Brachyspira pilosicoli colonization in experimentally infected mice can be facilitated by dietary manipulation

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The purpose of this study was to determine whether defined dietary manipulations would enhance colonization of mice experimentally challenged with the intestinal spirochaete Brachyspira pilosicoli. Weanling C3H/HeJ mice (n = 48) were fed either a standard balanced mouse diet or a diet supplemented with 50 p.p.m. zinc bacitracin (ZnB), with 50 % (w/w) lactose or with both supplements. Eight mice from each group were challenged orally with a human strain of B. pilosicoli (WesB), whilst four in each group acted as uninfected controls to evaluate the effects of the diets alone. The mice were kept for 40 days following challenge and faeces were collected every 3–4 days and subjected to culture and PCR for B. pilosicoli. Feeding ZnB reduced total volatile fatty acid production in the caecum. Feeding lactose alone doubled the weight of the caecum and its contents compared with control mice, and resulted in a relatively greater production of acetate, but a reduction in propionate and isovalerate production. These effects were negated by the addition of ZnB with the lactose. None of the mice fed the standard diet or receiving ZnB alone became colonized following experimental challenge. One of the mice receiving lactose was culture and PCR positive for B. pilosicoli on one sampling 1 week after inoculation, and one was positive on three samplings taken 20, 25 and 29 days after inoculation. All mice receiving both lactose and ZnB became colonized and remained so, with all samples being positive over the last seven samplings. The colonization was not associated with an end-on attachment of the spirochaete to the epithelial cells of the caecum, but colonized mice developed a specific humoral antibody response to the spirochaete.

INTRODUCTION

The anaerobic intestinal spirochaete Brachyspira pilosicoli colonizes the large intestine of many species, including pigs (Trott et al., 1996b), chickens (McLaren et al., 1997), dogs (Duhamel et al., 1998) and humans (Trivett-Moore et al., 1998). Colonization may be associated with a mild colitis and diarrhoea, a condition generally called intestinal spirochaetosis (IS). A characteristic but not universal feature of IS is the presence of large numbers of spirochaetes attached by one cell end to the colorectal epithelium, forming a ‘false brush border’ (Harland & Lee, 1967).

Colonization of humans by B. pilosicoli is common in developing countries, but rare in urban residents of developed countries (Brooke et al., 2001). These differences in prevalence may be related to differences in exposure to the spirochaete, for example as a result of the relative access of various population groups to good sanitation and uncontaminated water supplies. There also may be dietary influences on colonization. In pigs, infection with the related intestinal spirochaete Brachyspira hyodysenteriae, the agent of swine dysentery, is enhanced by the presence of non-starch polysaccharides (fibre) in the diet (Siba et al., 1996; Pluske et al., 1996), which increase fermentation in the large intestine. Similarly, the duration and extent of colonization by B. pilosicoli in young pigs are increased with the presence of fibre, or with the inclusion of viscous-forming non-fermentable compounds in the diet (Hampson et al., 2000; Hopwood et al., 2002). On the other hand, in layer chickens, the addition of 50 p.p.m. zinc bacitracin (ZnB) to the diet enhances colonization with B. pilosicoli, presumably as a result of the antimicrobial’s effects on the overall composition of the large intestinal microbiota (Jamshidi & Hampson, 2002).

To date, there have been no reports of natural colonization of mice by B. pilosicoli and, in previous studies in our...
laboratory, we have been unable to colonize various mouse strains following experimental challenge with different strains of the spirochaete (unpublished data). There is one published study in which mice (C3H/HeJ) were successfully colonized with *B. pilosicoli* (Sacco et al., 1997), and these animals were fed a specialized diet (Teklad diet TD 85420, Harlan Sprague–Dawley). The same diet previously has been shown to increase the susceptibility of mice to experimental colonization by *B. hyodysenteriae* (Nibbelink & Wannemuehler, 1992).

The aim of the current study was to investigate whether specific dietary manipulations could be used to enhance the colonization of mice with *B. pilosicoli*.

**METHODS**

**Ethics.** This experiment was conducted with the approval of the Murdoch University Animal Ethics Committee.

**Experimental diets.** The diets used were based on a defined commercial pelleted basal mouse diet (18.9 % protein, 5.2 % fat, 5 % crude fibre; digestible energy 14.5 MJ kg⁻¹; Glen Forrest Stockfeeders). The pellets were ground to a coarse powder in a blender, and the appropriate amounts of lactose (Sigma) and/or ZnB (Jurox) were added to obtain final concentrations of 50 % (w/w) lactose and/or 50 p.p.m. ZnB. To ensure uniformity, each diet was made by mixing the ground pellets and the additional components with tap water to form a dough. The dough was then flattened and dried into biscuits in a ventilated oven at 50 °C.

**Experimental design.** Forty-eight weanling C3H/HeJ female mice of 3 weeks of age were obtained from the Western Australian State Animal Resources Centre, Murdoch, and were randomly assigned to four dietary treatment groups. Each group was made up of 12 mice, each housed in standard laboratory mouse cages in groups of four. Mice of group 1 were fed the nutritionally balanced standard mouse diet. Mice of group 2 were fed the standard diet supplemented with 50 p.p.m. ZnB. Mice of group 3 were fed the standard diet supplemented with 50 % (w/w) lactose. Mice of group 4 were fed the standard diet with both 50 p.p.m. ZnB and 50 % (w/w) lactose. The diets and fresh water were available to the mice *ad libitum*.

**Preparation of challenge inoculum.** *B. pilosicoli* human strain WesB was obtained from frozen stock held in the culture collection at the Reference Centre for Intestinal Spirochaetes, Murdoch University. The strain was originally isolated from an Aboriginal child with diarrhoea, and previously has been used for the experimental infection of pigs (Trott et al., 1996a), chickens (Trott et al., 1995) and a human volunteer (Oxberry et al., 1998). Spirochaetes were propagated at 37 °C in Kunkle’s pre-reduced anaerobic broth, containing 2 % (v/v) fetal bovine serum and 1 % (v/v) ethanolic cholesterol solution (Kunkle et al., 1986). At mid-exponential phase growth, at a density of approximately 10⁸ cells ml⁻¹, the broth was centrifuged at 2500 g for 15 min at 4 °C, the cell deposit was resuspended in sterile broth, cell numbers were counted using a haemocytometer and the cell density was adjusted to approximately 10⁵ cells ml⁻¹ with sterile broth.

**Experimental infection.** All mice were fed on their respective diets for 7 days. For each group, eight mice (i.e. two cages per group) were used for experimental infection whilst four mice (one cage) were left uninfected. Water was removed from the mice to be infected and, 1 h later, 0.3 ml of spirochaete broth culture was administered by gastric intubation. Water was reinstated after 30 min. The inoculation procedure was repeated on the following 2 days.

**Faecal samples and culture.** Individual faecal samples from each mouse were collected every 3–4 days, starting 3 days after the last inoculation. Bacteriological swabs were inserted into the faeces and streaked onto selective trypticase soy agar (BBL) plates containing 5 % (v/v) defibrinated ovine blood, 400 μg spectinomycin ml⁻¹ and colistin and vancomycin each at 25 μg ml⁻¹. The plates were incubated for 7–10 days at 37 °C in an anaerobic environment generated using anaerobic Gaspak Plus sachets (BBL), before being examined. The presence of low, flat, spreading growth of spirochaetes on the plate was recorded, as was the presence of weak haemolysis around the growth. Spirochaete presence was confirmed by picking off areas of suspected spirochaetal growth, resuspending in PBS and examining the suspension under a phase-contrast microscope at 400× magnification.

**DNA preparation from isolation plates.** A species-specific PCR for *B. pilosicoli*, based on amplification of part of the 16S rRNA gene, was applied to growth harvested from the primary plate. The tip of a sterile wooden toothpick was used to stab the area of spirochaetal growth, and the adherent material was resuspended in 50 μl of ultra-pure water, then boiled for 30 s. A 2.5 μl aliquot of the boiled cells was added to the PCR. The PCR and its optimized conditions have been described previously (La et al., 2003).

**Post-mortem procedure.** Forty days after the last of the three inoculations, the mice were weighed, euthanased by methoxyflurane inhalation followed by cervical dislocation and then subjected to post-mortem examination. Blood was collected by cardiac puncture of the dead mice in the infected groups, and the serum was removed for serology. For the uninfected mice, the caeca were excised and weighed with their contents intact, and the contents were then collected and stored at −20 °C for subsequent analysis of volatile fatty acid (VFA) concentrations. For all mice, the luminal surface of the caecal wall was rubbed with a sterile bacteriological swab, which was cultured for *B. pilosicoli* in the same way as described for faecal swabs. Sections of the caecal wall were excised and placed in 10 % (v/v) buffered formalin for subsequent histological examination. The fixed tissue was processed through to paraffin blocks, cut at 4 μm and stained with haematoxylin and eosin.

**VFA analysis.** VFA concentrations in the caecal contents of the four groups of uninfected mice were determined by gas-liquid chromatography, as previously described (Pluske et al., 1996).

**Antigen preparation for ELISA.** One hundred millilitres of the homologous *B. pilosicoli* inoculum (~10¹⁰ cells ml⁻¹) was centrifuged at 2500 g for 15 min. The supernatant was discarded and the cell pellet resuspended in 1 ml sterile PBS. The cell suspension was placed on ice for 10 min before being sonicated three times for 30 s with a 2 min interval on ice between each sonication. The sonicated cells were centrifuged at 20 000 g for 10 min at 4 °C. The supernatant was transferred to a fresh microfuge tube and the whole-cell preparation was then quantified for total protein using the Bio-Rad protein assay according to the manufacturer’s instructions.

**Whole-cell ELISA.** Microtitre plates were coated with 100 μl per well of clarified whole-cell sonicate (1 μg ml⁻¹) in carbonate buffer (pH 9.6). Coating was allowed to occur at 4 °C overnight. Plates were blocked with 150 μl PBS-BSA (1 %, v/v) for 1 h at room temperature with mixing, and were then washed three times with 150 μl PBS-T (PBS plus 0.05 % Tween, v/v). Mouse sera were diluted 200-fold in 100 μl PBS-T-BSA (0.1 %, v/v) and incubated at room temperature for 2 h with mixing. Plates were washed as above, before 100 μl goat anti-mouse IgG (whole molecule)-HRP diluted 2000-fold in PBS-T was added. After incubation for 1 h at room temperature, the plates were washed and 100 μl TMB (3,3',5,5'-tetramethylbenzidine) substrate was added. Colour development was allowed to occur for 10 min at room temperature before being...
stopped with the addition of 50 µl 1 M sulphuric acid. The absorbance of each well was measured at 450 nm.

**Statistical analysis.** For the uninfected mice, group values for body weights and caecal weights and for individual VFAs and total VFAs at euthanasia were compared for significance using the Kruskal-Wallis test, with Dunn’s multiple comparison test used to determine significance between groups. A non-parametric test was used because of the small sample size. Total VFA concentrations were also compared between the combined uninfected mice of groups 2 and 4 (receiving ZnB) and those of groups 1 and 3 (not receiving ZnB) using Student’s t-test. For the four groups of infected mice, body weights and ELISA titres were compared by ANOVA, and group differences were then tested for significance using the Bonferroni multiple comparisons test.

**RESULTS AND DISCUSSION**

**Body weights**

The body weights of the uninfected mice at the time of euthanasia are presented in Table 1, and those of the infected mice are presented in Table 2. There were no significant differences between group weights for the uninfected mice, but those receiving ZnB alone (group 2) were the heaviest. In the infected mice, those of group 2 were significantly heavier than those of groups 3 and 4.

The dietary intake of the mice was not recorded, and the difference in weight observed may have been associated with an increased dietary intake in group 2. On the other hand, ZnB is fed to production animals as a growth promoter, and the increase in weight of mice in group 2 may have been attributable to some other specific effect of ZnB, for example in reducing the numbers or altering the composition of the microbiota within the gastrointestinal tract.

**Caecal weights**

The means ± SEM of the caecal weights of the uninfected mice in the four groups are presented in Table 1. The mice receiving lactose (group 3) had significantly heavier caeca and contents than those of the other three groups. Lactose was added to the diet with the intention of increasing caecal fermentation, and the greater size and heavier content

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Experimental group</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>20.17 ± 1.06</td>
<td>26.34 ± 0.49</td>
</tr>
<tr>
<td>Caecal weight</td>
<td>0.41 ± 0.07</td>
<td>0.37^a + 0.01</td>
</tr>
<tr>
<td>Concentration (mmol l⁻¹)</td>
<td>Acetic acid</td>
<td>12.33 ± 5.09</td>
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<td></td>
<td>Propionic acid</td>
<td>1.64^a ± 0.67</td>
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<td></td>
<td>Butyric acid</td>
<td>3.52 ± 0.49</td>
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<td></td>
<td>Isovaleric acid</td>
<td>0.31^a ± 0.07</td>
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<td></td>
<td>Caproic acid</td>
<td>0.35 ± 0.04</td>
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<tr>
<td>Total VFA</td>
<td>18.15 ± 6.05</td>
<td>8.27 ± 2.42</td>
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**Table 2.** Body weights at euthanasia and ELISA titres against *B. pilosicoli* in experimentally infected mice

Within a row, means with the same superscript differ at the 5 % level of significance (ANOVA, with Bonferroni multiple comparisons test). Values are means ± SEM.

<table>
<thead>
<tr>
<th>Measurement</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>22.15 ± 0.76</td>
<td>25.10^ab ± 0.52</td>
</tr>
<tr>
<td>ELISA titre (A₄₅₀)</td>
<td>0.34^a ± 0.16</td>
<td>0.23^b ± 0.11</td>
</tr>
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</table>
Lactose was used for this purpose because it is known that \( \beta \)-galactosidase activity in the small intestine diminishes rapidly following weaning (Hampson & Kidder, 1986); it was therefore reasoned that the sugar would be incompletely digested and absorbed in the small intestine of weaned mice. As a result, the lactose would enter the large intestine and act as a substrate to induce increased fermentation, and potentially enhance proliferation of intestinal spirochaetes (Pluske et al., 1996; Hampson et al., 2000). The Teklad diet that previously has been shown to enhance proliferation of \( B. \) pilosicoli in mice was not available for the current study, but, as it contains 63 % glucose, it might also act by stimulating fermentation. In the current experiment, lactose was used rather than glucose because, in a previous experiment, when mice were given glucose through the drinking water, they failed to show enhanced colonization with \( B. \) hyodysenteriae (Nibbelink & Wannemuehler, 1992).

The fourth diet contained both ZnB and lactose, because it was reasoned that there might be a cumulative effect on colonization if both additives were included in the diet. Unexpectedly, the combination of lactose and ZnB appeared to neutralize the expected effects of the two individual components, in that there was no increased body weight (associated with ZnB) or increased caecal weight (associated with lactose).

**VFA concentration**

The mean \( \pm \) SEM concentrations of individual VFAs and the overall VFA content of the caecal contents of the four groups of uninfected mice are presented in Table 1. Only one mouse (group 3) had any isobutyric acid recorded (2.37 mmol l\(^{-1} \)). Overall, the mice in groups 2 and 4, receiving ZnB (+ lactose in group 4), had lower total VFA concentrations than the mice in groups 1 and 3, although these differences were not statistically significant. When the results for individuals in groups 2 and 4 were combined (9.03 \( \pm \) 1.31 mmol l\(^{-1} \)) and compared with those for groups 1 and 3 combined (19.18 \( \pm \) 3.3 mmol l\(^{-1} \)), the difference was highly significant \((P = 0.007)\). Presumably, the reduced fermentation was a result of the antimicrobial effects of ZnB. Bacitracin is bacteriocidal to Gram-positive bacteria, although it has little effect against Gram-negative bacteria (Prescott, 2000).

Mice fed on the diet supplemented with lactose alone (group 3) had more acetic acid and less propionic and isovaleric acids than mice in the other groups. As the addition of 50 % (w/w) lactose to the diet substituted for other dietary components, including protein, it might be argued that these changes in end products were a result of reduced availability of protein as a substrate, rather than the increased availability of lactose. This seems unlikely, however, as the same effects were not seen in mice of group 4, which also consumed a diet containing 50 % lactose. The mice in group 4, and those in group 2, had lower acetic and caproic acid concentrations than the mice in the other two groups. This difference is likely to be attributable to the consumption of ZnB by both groups of mice and its influence on the metabolic activity of the caecal microbiota.

**Colonization**

None of the faecal samples from the experimentally infected mice in groups 1 or 2 were culture or PCR positive at any point over the experimental period. In group 3, one mouse was culture positive for \( B. \) pilosicoli on one sampling, 1 week after the last inoculation, and one was positive on three samplings taken 20, 25 and 29 days after the last inoculation. From the second sampling onwards, all mice in group 4 had positive faecal cultures and were PCR positive for all or most of the experimental period, with 90 % of samples collected being positive. Caecal samples from four mice in group 4 were culture and PCR positive at post-mortem. No specific histological changes were detected in the caeca of any mice, and spirochaetes were not seen attached to caecal enterocytes.

The lack of colonization of mice on the standard mouse diet suggests that this species has little natural susceptibility to colonization by \( B. \) pilosicoli. It was unclear why addition of 50 p.p.m. ZnB did not enhance colonization, even though it does so in chickens (Jamshidi & Hampson, 2002), but it may have resulted from differences in the composition of the resident caecal microbiota of mice compared with chickens and the relative effects of ZnB on this microbiota. Work undertaken with mice experimentally infected with \( B. \) hyodysenteriae has suggested that there are different components of the caecal flora that either naturally enhance or inhibit colonization by the spirochaete (Suenaga & Yamazaki, 1986; Hayashi et al., 1990). The same may apply to \( B. \) pilosicoli colonization.

The increased fermentation induced by eating high concentrations of lactose did not provide an intestinal environment that greatly enhanced colonization by the spirochaete, since only two mice showed transient faecal shedding. On the other hand, addition of both lactose and ZnB had a pronounced positive influence on colonization. Caecal fermentation in this group tended to be similar overall to that of the mice on the standard diet, so it would appear that changes to specific components of the caecal microbiota, and/or to their metabolic activities, rather than to the overall metabolic activity of the microbiota, are important in facilitating colonization of \( B. \) pilosicoli in mice. Further work is required to characterize these predisposing changes.

The colonized mice did not show any obvious signs of disease, although their body weights tended to be less than those of the other non-colonized groups. No pathological changes were seen in the caeca, nor was attachment of spirochaetes to the epithelium observed. Interestingly, in the study by Sacco et al. (1997), a \( B. \) pilosicoli isolate of human origin failed to attach to the caecal epithelium of mice, but strains of avian and porcine origin did attach. Further work is
required to determine whether other human and animal strains of \textit{B. pilosicoli} show specificity in relation to their attachment to murine caecal epithelium.

**ELISA titres**

The mean ± SEM of the ELISA titres for the four groups of infected mice are shown in Table 2. The group values were highly significantly different ($P < 0.0001$), with the titres in mice of group 4 being highly significantly greater ($P < 0.001$) than those of the other three groups. No other differences were significant.

The development of a significant circulating antibody response against \textit{B. pilosicoli} in the colonized mice was unexpected. In previous studies, pigs that were experimentally colonized with \textit{B. pilosicoli} failed to develop a systemic immune response (Hampson et al., 2000), and we have observed a similar lack of response in experimentally colonized chickens (unpublished data). It is unclear why the mice showed a systemic immune response, particularly in the absence of caecal inflammation, but it is possible that the organisms were translocated across the epithelial barrier and stimulated the local lymphoid material, or even disseminated into the general circulation. Previously, \textit{B. pilosicoli} has been identified in the bloodstream of a series of debilitated human patients (Trott et al., 1997), but, to date, a similar occurrence has not been reported in animals. The possibility of spirochaetal translocation in the mouse model of \textit{B. pilosicoli} infection deserves further study.

In conclusion, this study has demonstrated that a human strain of \textit{B. pilosicoli} is capable of colonizing mice fed with a standard mouse diet supplemented with both lactose and ZnB. The work appears reproducible as, in a subsequent study, we found that a group of eight mice fed this diet and experimentally infected with the same strain of \textit{B. pilosicoli} all became colonized with the spirochaete (unpublished data). The availability of this convenient murine model of \textit{B. pilosicoli} infection should enhance future studies on the pathogenesis of IS.

**ACKNOWLEDGEMENTS**

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