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Bifidobacterium Infantis 35624 Protects Against *Salmonella*-Induced Reductions in Digestive Enzyme Activity in Mice by Attenuation of the Host Inflammatory Response

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OBJECTIVES: *Salmonella*-induced damage to the small intestine may decrease the villi-associated enzyme activity, causing malabsorption of nutrients and diarrhea, and thus contribute to the symptoms of infection. The objective of this study was to determine the mechanism by which different doses and durations of *Salmonella* infection and lipopolysaccharide (LPS) affect brush border enzyme activity in the mouse, and to determine if the probiotic *Bifidobacterium longum* subspecies *infantis* 35624 could attenuate the intestinal damage.

METHODS: BALB/c mice were challenged with *Salmonella enterica* serovar Typhimurium UK1 at various doses (10^2–10^8 colony-forming unit (CFU)) and durations (10^6 CFU for 1–6 days). Mice were also treated with *B. longum* subsp. *infantis* 35624 for 2 weeks before and during a 6-day *S.* Typhimurium challenge (10^6 CFU), or before injection of LPS. The small intestine was assessed for morphological changes, mRNA expression of cytokines, and activity of the brush border enzymes sucrase–isomaltase, maltase, and alkaline phosphatase.

RESULTS: *S.* Typhimurium infection significantly reduced the activity of all brush border enzymes in a dose- and time-dependent manner ($P < 0.05$). This also occurred following injection of LPS. Pre-treatment with *B. longum* subsp. *infantis* 35624 prevented weight loss, protected brush border enzyme activity, reduced the small intestinal damage, and inhibited the increase in interleukin (IL)-10 and IL-8 expression due to *Salmonella* challenge.

CONCLUSIONS: *Salmonella* infection reduces the small intestinal brush border enzyme activity in mice, with the level of reduction and associated weight loss increasing with dose and duration of infection. *B. longum* subsp. *infantis* 35624 treatment attenuated the effect of *Salmonella* infection on brush border enzyme activity and weight loss, which may be due to modulation of the host immune response.

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INTRODUCTION

The gastrointestinal tract can be affected by a number of pathogens that disturb the normal function and cause diarrhea, with salmonellosis being one of the most common foodborne diseases. Diarrheal diseases are a leading cause of childhood morbidity and mortality in developing countries; however, the use of antibiotic therapy for salmonellosis is being questioned because of the emergence of multidrug-resistant strains of *Salmonella*.[1] Alternative therapies and preventative strategies are therefore required.

The presence of pathogens within the gastrointestinal tract can cause a number of host immune responses and pathological effects, including the modification of epithelial function to enhance penetration across the epithelial barrier (reviewed in Lu and Walker[2]). Salmonella is one such pathogen that attaches to the epithelial cells in the gastrointestinal tract, resulting in degeneration of the microvilli and membrane ruffling (reviewed in Lu and Walker[2]). This affects small intestinal function, resulting in decreased brush border enzyme activity.[3–5] Brush border enzymes, such as sucrase–isomaltase and maltase, play an important role in the digestion of carbohydrates and therefore reduced activity results in undigested food in the intestine, causing maldigestive diarrhea,[6] which contributes to the gastrointestinal symptoms of *Salmonella* infection. The underlying mechanisms for decreased enzyme activity have not been completely determined. Many studies have only assessed enzyme activity of the ileum as *Salmonella* is known to appear in the terminal ileum, cecum, and colon;[7] however, since the jejunum is a main area for nutrient digestion and uptake, it is important to assess if infection also affects jejunal brush border enzymes. In addition, studies have mainly assessed

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the effect of infection on the disaccharidase enzymes involved in carbohydrate digestion, including sucrase–isomaltase and maltase. Intestinal alkaline phosphatase (ALP) is a brush border enzyme unrelated to carbohydrate digestion that catalyses the hydrolysis of phosphomonoesters in an alkaline environment, and is thought to participate in regulating fat absorption.[8] This enzyme is therefore also important to assess in disease states, as an alteration to its activity may affect fat digestion.

There is increasing interest in the use of probiotics for disease prevention and health benefits. *In vitro* studies suggest that certain probiotic strains can inhibit *Salmonella* growth,[9–11] adhesion[12] and cell invasion,[13] and alter immune responses, e.g., by reducing interleukin (IL)-8 secretion[14] and decreasing tumor necrosis factor (TNF)-α production in the small intestine.[15] Few *in vivo* studies have been performed, but report increased survival, reduced intestinal damage,[16,17] and decreased translocation of *Salmonella* to the liver and spleen.[18,19] In addition, the probiotic *Bifidobacterium infantis* 35624 reduces the inflammatory activity associated with *Salmonella* infection by the induction of T regulatory cells.[20,21] No study has assessed the effect of probiotics on brush border enzyme activity following *Salmonella* infection. It was hypothesized that *Salmonella* would significantly reduce the digestive enzyme activity of the gastrointestinal tract, and that treatment with the probiotic *B. longum* subspecies *infantis* 35624 would attenuate the activation of the inflammatory immune response and reduce the gastrointestinal damage. The aims of the following studies were therefore to establish the effect of different doses and duration of *Salmonella* infection on small intestinal function in mice and to determine the effect of *B. longum* subsp. *infantis* 35624 treatment on disease pathology.

Methods

Studies were conducted in specific pathogen-free BALB/c mice (>10 weeks; Harlan UK, Bicester, UK). Mice were housed in a 12 h light:12 h dark cycle at 21 °C. They were fed a standard pellet diet and water *ad libitum*. All studies were approved by the animal ethics committee of University College Cork (Cork, Ireland).

Bacterial culture. Challenge doses of *Salmonella* for the following studies were prepared by inoculating trypsin soy broth (Oxoid, Basingstoke, UK) with *Salmonella enterica* serovar Typhimurium UK1 and growing it aerobically overnight (37 °C). The broth was centrifuged (1,600 × *g*, 5 min) and the pellet was resuspended in phosphate-buffered saline (PBS). The colony count was determined by serial dilution in PBS and spread plating on trypsin soy agar (Oxoid, Basingstoke, UK). Agar plates were aerobically incubated (37 °C) for 24 h, following which the bacteria was enumerated and the appropriate inoculation concentrations could be prepared by dilution of the culture in PBS.

The probiotic used in the following studies was freeze-dried *B. longum* subsp. *infantis* 35624 (Alimentary Health Ltd, Cork, Ireland), which was administered to mice via drinking water that was refreshed daily. The quantity of *B. longum* subsp. *infantis* 35624 added to the drinking water allowed mice to consume approximately 10^8 colony-forming units (CFU) per day. For the study assessing quantification of the bifidobacteria within the mouse gastrointestinal tract, a freeze-dried rifampicin-resistant *B. longum* subsp. *infantis* 35624 (spontaneous rifampicin-resistant variant) was used.

Effect of *Salmonella* dose and disease duration on small intestinal function. To determine the effect of infection dose on intestinal function, mice were orally inoculated by placing a pipette tip in the mouth and administering 20 μl of 10^2, 10^4 or 10^6 CFU *S.* Typhimurium UK1 ($n=10$ per group). A further two groups ($n=8$ per group) received 10^7 or 10^8 CFU. All mice were killed at day 6 post-challenge. The effect of duration of infection was determined by challenging mice with 10^6 CFU *S.* Typhimurium UK1 and being killed at days 1 ($n=10$), 3 ($n=10$) and 6 ($n=10$) post-challenge. Results were compared with non-infected mice ($n=10$). All *S.* Typhimurium UK1 challenge concentrations were confirmed by spread plating on to trypsin soy agar as described above.

Mice were monitored and weighed throughout the study. At the study end point, mice were killed via cervical dislocation, and the small intestine was removed and divided into jejunum (proximal half) and ileum (distal half). The distal 1 cm sections of the jejunum and ileum were stored in cassettes in 10% neutral-buffered formalin (Sigma-Aldrich, St Louis, MO, USA) for histology. The neighboring 2 cm sections were stored at −80 °C until analyzed for enzyme activity. The remaining small intestine, spleen, and liver were used for *Salmonella* quantification. To investigate the effect of duration of *Salmonella* infection on intestinal gene expression of cytokines and chemokines, a subgroup of mice from the duration of infection study ($n=5$ per group) had an additional 1 cm tissue section removed from the jejunum and stored in RNAlater (Ambion, Warrington, UK) at −80 °C until it was processed for RNA extraction.

Colonization site of *B. longum* subsp. *infantis* 35624 and effect on small intestinal function. To determine the colonization site for *B. longum* subsp. *infantis* 35624, healthy mice ($n=6$) were fed approximately 10^8 CFU per day rifampicin-resistant *B. longum* subsp. *infantis* 35624 in drinking water for 3 weeks (refreshed daily). After 3 weeks, mice were euthanized. The liver, spleen, small intestine (proximal, mid and distal), cecum, and large intestine (proximal and distal), as well as the removed luminal contents, were collected. Samples were mechanically disrupted in PBS in stomacher bags (Seward, Thetford, UK), and serial dilutions were plated on reinforced clostridial agar (Merck, Darmstadt, Germany) containing 0.05% L-cysteine hydrochloride (Sigma-Aldrich) and 50 mg/ml rifampicin (Sigma-Aldrich). Plates were incubated for 48 h (37 °C) in an anaerobic chamber. The use of rifampicin eliminated the growth of the other intestinal bacteria on the plate, and allowed enumeration of the *B. longum* subsp. *infantis* 35624, which was expressed as CFU/g sample.

To determine the effect of *B. longum* subsp. *infantis* 35624 on brush border enzyme activity, mice were given 10^8 CFU *B. longum* subsp. *infantis* 35624 or regular water ($n=10$ per group). Following 3 weeks of administration, mice were

euthanized and sections of jejunum were analyzed for enzyme activity.

Effect of *B. longum* subsp. *infantis* 35624 pre-treatment on *Salmonella* pathology. Mice ($n=6$) were administered 10^8 CFU per day *B. longum* subsp. *infantis* 35624 (Alimentary Health Ltd) in drinking water for 2 weeks before and for 6 days following challenge with *Salmonella*. *S.* Typhimurium (10^6 CFU) was orally administered and mice were euthanized 6 days post-challenge. The jejunum was removed and stored for histology, RNA extraction and analysis of enzyme activity. The remaining small intestine, spleen, and liver were collected for quantification of *S.* Typhimurium. Results were compared with age-matched healthy control mice ($n=6$) and *S.* Typhimurium UK1-infected mice (10^6 CFU, $n=6$) that received no probiotics.

To investigate the mechanisms underpinning the *Salmonella*-induced decrease in digestive enzyme activity, mice were challenged with lipopolysaccharide (LPS) from *Salmonella*. LPS was administered via the intraperitoneal route (rather than orally) to exclude any mechanism that may involve morphological alterations to the apical side of the epithelial cells. Mice were injected (intraperitoneally) with 2 mg/kg LPS (Sigma-Aldrich) following 3 weeks of 10^8 CFU per day *B. longum* subsp. *infantis* 35624 ($n=9$) or water alone ($n=9$). At 3 h following LPS, mice were euthanized. The jejunum was removed and analyzed for brush border enzyme activity. The results were compared with age-matched non-challenged mice ($n=9$).

Histology. Following storage in 10% neutral-buffered formalin, intestinal samples were processed and embedded in paraffin. Cross-sections were cut at 3 μm, attached to glass slides, and stained with hematoxylin and eosin. Villus height and crypt depth were determined from more than five sites. Only well-orientated villi and crypts were assessed and measurements were made in a blinded manner by two observers (Olympus BX51 upright microscope with Olympus DP Soft imaging capturing software).

Enzyme analysis. Intestinal tissue samples were homogenized in PBS (T10 basic Ultra-Turrax, IKA, Staufen, Germany), centrifuged (2,000 × *g*, 10 min, 4 °C), and the supernatant was used in the assays. The samples were assayed for sucrase–isomaltase and maltase,[22] and ALP[23] activity using published methods but modified for a 96-well plate. Enzyme activity was expressed as specific activity (units per milligram of protein (assessed with the Bradford protein assay)[24]). For disaccharidases, 1 U was defined as the activity that produces 1 μmol glucose per min per mg protein. For ALP, 1 U was defined as the activity that hydrolyses 1 μmol *p*-nitrophenol phosphate per min per mg protein.

***Salmonella* quantification from mouse tissue.** Tissues were homogenized in PBS in stomacher bags, and DNA was extracted (DNAeasy tissue kit; Qiagen, Crawley, UK) and quantified (NanoDrop Technologies, Wilmington, DE). Levels of *Salmonella* were quantified with real-time polymerase chain reaction using the LightCycler TaqMan Master kit (Roche Diagnostics, East Sussex, UK). DNA (20 ng) was used in the reaction with universal probe library and primers for the invasion gene specific to *S.* Typhimurium UK1 (designed using Roche Probelibrary system; Roche; Table 1). Cycling conditions were 95 °C for 10 min, and 45 cycles of 95 °C for 10 s, followed by 55 °C for 30 s and 72 °C for 30 s. Absolute quantification of *Salmonella* was performed by relating the polymerase chain reaction signal to a standard curve of *S.* Typhimurium UK1 DNA.

Gene expression. Tissue sections were homogenized in lysis buffer (Stratagene, Milton Keynes UK) using MagNA Lyser Green Beads (Roche), and RNA was extracted (Absolutely RNA Miniprep kit; Stratagene) and quantified (Nanodrop). RNA quality was assessed with the RNA 2100 Pico Labchip Kit (Agilent Technologies, Amstelveen, The Netherlands). To prepare cDNA, RNA (1 μg) was annealed to random primers (150 ng) (Roche) and then reverse transcribed at 42 °C for 50 min in a reaction containing 25 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate mix, 5 × reaction buffer, RNase inhibitor, and reverse transcriptase enzyme (Promega, Southampton, UK). The reaction was terminated by heat inactivation (70 °C, 15 min).

Quantitative real-time reverse transcriptase-polymerase chain reaction was performed using the LightCycler TaqMan Master kit (Roche). cDNA (5 μl) was used in the reaction with 1 μl of each primer and 0.2 μl universal probe library. Cycling conditions were as described for *Salmonella* quantification.

Table 1 Primer sequences for mouse mRNA targets with probe library number (Roche Diagnostics)

Primer	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	ProbeLibrary#
S. Typhimurium UK1 invasion gene	TGTCCTCCGCTCTGTCTACTT	ATCAACAATGCGGGGATCT	9
Sucrase-isomaltase	CACAATGCTGAAGGCTATGC	TGCCTTGATGTGTTTACCAAAA	32
Maltase-glucoamylase	ATTCAAGTTCGCCGAAAGAG	TGAAGGTGAAGCCGAGGA	95
Alkaline phosphatase	TCAGACATCAGCTAAGAACCTCA	TCCAACTGCCCCTTTAGGAT	83
IFN-γ	ATCCTGGAGGAACTGGCAAAA	TTCAAGACTTCAAAGAGTCTGAGGT	21
TNF-α	CTGTAGCCCACGTCGTAGC	TTGAGATCCATGCCGTTG	25
TGF-β	GAGCTGCTTATCCCAGATTCA	GGCAGTGGAGACGTCAGATT	20
IL-1β	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG	78
KC	ATAATGGGCTTTTACATTCTTTAACC	AGTCCTTTGAACGTCTCTGTCC	2
IL-10	CAGAGCCACATGCTCCTAGA	GTCCAGCTGGTCCTTTGTTT	41

IFN-γ, interferon-γ; IL, interleukin; KC, keratinocyte chemoattractant: murine homolog for human IL-8; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

Primers for mouse sucrase–isomaltase, maltase, ALP, interleukin (IL)-1β, IL-10, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, keratinocyte chemoattractant (KC: murine homolog for human IL-8), and interferon (IFN)-γ were designed using Roche ProbeLibrary system (Table 1). Amplification of β-actin was included as an endogenous control. The parameter threshold was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passed a fixed threshold above the baseline value. Relative mRNA expression levels were calculated with the formula $2^{-\Delta\Delta CT}$, where the threshold cycle (C_T) of the target gene is normalized to β-actin expression and relative to the control group.

Data analysis. Data are expressed as mean ± s.e. Comparison between control and challenged mice was performed with a one-way analysis of variance, with a P-value <0.05 considered significant. Where significant, the Holm–Sidak *post-hoc* test was performed to determine the differences compared with the control mice. Correlations were performed with a Spearman rank test. All statistical analyses were performed with SigmaStat (version 3.0.1a; SPSS Inc., Chicago, IL).

RESULTS

***Salmonella* dose on small intestinal function.** Following 6 days of *Salmonella* infection all mice had diarrhea and weight loss, with the weight loss greater in the mice challenged with 10^6, 10^7, and 10^8 CFU compared with healthy controls (weight change compared with baseline: non-infected mice = 1.41 ± 0.87%, 10^2 CFU = −1.95 ± 2.50%, 10^4 CFU = −2.84 ± 1.19%, 10^6 CFU = −7.04 ± 2.62%, 10^7 CFU = −11.37 ± 2.66%, 10^8 CFU = −10.50 ± 1.38%, $P<0.001$). Two mice that had been challenged with 10^8 CFU had to be euthanized early because of severity of disease and therefore were excluded from the analysis. Quantification of *Salmonella* showed no differences in the levels recovered from the liver, spleen, or small intestine from the different challenge groups (Table 2). No *Salmonella* was recovered from the non-challenged mice. There was a negative correlation between infection dose with jejunal villi length ($r=-0.764$, $P<0.001$) and crypt depth ($r=-0.518$, $P<0.001$), with villi and crypt length reduced to 70% of control levels. These structural alterations were restricted to the jejunum as changes to the ileum did not reach significance ($P>0.05$).

Infection with *Salmonella* significantly reduced enzyme activity. Sucrase–isomaltase, maltase, and ALP activity was reduced in the jejunum ($P<0.001$; Figure 1a) when mice were challenged with 10^4 CFU and higher doses of *Salmonella*, and there were significant reductions in sucrase–isomaltase and maltase in the ileum for challenge doses of 10^6 CFU and above ($P<0.01$; Figure 1b). This occurred in a dose-dependent manner for both the jejunum and ileum (jejunum: sucrase–isomaltase $r=-0.679$, maltase $r=-0.629$, ALP $r=-0.553$, $P<0.001$; ileum: sucrase–isomaltase $r=-0.496$, maltase $r=-0.480$, $P<0.001$), and was correlated with villi length in the jejunum (sucrase–isomaltase $r=0.377$, maltase $r=0.368$, ALP $r=0.318$, $P<0.05$). There was also a negative correlation between jejunal enzyme activity and weight loss (sucrase–isomaltase $r=-0.412$, maltase $r=-0.359$, ALP $r=-0.385$, $P<0.05$).

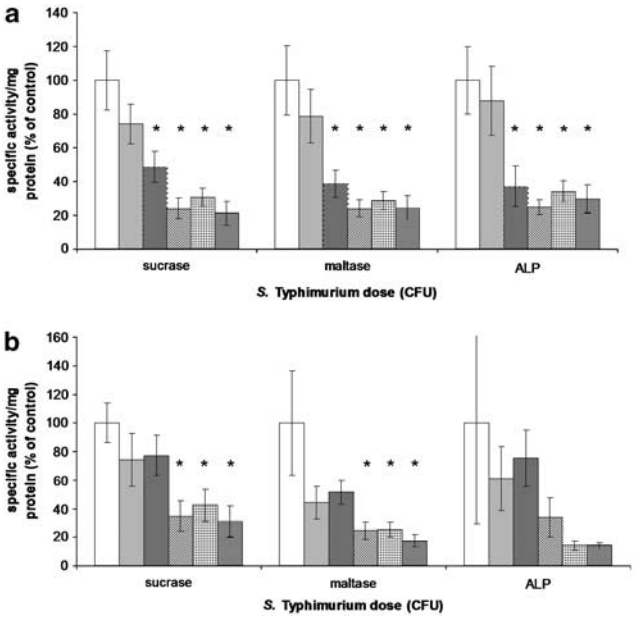

Figure 1 Brush border enzyme activity of (**a**) the jejunum and (**b**) the ileum following 6 days of challenge with different doses of *S.* Typhimurium UK1: non-infected (white, $n=10$), 10^2 (light gray, $n=10$), 10^4 (dark gray, $n=10$), 10^6 (diagonal stripe, $n=10$), 10^7 (thatched, $n=8$) and 10^8 (horizontal stripe, $n=6$) colony-forming unit (CFU). The results are expressed as % of non-infected value. Data are mean ± s.e., with *representing $P<0.05$ compared with non-infected mice. ALP, alkaline phosphatase; CFU, colony-forming unit.

Table 2 Levels of *S.* Typhimurium UK1 ($\log_{10}$ CFU/g tissue) in the liver, spleen, and small intestine of mice following challenge with 10^2, 10^4, 10^6, 10^7, and 10^8 CFU *S.* Typhimurium UK1

	***S.* Typhimurium UK1 inoculation dose**				
	10^2 CFU ($n=10$)	**10^4 CFU ($n=10$)**	**10^6 CFU ($n=10$)**	**10^7 CFU ($n=8$)**	**10^8 CFU ($n=6$)**
Liver	4.33 ± 0.95	5.19 ± 0.88	6.08 ± 0.44	6.37 ± 0.47	6.69 ± 0.28
Spleen	6.55 ± 0.31	6.87 ± 0.20	6.69 ± 0.43	6.43 ± 0.96	7.39 ± 0.26
Small intestine	6.26 ± 0.24	6.28 ± 0.24	5.27 ± 0.78	4.77 ± 0.68	4.58 ± 0.32

CFU, colony-forming unit.
Data are mean ± s.e. There were no significant differences between *Salmonella* recovered between the different challenge doses ($P>0.05$).

Infection duration on small intestinal function. Duration of *Salmonella* infection was associated with weight loss ($r=-0.521$, $P<0.001$) and became significant at day 6 compared with non-infected mice (weight change compared with baseline: non-infected mice $=1.41\pm0.87\%$, day $1=-2.16\pm0.97\%$, day $3=-1.32\pm0.71\%$, day $6=-6.64\pm2.34\%$, $P<0.005$). Following 24 h of infection, *Salmonella* was detected in the liver, spleen, and small intestine, and these levels did not significantly change over the 6 days of infection (Table 3; $P>0.05$). There were significant changes in the enzyme activity, with sucrase–isomaltase, maltase, and ALP activity in the jejunum significantly reduced at all days of infection ($P<0.0001$; Figure 2a), whereas in the ileum the changes were more variable, with a decrease in maltase activity at all days post-infection, but a decrease in sucrase–isomaltase activity after only 3 days (Figure 2b). There was a negative correlation between the activity of all enzymes and duration of infection in the jejunum (sucrase–isomaltase $r=-0.679$, maltase $r=-0.632$, ALP $r=-0.510$, $P<0.005$), and for sucrase–isomaltase and maltase in the ileum (sucrase–isomaltase $r=-0.748$, maltase $r=-0.470$, $P<0.005$).

Infection with *Salmonella* increased IL-10, KC, interferon-γ, and transforming growth factor-β mRNA expression, with most peaking by day 1 post-infection and remaining elevated ($P<0.05$; Figure 3). There were slight increases in TNF-α and IL-1β expression; however, these did not reach statistical significance ($P>0.05$; Figure 3).

Table 3 Levels of *S. Typhimurium* UK1 ($\log_{10}$ CFU/g tissue) in the liver, spleen, and small intestine of mice following challenge with 10^6 *S. typhimurium* UK1 at days 1, 3, and 6

	Days post-infection		
	Day 1	**Day 3**	**Day 6**
Liver	4.25 ± 0.95	4.13 ± 0.95	3.76 ± 0.89
Spleen	6.41 ± 0.29	6.24 ± 0.33	5.40 ± 0.71
Small intestine	5.43 ± 0.67	5.96 ± 0.36	4.54 ± 0.66

CFU, colony-forming unit.
Data are mean ± s.e. $N=10$ per group. There were no significant differences between *Salmonella* recovered between the different challenge durations ($P>0.05$).

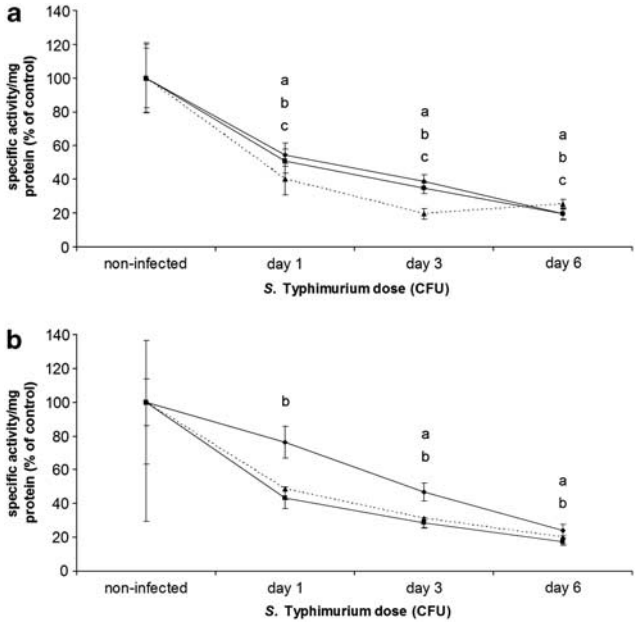

Figure 2 Brush border enzyme activity of (**a**) the jejunum and (**b**) the ileum over 6 days of infection with 10^6 *S.* Typhimurium UK1 ($n=10$ per group). The results are expressed as % of non-infected value, with diamonds representing sucrase–isomaltase activity, squares representing maltase activity and the broken line representing alkaline phosphatase activity. Data are mean ± s.e., with "a" indicative of $P<0.05$ compared with the sucrase–isomaltase non-infected values, "b" indicative of $P<0.05$ compared with the maltase non-infected values and "c" indicative of $P<0.05$ compared with the alkaline phosphatase non-infected values. CFU, colony-forming unit.

Colonization site of *B. longum* subsp. *infantis* 35624 and the effect on small intestinal function. Viable *B. longum* subsp. *infantis* 35624 was not detected either in the small intestinal tissue or contents, or in the spleen or in the liver. It was detected in the cecal tissue and contents (at similar levels), but was at 10^2- to 10^3-fold higher levels in the proximal and distal colon luminal contents compared with their corresponding tissue sections (Figure 4). Consumption of *B. longum* subsp. *infantis* 35624 (for 3 weeks) did not significantly alter the brush border enzyme activity ($P>0.05$)

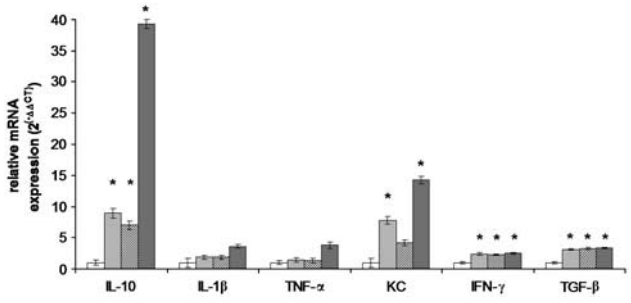

Figure 3 mRNA expression of cytokines and chemokines in the jejunum of non-infected mice (white column), and *S.* Typhimurium UK1-infected mice at day 1 (light gray), day 3 (striped) and day 6 (dark gray). Data are mean ± s.e., with values expressed relative to non-infected values. *$P<0.05$ compared with control values. $N=5$ per group.

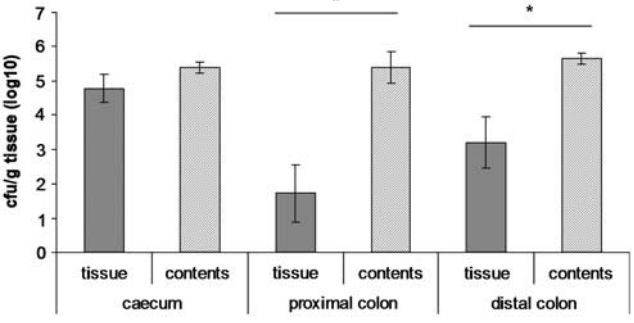

Figure 4 Levels of viable *B. longum* subsp. *infantis* 35624 in the cecum, proximal and distal colon (gray), and in the corresponding luminal contents (striped). Data are mean ± s.e., with *indicating $P<0.05$. $N=6$ per group. CFU, colony-forming unit.

or the villi or crypt length of the jejunum ($P>0.05$, data not shown).

Effect of *B. longum* subsp. *infantis* 35624 pre-treatment on *Salmonella* pathology. The jejunum is an area of high nutrient uptake, and as greater physiological alterations were occurring here following infection, we only assessed this site for the subsequent probiotic treatment studies. *Salmonella* infection caused weight loss, which was evident from day 3 post-infection ($P<0.05$; Figure 5). Pre-treatment with *B. longum* subsp. *infantis* 35624 prevented the *Salmonella*-associated weight loss, with no differences between weight of this group and non-infected mice. *B. longum* subsp. *infantis* 35624 also attenuated the reduction in enzyme activity, with sucrase–isomaltase activity significantly greater than the non-treated *Salmonella*-infected mice ($P<0.005$; Figure 6). *B. longum* subsp. *infantis* 35624 reduced the *Salmonella*-induced decrease in the villi length (villi length as the percentage of control: *Salmonella* $=71.3\pm5.3\%$; *B. longum* subsp. *infantis* 35624 $=87.7\pm6.6\%$), but did not alter the levels of *Salmonella* in the spleen, liver, or small intestine (Table 4; $P>0.05$).

Salmonella infection increased mRNA expression of cytokines and chemokines of the jejunal tissue (Figure 7). *B. longum* subsp. *infantis* 35624 reduced the increase in IL-10 and KC expression ($P<0.05$). *Salmonella* infection also decreased mRNA expression of sucrase–isomaltase and ALP, while *B. longum* subsp. *infantis* 35624 prevented some of the decrease in sucrase–isomaltase expression ($P>0.05$ compared with healthy levels).

At 3 h following LPS injection, mice exhibited diarrhea associated with a decrease in enzyme activity ($P<0.005$; Figure 8), which was an average of 37% of healthy levels. Treatment with *B. longum* subsp. *infantis* 35624 for 3 weeks before the LPS challenge reduced this degree of change so that brush border enzyme levels were between 56 and 66% of healthy levels and not significantly different to unchallenged mice ($P>0.05$; Figure 8).

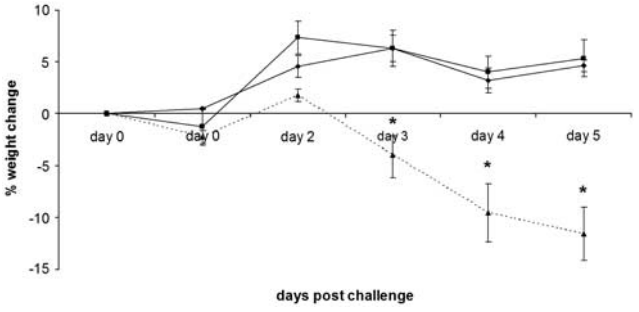

Figure 5 Weight change in non-infected mice (diamonds), *S.* Typhimurium UK1-infected mice with *B. longum* subsp. *infantis* 35624 pre-treatment (squares), and *S.* Typhimurium UK1-infected mice (broken line). The values are mean percentage weight change compared with the baseline weight ± s.e. $*P<0.05$ compared with non-infected mice. $N=6$ per group.

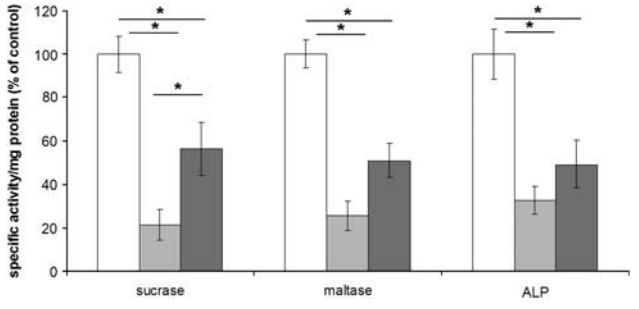

Figure 6 Brush border enzyme activity in non-infected mice (white), *S.* Typhimurium UK1-infected mice (light gray) and in *B. longum* subsp. *infantis* 35624-pre-treated *S.* Typhimurium UK1-infected mice (dark gray). The results are expressed as % of non-infected value. Data are mean ± s.e., with * representing $P<0.05$ between indicated groups. $N=6$ per group. ALP, alkaline phosphatase.

Table 4 Levels of *S.* Typhimurium UK1 ($\log_{10}$ CFU/g tissue) in the liver, spleen, and small intestine of mice challenged with 10^6 *S.* Typhimurium UK1 that had been pre-treated with water or 10^8 CFU/day *B. longum* subsp. *infantis* 35624

	Treatment	
	Water	***B. infantis* 35624**
Liver	6.88 ± 0.38	6.46 ± 0.20
Spleen	7.83 ± 0.54	6.73 ± 0.41
Small intestine	5.80 ± 0.69	5.74 ± 0.42

CFU, colony-forming unit.
Data are mean ± s.e. $N=6$ per group. There were no significant differences between *Salmonella* recovered between the different treatments ($P>0.05$).

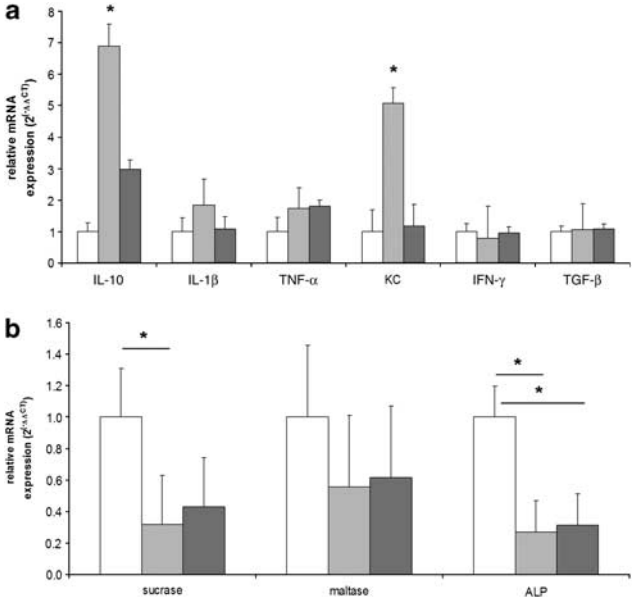

Figure 7 mRNA expression of (**a**) cytokines and chemokines and (**b**) brush border digestive enzymes in the jejunum for non-infected mice (white column), *S.* Typhimurium UK1-infected mice (light gray), and *B. longum* subsp. *infantis* 35624-pre-treated *S.* Typhimurium UK1-infected mice (dark gray). Data are mean ± s.e., with values expressed relative to non-infected values. $*P<0.05$ compared with non-infected values. $N=6$ per group. ALP, alkaline phosphatase; IFN, interferon; IL, interleukin; KC, keratinocyte chemoattractant; TGF, tumor growth factor; TNF, tumor necrosis factor.

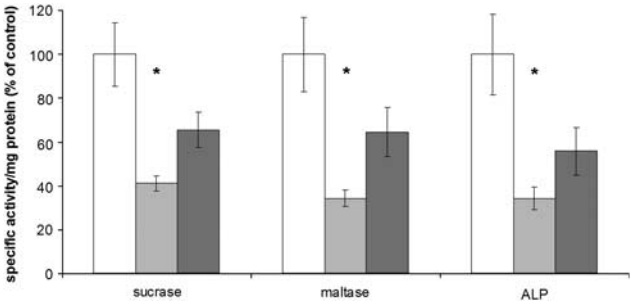

Figure 8 Jejunal brush border enzyme activity in non-infected mice (white), in lipopolysaccharide (LPS)-challenged mice (light gray) and in *B. longum* subsp. *infantis* 35624-pre-treated LPS-challenged mice (dark gray). The results are expressed as % of non-infected value. Data are mean ± s.e., with *representing $P<0.05$ compared with non-infected values. $N=9$ per group. ALP, alkaline phosphatase.

DISCUSSION

Salmonella is a leading cause of bacterial gastroenteritis that disturbs the normal function of the gut and results in diarrhea; however, the molecular mechanisms by which *Salmonella* interacts with the host to cause disease are not well understood. This study has shown that *S.* Typhimurium UK1 decreases brush border enzyme activity in a dose- and time-dependent manner. These changes are most likely due to a combination of small intestinal morphological alterations and the host immune inflammatory response possibly altering gene expression and protein activity of the enzymes. These physiological alterations and the associated weight loss also occurred with LPS administration, but were attenuated by pre-treatment with the probiotic *B. longum* subsp. *infantis* 35624.

The small intestinal brush border enzymes are often affected in diseases involving enteropathy of the gastrointestinal tract. Following entry of *Salmonella* into the host, the pathogen attaches to the intestinal surface[25] and causes local degeneration of the intestinal microvilli[26] and stunting of the villi.[7] *Salmonella* can then pass through the epithelium and spread to the lymph nodes, liver, spleen, and blood.[27] This study has shown that *Salmonella* causes significant damage to the small intestine of mice. As also seen with humans, infection caused weight loss and diarrhea. Examination of the small intestine showed functional changes to the entire length, with villi blunting and reductions to all brush border enzymes. The jejunum showed more damage, which is significant for health as this is a main area for nutrient digestion. It has been previously shown that *Salmonella* infection reduces brush border enzyme activity,[3–5] but this was mainly assessed in the ileum, with measurements limited to lactase. In our study, we found that all enzyme activity was reduced, showing that changes are not limited only to the disaccharidases. However, it is not clear whether this is due to morphological damage or changes in mRNA expression. It has been previously assumed that changes to brush border enzymes are due to villi damage from *Salmonella* entry, yet a study has shown that these physiological changes can occur in the absence of obvious histology changes.[28] A previous study showed *S.* Typhimurium enterotoxin administration in a rabbit ileal loop model reduced sucrase and lactase activity, but did not affect mRNA expression.[5] In this study, *Salmonella* infection caused a reduction in sucrase–isomaltase and ALP expression but not maltase.

Enzyme activity as well as body weight of the *Salmonella*-infected mice decreased in a dose- and duration-dependent manner. In the jejunum, the activity of all enzymes assessed had decreased by day 1, whereas in the ileum the functional changes were smaller, with sucrase–isomaltase levels reduced at day 3 post-infection and maltase affected by day 6. In humans, the incubation period of *Salmonella* is typically 6–48 h and is followed by abdominal pain and diarrhea. Following oral administration in animals, *Salmonella* has a rapid transit through the intestine. Oral *S.* Typhimurium challenge of mice causes high numbers of bacteria in the cecum within the first few hours, with less in the small intestine.[27] Another study reported that the jejunum and ileum are positive for *S. enteritidis* by 8 h post-inoculation, which peaks by 24–36 h.[29] Following intestinal colonization the infection becomes systemic, with bacteria appearing in the spleen by day 3, with numbers rapidly increasing over the next 4 days.[27] By using quantitative polymerase chain reaction, we were able to detect *Salmonella* in the spleen and in the liver by 24 h post-challenge. These levels and those in the small intestine did not change significantly over the 6 days.

This study challenged mice with different doses of *Salmonella* and investigated the outcome on bacterial colonization and translocation, weight loss, and small intestinal function. It is generally believed that a large inoculum (10^5–10^{10} bacteria) is required to initiate infection, to overcome the stomach acidity and to compete with the normal gut microbiota.[30] However, we found decreases in enzyme activity with lower doses (10^4 bacteria), and that by day 6 post-infection, levels recovered from the spleen and small intestine were the same, regardless of the initial dose. This is in agreement with a study that found that initial penetration at 6 h was dependent on dose, but by day 3 there was no significant difference in *S.* Typhimurium recovered from the spleen.[27]

Salmonella infection causes structural and functional changes to epithelial cells, thought to be mediated by bacterial toxins. Epithelial cells sense the microbial environment and prime the host immune response by releasing cytokines in response to bacterial signals. Immune functions may play a role as inflammatory cells and cytokines can infiltrate the intestinal tract during enteric bacterial infections. Studies show increases in pro-inflammatory cytokines and chemokines in response to *Salmonella* infection, including increased TNF-α[31] and IL-8.[32,33] In this study, we found that *Salmonella*-infected mice had an upregulation of the pro-inflammatory cytokines and chemokines interferon-γ and KC (IL-8), as well as an increase in IL-10 and transforming growth factor-β expression in the jejunum. This is in agreement with previous studies.[34–36] Other studies have also reported an increase in TNF-α following *Salmonella* infection, [31] but we did not observe this in the mouse jejunum. The reason for this discrepancy is likely to be that the upregulation of TNF-α is an early transient induction. TNF-α plays an important role in host defense against infectious agents, but also mediates some of the pathology, with increases occurring within 6 h post-infection.[31] We did not measure cytokine expression until 24 h post-challenge, and therefore may have missed this early immune response.

The role of T lymphocytes and cytokines in the *Salmonella*-related pathology of brush border microvilli rather than villi is largely unknown;[28] however, the upregulation of pro-inflammatory cytokine expression may be an underlying mechanism for the observed decrease in enzyme activity. Some cytokines, including IL-6 and interferon-γ, have been found to downregulate the gene expression of sucrase–isomaltase.[37] This study also reported that TNF-α induced a small increase in sucrase–isomaltase expression, while IL-1β had no effect on the enzyme. Expression of the disaccharidase lactase was not affected by any of the cytokines.[37] This suggests that the effect of each cytokine on enzyme expression is specific and selective, and this may explain why changes to the gene expression of maltase in this study were not observed with infection.

To further investigate the mechanisms underlying the reduction in brush border enzyme activity, mice were injected with *Salmonella* LPS. This caused a similar decrease in enzyme activity to that found with oral administration of *Salmonella*. LPS is a major surface component of the outer membrane and is an important virulence factor of *S.* Typhimurium.[38] Bacterial LPS is the principal component in the pathogenesis of endotoxic shock, and evokes an acute phase response, resulting in excessive production of pro-inflammatory cytokines.[39] Studies have also reported LPS to decrease digestive enzyme activity *in vitro* and *in vivo*.[40,41] Our results support the concept that decreases in enzyme activity are not due to *Salmonella* damage of cells alone (as the LPS was not administered on the apical side of the intestine), but rather related to the host immune response. The pro-inflammatory signaling events from *Salmonella* are also thought to be triggered on the basolateral surface of the epithelial cells through toll-like receptors.[42,43]

There are several studies that investigated probiotic treatment on *Salmonella* infection; however, no study determined whether probiotics can attenuate the damage caused to the small intestinal enzymes. Probiotics improve clinical outcomes in *Salmonella* infection by increasing survival, decreasing weight loss, reducing intestinal damage,[16,17] and decreasing *Salmonella* translocation,[18,19] but this is the first study to demonstrate that probiotics reduce the alterations caused to the brush border enzymes. *B. longum* subsp. *infantis* 35624 also prevented weight loss and decreased the pathology associated with *Salmonella* infection. The mechanisms for the beneficial effects of probiotics are largely unknown; however, it could include reducing intestinal infections by decreasing adhesion and invasion,[12,13] competing for nutrients within the intestinal lumen,[44] or by modulating host immune response.[14,15,45] In this study, *B. longum* subsp. *infantis* 35624 reduced the small intestinal damage associated with *Salmonella* infection. It is unlikely that these changes are from decreased adhesion, because *B. longum* subsp. *infantis* 35624 did not change the levels of *Salmonella* recovered from the small intestine. This is in agreement with some studies, which found that despite probiotics causing a decrease in disease severity, they do not alter the intestinal or fecal levels of *Salmonella*.[16,46] The effects of the probiotic treatment were also not due to decreased *Salmonella* translocation and dissemination, because there were no significant changes to the *Salmonella* levels in the liver and spleen; however, it is possible that changes to *Salmonella* levels could have been found in the mesenteric nodes, but this was not assessed. The beneficial effects of the probiotic were also unlikely to be due to *B. longum* subsp. *infantis* 35624 interacting with *Salmonella* or competing for nutrients within the small intestine because the probiotic was not detected in the small intestine, only within the cecum and large intestine (mainly luminal contents), nor was the probiotic enhancing the levels of brush border enzyme activity. The lack of *B. longum* subsp. *infantis* 35624 in the small intestine was unexpected as high levels were found in the cecum, but this could be explained either by the probiotic having a fast transit time through the small intestine or perhaps levels in the small intestine were below the sensitivity of our measuring technique and therefore its presence, even in low quantities could be having a biological effect. However, more likely we propose that the attenuation of the small intestinal damage is from an altered host immune response. This is supported by the finding that *B. longum* subsp. *infantis* 35624 reduced the intestinal damage caused not only by *Salmonella*, but also by LPS, a chemical agent that was not present on the luminal side of the gastrointestinal tract. Previous work also shows that bifidobacteria can stimulate the immune system and decrease intestinal inflammation[47] via induction of T regulatory cells.[20,21]

Probiotics are considered a potentially important strategy to modulate inflammatory responses in the gastrointestinal tract as they can enhance the host immune response and positively affect indigenous microbiota. In this study, *B. longum* subsp. *infantis* 35624 suppressed the expression of KC (IL-8) and reduced IL-10. IL-8 secretion correlates with the invasion of epithelial cells by *Salmonella*[48] and is a mediator of mucosal inflammation,[49] whereas IL-10 is an immunosuppressive compound that is triggered by *Salmonella* infection and depresses resistance to infection by blocking an effective immune response.[35] Decreased IL-8 with *B. longum* subsp. *infantis* 35624 treatment agrees with previous findings;[14] however, decreased IL-10 may be due to a direct effect of the probiotic on the *Salmonella*, such as decreasing its ability to trigger immunosuppressive activity. The reduced IL-8 and IL-10 expression observed with probiotic treatment may contribute to the attenuation of damage to the brush border enzymes as well as the decrease in weight loss, as there is an association between weight loss and enzyme activity, suggesting that malnutrition is contributing to the weight loss in mice.

Salmonella is one of the most extensively characterized bacterial pathogens and is a leading cause of bacterial gastroenteritis. Despite this, not much is understood at a molecular level as to how *Salmonella* interacts with the host to cause disease. This study has shown how gastrointestinal and immune functions are altered with different levels and durations of infection, with systemic infection and functional changes to the small intestinal enzymes occurring as early as 24 h post-infection. In addition, this is the first study to show the beneficial effects of a probiotic on enzyme activity following *Salmonella* challenge. The exact mechanisms are still to be determined, but it is likely due to modulation of the host immune system. Bifidobacteria are an attractive choice as probiotics as they make up one of the predominant

populations of the normal human colonic microbiota;[16] however, improved knowledge of the molecular mechanisms will allow for the selection of the best probiotic species, strains or substrates to protect against specific pathogens.

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CONFLICT OF INTEREST

Guarantor of the article: Erin L. Symonds, PhD.
Specific author contributions: Involved with the design of the trial, collected and analyzed samples throughout all studies, analyzed the data, and drafted and revised the paper: Erin L. Symonds; involved with the design and conduct of the trial, assisted with data analysis, and revised the draft paper: Caitlin O'Mahony; involved with the design and conduct of the trial and revised the draft paper: Susan Lapthorne; involved with the design and conduct of the trial and revised the draft paper: David O'Mahony; involved with the design and conduct of the trial and revised the draft paper: John Mac Sharry; involved with the design of the trial, assisted with data interpretation, and revised the draft paper: Liam O'Mahony; assisted with data interpretation revised the draft paper: Fergus Shanahan.
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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- *Salmonella* is one of the most common foodborne diseases that cause diarrhea.
- *Salmonella* pathology includes intestinal damage and functional changes, as well as alterations to the host immune response.
- There is a need for preventative therapy.

WHAT IS NEW HERE

- *Salmonella* infection in mice reduces the activity of the small intestinal brush border enzymes, sucrase–isomaltase, maltase, and ALP, in a dose- and time-dependent manner.
- Intraperitoneal administration of LPS from *Salmonella* also decreases small intestinal brush border enzyme activity.
- Pre-treatment with the probiotic *B. longum* subspecies *infantis* 35624 prevents the weight loss and attenuates the changes to brush border enzyme activity and pro-inflammatory cytokine expression induced by *Salmonella* infection.

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