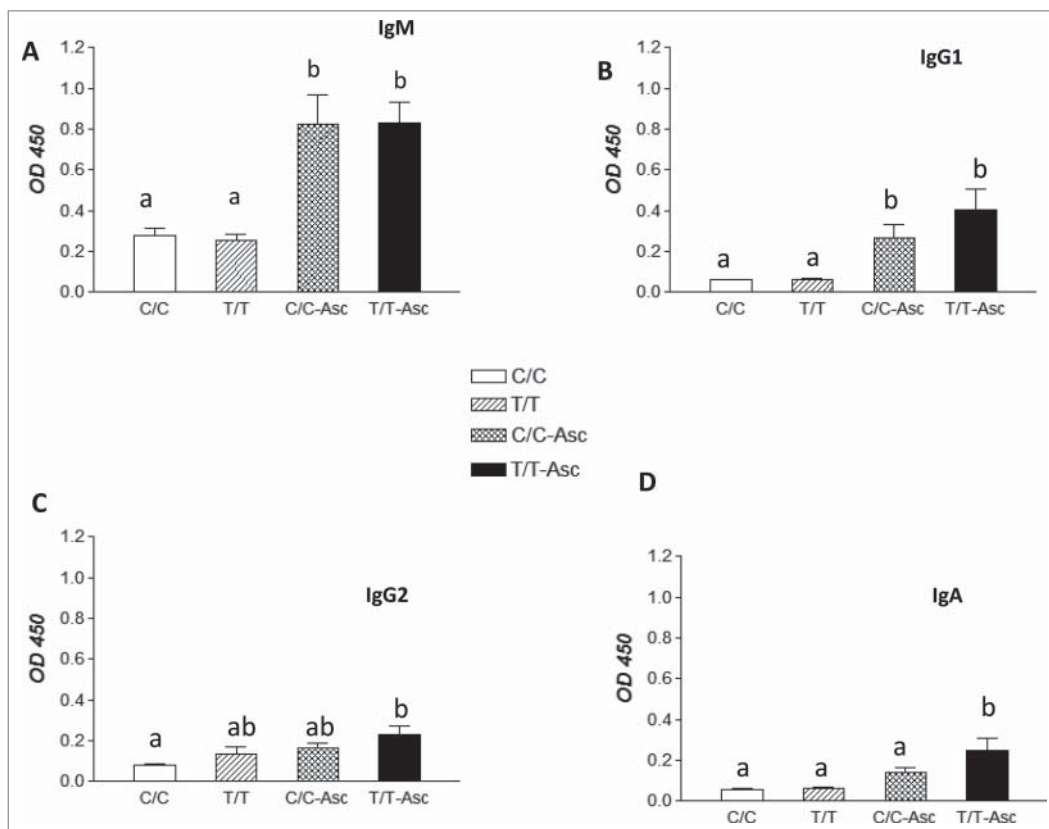
Figure 7. Anti-*Ascaris* L3/L4 antibodies in sera of pigs 21 days post inoculation (p.i.) with infective *A. suum* eggs. Immunolon plates were coated with *A. suum* L3/L4 antigen extract. OD_{450} readings are means ± SE. Different letters denote differences among treatments after ANOVA($p < 0.05$) in (A) IgM, (B) IgG1, (C) IgG2, and (D) IgA.

worm expulsion during the period of self-cure in mice,[30] and may be a more sensitive indicator of functional mast cell activity in the pig intestine.[29] Concomitant treatment of *A. suum*-infected pigs with *Bb12* attenuated the hyper-secretory response to histamine (Figure 4) and the reduced sodium (Na)-linked glucose transport that contributes to parasite-induced edema in the intestinal lumen (Figure 5).[31] These changes suggest that *Bb12* can modulate intestinal function without affecting host protection since parasite clearance from the proximal to distal small intestine was not significantly changed.

It is generally accepted that nematodes like *A. suum* are immunogenic and several studies have demonstrated high serum levels of *A. suum* antigen-specific antibodies after infection.[32-34] In this study, there was a significant parasite-induced increase in serum IgM and IgG1 anti-*Ascaris* L3/L4 antibodies at 21 days p.i. that was not affected by feeding *Bb12*, while *Bb12* slightly enhanced IgA anti-*Ascaris* L3/L4 antibodies. The IgG1 and IgG2 anti-*Ascaris* L3/L4 antibody responses detected in the ileal fluid of *A. suum*-infected pigs were marginally but significantly

increased in pigs fed *Bb12*. Probiotics have been shown to enhance humoral immunity to viral infection[35,36] and the capacity to increase immune responses to vaccine antigens.[37,38] Specific antibody responses to *Bb12* antigen were observed only in ileal fluid of *Bb12*-treated pigs infected with *A. suum* (data not shown), suggesting a synergism that may be due to the enhanced polyclonal expansion of B cells commonly observed during parasitic infection or effects on antigen access and processing in the intestinal mucosa.

Changes in gene expression in the intestinal mucosa observed after infection with *A. suum* were characteristic of a type-2 cytokine response with significant up-regulation of *IL9, IL13*, mast cell-associated activation markers such as *FCER1A* and *TPSAB1*, and goblet cell-specific *RETNLB* (P<0.05) (Figure 6). These changes reflect the stereotypical response to intestinal helminths observed in pigs infected with *A. suum*[31,39] and in mice infected with several different gastrointestinal nematode parasites.[40] In the current study, all pigs born to *Bb12*-treated sows received oral *Bb12* from birth and had high numbers of copies of
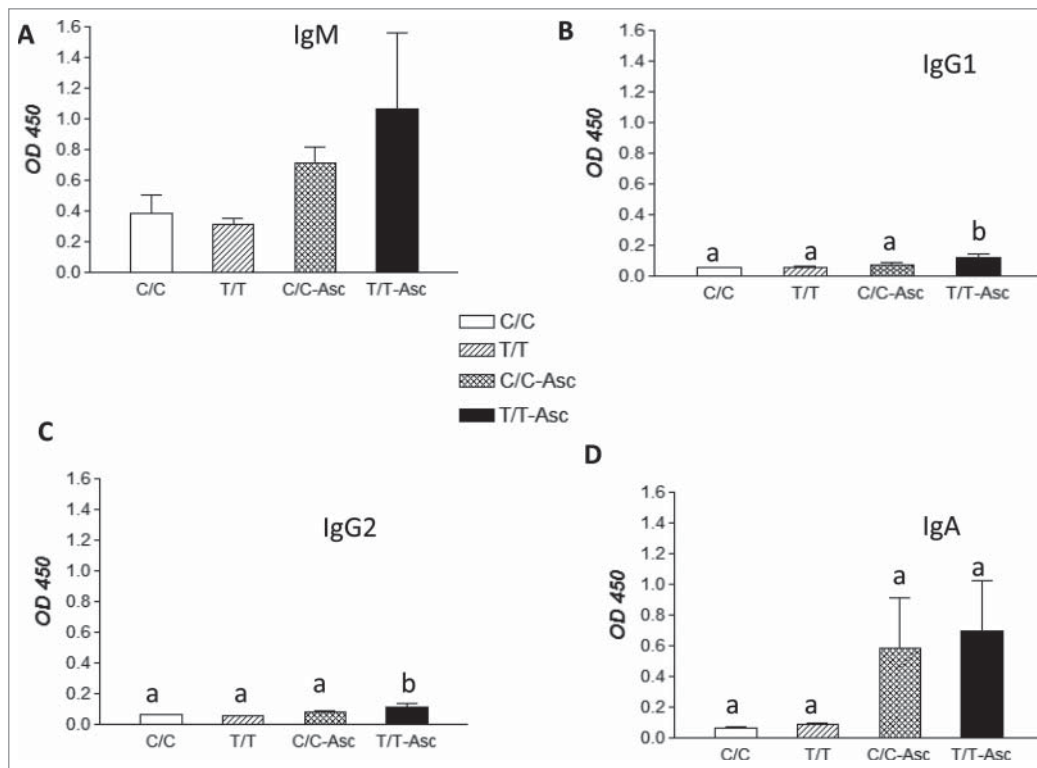
Figure 8. Anti-*Ascaris* L3/L4 antibodies in ileum fluid of pigs 21 days post inoculation (p.i.) with infective *A. suum* eggs. Immunolon plates were coated with *A. suum* L3/L4 antigen. OD$_{450}$ readings are means $\pm$ SE, different letters denote differences among treatments after ANOVA ($p < 0.05$) in (A) IgM, (B) IgG1, (C) IgG2, and (D) IgA.

the *Bb12 tuf* gene detected by PCR in the intestinal contents after six and nine weeks of continuous feeding; infection with *A. suum* did not affect *Bb12* copy number in the feces or proximal colon contents after infection. Treatment of pigs with *Bb12* induced a significant down-regulation of *IL3, TBX21, and RETNLB* expression in tissue from the proximal colon, and reduced *IL4* gene expression in jejunum (Figure 6); no changes were observed in the MLN of *Bb12*-treated pigs except for reduced *IGHE* gene expression (data not shown). These data indicated that *Bb12* alone exerts a relatively modest localized immune modulating effect on intestinal mucosa of treated pigs. In contrast, *Bb12*-treated pigs infected with *A. suum* showed

Table 2. Mean values for eosinophils and goblet cell distribution in jejunum lamina propria.

Treatment group	Goblet cell	Eosinophil
C/C control	13.6 $\pm$ 1.2	1.6 $\pm$ 0.3[b]
C/C infected	17.9 $\pm$ 1.5	6.2 $\pm$ 1.6[a]
T/T control	14.6 $\pm$ 1.8	2.6 $\pm$ 0.4[b]
T/T infected	15.7 $\pm$ 1.4	2.5 $\pm$ 0.3[b]

Treatment groups: C/C (*Ascaris*) infected, T/T control, and T/T (*Ascaris*) infected are compared to C/C control. Statistical significance is indicated by[a] ($P \leq 0.05$).

a significant alteration in the expression of several genes associated with a type-2 response to *A. suum* (Figure 6). Up-regulation of *IL13* was maintained with a concomitant down-regulation of the *IL13RA1* chain that is the functional component of the IL-4 receptor complex for IL-13 binding. This finding is consistent with that reported in pigs infected with *Trichuris suis*[41] but suggests that feeding *Bb12* can modulate IL-13 signaling capacity. The expression of *IL25* and *RETNLB*, two genes associated with the regulation of type 2 cytokine-dependent immunity against parasites, were significantly up-regulated ($p<0.05$) in the jejunum of *A. suum*-infected pigs fed *Bb12*. *RETNLB*, characteristically expressed by goblet cells in the intestine, has been associated with protection against helminths with maximal secretion of RELM beta protein correlated with enhanced Th2 cytokine expression and worm expulsion.[42-45] *IL-25* also has been identified as a critical mediator of type 2 immunity since it is required for regulation of inflammation in gastrointestinal tract.[43,46,47] There also was a significant decrease in *SOCS3* expression ($P<0.05$), a major negative feedback regulator of signal transducer, that affects production of the immune regulatory cytokines

TGFβ1 and IL-10 through modulation of *STAT3*[48] and regulation of IL-17 secreting cells.[49] A significant down-regulation in *IL10* gene expression in the jejunum of *A. suum*-infected pigs fed *Bb12* indicated a reduced IL-10 dependent response to infection with *A. suum*.[50] It should be noted, however, that changes in these regulatory genes could represent a *Bb12*-induced palliative mechanism that reduced local *A. suum* induced-eosinophilia in the intestine with no significant effect on parasite expulsion (Table 2). Additional studies that include repetitive and longer exposure to infection, and the effects on memory responses and associated pathology, would be required to test this hypothesis.

Reducing *IFN-γ*-dependent responses to non-pathogenic bacteria while mounting an appropriate Th1-mediated response to pathogenic bacteria is key to maintenance of physiological homeostasis.[51] The down-regulation of *IFNγ* and *IL10* mRNA expression in *Bb12*-treated pigs infected with *A. suum* could represent a beneficial effect of probiotics on intestinal mucosa perturbed by nematode infection. Certain probiotic bacteria reduce pro-inflammatory *IFN-γ* production independent of *IL10*.[51,52] The significant down-regulation of *TLR9* in the jejunum of *A. suum*-infected pigs exposed to *Bb12* may also limit bacterial-induced inflammation of the intestinal mucosa.[53]

Protease-activated receptor-1 (PAR-1, *F2R*) expression is activated by proteases in a variety of tissues including the gastrointestinal tract.[54] Helminth-induced activation of PAR-1 is dependent on IL-13-activated STAT6 signaling pathway independent of IL4.[55] Activation of PAR-1 induces Cl$^-$ secretion by intestinal epithelial cells through cyclooxygenase-dependent pathways.[56,57] Although the mechanism is unknown, immune regulation of PAR-1 and COX2 activity or expression in intestinal mucosa represents novel therapeutic targets given that many reactive allergens and helminths express protease moieties that enhance PAR-1 activation. The significant down-regulation observed in gene expression for both -*F2R* (5-fold) and *PTGS2* (2-fold) in pigs infected with *A. suum* and fed *Bb12* indicates a potentially novel effect of probiotic treatment on intestinal inflammation associated with barrier dysfunction,[57,58] such as is observed in inflammatory bowel disease and celiac disease.

Cytokines directly affect epithelial functions including barrier stability[59-61] and electrolyte transport across the intestinal mucosa.[6,30,62] Various stimuli including cytokines, allergens, and bacterial products enhance mucosal permeability by affecting tight junction (TJ) integrity.[59,63] Infection of pigs with *A. suum* or treatment with *Bb12* had no effect on epithelial barrier function (resistance) or Cl$^-$ secretory responses to acetylcholine and prostaglandin E2. *Bb12* treatment, however, abrogated the histamine-induced Cl$^-$ secretory response to *A. suum*. In addition, infection with *A. suum* or treatment with *Bb12* did not change expression for the Cl$^-$ channel gene *CLCA1* and paracellular permeability genes *TJP2, TJP3,* and *OCLN*. There was, however, a 2-fold down-regulation of *TJP1* in the jejunal mucosa of *Bb12*-treated pigs infected with *A. suum*.

Glucose is the single most abundant actively transported nutrient in human diets. It is transported across the apical or luminal surface via Na-linked glucose transporter SGLT1 (*SLC5A1*), and the basolateral membrane through the facilitated glucose exchanger GLUT2 (*SLC2A2*).[64] Trans-epithelial ion transport results in the generation of a negative potential difference (PD) due the active absorption of Na$^+$ ions. The current that is necessary to bring the PD continuously to zero voltage is called the short circuit current (I_{sc}) and is the real time measurement of the net electrogenic transport across an epithelium.[65] Pigs infected with *A. suum* and treated with *Bb12* had a reduction in I_{sc}, indicating a decreased Na-linked transport of glucose. Gene expression for the luminal surface transporter SGLT1 *(SLC5A1)* did not increase in these pigs suggesting that other mechanisms may be responsible for the reversal of the normal intestinal response to parasite infection. In rodent models of parasite infection, epithelial glucose absorption has been shown to be regulated by macrophages without affecting its expression in the intestine.[66]

While changes in gene expression related to both pro- and anti-inflammatory pathways were relatively minor in pigs treated with *Bb12*, the effects of feeding *Bb12* were more pronounced in pigs following infection with *A. suum*. This pattern is similar to that observed during infection with *Salmonella* or *E. coli* in animals treated with probiotics[67,13,14] suggesting that probiotic efficacy is best observed in tissues exposed to an inflammatory stimulus.

We have demonstrated that *Bb12* regulates an efficient type-2-dependent response to parasite infection in the intestine by reducing local

intestinal eosinophilic infiltration, the histamine-induced Cl^- secretory response, and blocking the inhibitory effects of infection on glucose absorption, while enhancing acquired antibody responses to infection. These data suggest that feeding *Bb12* can ameliorate dysregulated type-2 diseases such as allergy and chronic inflammation, and can enhance systemic and local antibody production to certain infections and, by extension, to appropriate immunization strategies.

Material and methods

Animals: All pigs used in these studies were cross-bred Yorkshire X Poland-China pigs (males and females) born and maintained at the Beltsville Agricultural Research Center swine facility under management conditions approved by the Beltsville Area Animal Care and Use Committee. Six sows were bred by artificial insemination after hormonal synchronization. Pregnant sows were treated with oral administration of either *Bb12* probiotic or placebo (maltodextrin vehicle without *Bb12*) starting the last third of gestation, during nursing, and until weaning of the piglets at three weeks after birth. Three sows received a daily oral dose of 2.2 g of freeze-dried *Bb12* (10 billion CFU/g) provided by Christian Hansen, Denmark, as a 100% pure lyophilized product dissolved in 10 mL of sterile PBS, and three additional sows received a daily oral dose of placebo only. Both products (placebo and probiotic) were microbiologically tested throughout the experiment as previously described.[20] Piglets born from *Bb12*-treated sows also were treated with a daily probiotic dose of 1.1 g (10 billion CFU/g) of *Bb12* starting shortly after birth until approximately 2.5 months of age (Treated/Treated = T/T group). Piglets born from placebo-treated sows received a daily dose of 1.1 g of placebo preparation starting at birth until the end of the experiment at approximately 2.5 months of age (Control/Control = C/C group). All piglets were weaned at week three after birth and allocated to individual pens with feed and water *ad libitum*. To prevent cross-contamination among treatment groups (n = 6-8/group), T/T and C/C pigs were housed in pens at opposite sides of the barn.

In a second study, four sows were also treated with oral administration of either *Bb12* probiotic (n = 2) or placebo (maltodextrin vehicle without *Bb12*, n = 2) as in the first study. Similarly, seven pigs derived from each treatment group were allocated to individual pens to continue the dietary treatment for 2.5 months before collection of samples.

Inoculation with Ascaris suum

The acquisition and preparation of infective *A. suum* eggs, oral inoculation, and management of pigs have been described.[28] In the first experiment, eight piglets from the T/T group and six piglets from the C/C group were inoculated with an oral dose of 2.5×10^4 *A. suum* eggs, and euthanized at day 21 post inoculation (p.i.). The remaining six animals of the T/T and C/C treatment groups were non-infected controls. Infected pigs were segregated on solid floors from non-infected (control) pigs by a center island that restricted water and particle flow between the opposite ends of the barn. "White spot" liver lesions, an indicator of active migration of *A. suum* larvae through the liver, were detected in inoculated pigs, but not control non-infected pigs at necropsy. This observation indicated that none of the control pigs were inadvertently infected.

In a second experiment, pigs born from four sows were split into the *Bb12* T/T group (n = 7) and placebo C/C group (n = 7) before oral inoculation with 2.5×10^4 *A. suum* eggs, and euthanized 17 days p.i. to collect *A. suum* L4 from the small intestine prior to natural expulsion.[32]

Parasite counts

Parasite expulsion is indicated by the displacement of L4 from the proximal to the distal small intestine between 17 and 21 days p.i., and six equivalent sections of the small intestine of each pig were examined for L4.[68] Protective immunity to *A. suum* was determined by comparing the number and location of L4 in the intestines of *Bb12*-treated versus placebo-treated pigs at 21 days p.i., for pigs in the first experimental group, and 17 days p.i for pigs in the second experimental group.

Quantitative detection of Bb12 in fecal samples and intestinal contents

A 5 g fecal sample was collected in a sterile 50mL conical tube at weaning, three weeks after weaning (pre-

infection) and 17 or 21 days p.i. DNA from the fecal samples or proximal colon contents collected at necropsy was isolated using the QIAamp DNA Stool mini kit (Qiagen, Valencia, CA) as previously described.[20] The abundance of *Bb12* in treated-pigs was determined using the primer-probe set against the *Bifidobacterium animalis subspecies lactis tuf* gene.[20]

Relative mRNA host gene expression by quantitative real time PCR

Tissue sections from liver, jejunum, proximal colon, and mesenteric lymph nodes (MLN) collected at necropsy were frozen immediately in liquid nitrogen and stored at⁻70°C until RNA extraction. Tissue RNA was extracted after homogenization in Trizol reagent (Invitrogen, Gaithersburg, MD), and quantitative real time PCR was performed on cDNA synthesized from each sample using 10 μg of total RNA as previously described.[69] Sequences for all probes and primers selected for real time PCR assays were obtained from the Porcine Translational Research Database (http://www.ars.usda.gov/Services/docs.htm?docid=6065).[70]

Fluorescence signals were processed after amplification and were considered positive if the fluorescence intensity was greater than 20-fold or more than the standard deviation of the baseline fluorescence. Gene expression was normalized based upon a constant amount of RNA and cDNA amplified.[31,71,72] Relative quantification of target gene expression was evaluated by comparing C_t values from cDNA processed from non-infected pigs and *A. suum*-infected pigs at 17 or 21 days p.i. after normalization with the housekeeping gene RPL32. Up- or down-regulation in gene expression is denoted by fold changes in C_t values.

Measurement of epithelial cell function ex vivo

Four segments of mucosa (0.126 cm^2) were stripped of muscle and mounted in Ussing chambers exposed to 10 mL of Kreb's buffer.[31] Briefly, agar salt bridges and electrodes were used to measure potential difference. The tissues were short-circuited at 1V (DVC World Precision Instruments, Sarasota, FL) every 50 s, and the short circuit current (I_{sc}) continuously monitored. In addition, every 50 s the clamp voltage was adjusted to 1 V for 10 s to allow calculation of tissue resistance using Ohm's law. Following a 15min equilibration period, basal I_{sc} representing the net Cl$^-$ ion flux before stimulation (baseline), and tissue resistance

were measured. After a second 15 min period, concentration-dependent changes in I_{sc} were determined in response to the cumulative addition of 5-hydroxytryptamine (5-HT), acetylcholine (ACH), histamine, or PGE$_2$ to the serosal side of the intestine. Following re-equilibration, concentration dependent changes in I_{sc} were measured in response to the cumulative addition of glucose to the mucosal side. All responses from treated-tissue segments from control and *A. suum* infected-pigs were averaged to yield a mean response per treatment group in the first experiment. Mucosal segments from jejunum also were collected in the second experiment for measurement of similar physiological responses.

Detection of parasite antigen-specific IgA, IgG, and IgM antibodies

Specific antibodies against *A. suum* were measured in the first experiment only. ELISA plates (Immunolon, Nunc, Thermo Fisher Scientific, Waltham, MA) were coated with 100 uL of 5 ug/mL of *A. suum* 3rd to 4th stage larvae (L3/L4) extract[73] or 0.1 ug of lyophilized *Bb12* in coating buffer (Sodium Carbonate buffer, pH 9.5). After an overnight incubation at 4°C, plates were washed six times (0.05% Tween 20 in 1X-PBS) and blocked with 0.05% Tween 20, 0.5% BSA in PBS for 30 minutes. Plates were washed and used for testing 100 uL of serum diluted at 1:3,000 and 1:6,000 (0.05% Tween 20 in PBS) or ileum fluid (contents obtained from the terminal 60 cm of pig intestine were centrifuged at 4C and 800 X g for 10 minutes to separate clear fluid from particulates). Test samples were run in duplicates and incubated for two hr at room temperature. After six washes, 100 uL of HRP-conjugated porcine anti-IgM, anti-IgA, anti-IgG1 or anti-IgG2 (Serotec, Raleigh, NC) were added at a 1:10,000 dilution and incubated for one hr. After a final wash, 100 uL of TMB-One substrate solution (Promega, Madison, WI) was added. Total anti-L3/L4 or anti-*Bb12*-specific antibody isotypes were determined after reading plates with an optical density (OD) at 450nm after 20 minutes. Reaction was stopped by addition of 1M HCl solution.

Histological and morphometric analysis of jejunum

Cross-sections of jejunum from the first experiment were fixed in 10% neutral buffered formalin and submitted to the University of Minnesota Diagnostic

Laboratory (St. Paul, MN) for histological analysis. Routine paraffin-embedded tissue blocks were sectioned at 4 μm and stained with hematoxylin and eosin (H&E). Villus height and crypt depth were measured by an observer blinded to treatment. The mean length of 15 well-oriented and representative villi and crypts were measured respectively from each pig. In addition, toluidine blue staining was performed for characterization of cellular components in the intestinal sections. Goblet cells and eosinophils were counted and mean values reported after evaluation of five independent fields under 40X magnification. Measurements were taken using an Olympus BX40 microscope (Olympus Optical Co., LTD, Japan) equipped with a Spot, InSight Color Model 3.20 digital camera and imaging software (Diagnostic Instruments Inc., Sterling Heights, MI).

Processing of data and statistical analysis

Bb12 in feces and the intestinal contents collected at necropsy were determined by quantitative detection of *Bb12-tuf*-gene copies. Data were log-transformed and C_t values compared among dietary treatment groups. Contrast statements were run to compare the effect of parasite infection among treatment groups. Data were transformed to log base to represent bacterial copy numbers per gram (cpg) of *Bb12* using the *tuf* gene as the bacterial marker (mean ± standard error). Statistical results were noted for mean values from each treatment group where any non-identical letters[(abc)] indicated significant differences at P≤0.05. The effect of infection with *A. suum* on tissue mRNA gene expression (C_t values) and cell numbers were evaluated using one-way ANOVA analysis and comparing values obtained in samples from *A. suum*-infected and non-infected pigs. Fisher's LSD post-hoc test was applied to access differences between treatment groups at 17 and 21 days p.i. P≤0.05 was considered statistically significant for all analyses (Statview 5.0 for Macintosh Abacus Concepts, Berkeley, CA). Differences in mucosal resistance were assessed for both experiments using a one-way ANOVA and concentration-dependent changes in I_{sc} or smooth muscle contractility were assessed by multiple ANOVA. All ANOVA tests were followed by t-tests to evaluate differences between means.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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