Oral treatment of chickens with *Lactobacillus reuteri* LM1 reduces *Brachyspira pilosicoli*-induced pathology

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Avian intestinal spirochaetosis (AIS) results from the colonization of the caeca and colon of poultry by pathogenic *Brachyspira*, notably *Brachyspira pilosicoli*. Following the ban on the use of antibiotic growth promoters in the European Union in 2006, the number of cases of AIS has increased, which, alongside emerging antimicrobial resistance in *Brachyspira*, has driven renewed interest in alternative intervention strategies. *Lactobacillus*-based probiotics have been shown to protect against infection with common enteric pathogens in livestock. Our previous studies have shown that *Lactobacillus reuteri* LM1 antagonizes aspects of the pathobiology of *Brachyspira* in *vitro*. Here, we showed that *L. reuteri* LM1 mitigates the clinical symptoms of AIS in chickens experimentally challenged with *B. pilosicoli*. Two groups of 15 commercial laying hens were challenged experimentally by oral gavage with *B. pilosicoli* B2904 at 18 weeks of age; one group received unsupplemented drinking water and the other received *L. reuteri* LM1 in drinking water from 1 week prior to challenge with *Brachyspira* and thereafter for the duration of the study. This treatment regime was protective. Specifically, *B. pilosicoli* was detected by culture in fewer birds, bird weights were higher, faecal moisture contents were significantly lower (*P*, 0.05) and egg production as assessed by egg weight and faecal staining score was improved (*P*, 0.05). Also, at post-mortem examination, significantly fewer *B. pilosicoli* were recovered from treated birds (*P*, 0.05), with only mild–moderate histopathological changes observed. These data suggest that *L. reuteri* LM1 may be a useful tool in the control of AIS.

INTRODUCTION

Avian intestinal spirochaetosis (AIS) is a disease in poultry that arises from the colonization of the caeca and colon of birds by the anaerobic spirochaete *Brachyspira* (Swayne & McLaren, 1997; Stephens & Hampson, 2001). Of the seven documented species, three are considered pathogenic in poultry, as demonstrated by experimental challenge with *Brachyspira alvinipulli* (Stanton et al., 1998), *Brachyspira intermedia* (Hampson & McLaren, 1999) and *Brachyspira pilosicoli* (Stephens & Hampson, 2002b). *B. pilosicoli* has a wide host range, also causing intestinal spirochaetosis in pigs (Trott et al., 1996) and humans (Tsinganou & Gebbers, 2010), with potential for zoonosis (Hampson et al., 2006a). AIS in layer and broiler breeder flocks has been associated with a delayed onset of lay, reduced egg weights, diarrhoea, faecal staining of eggshells, reduced growth rates, increased feed consumption and non-productive ovaries (Davelaar et al., 1986; Griffiths et al., 1987; Swayne et al., 1992; Feberwee et al., 2008). Colonization by *B. pilosicoli* has been characterized by an ability of the organism to form end-on attachments to and invade the intestinal epithelia, forming dense fringes that penetrate between enterocytes associated with inflammatory responses (Jensen et al., 2000, 2001; Feberwee et al., 2008).

**Abbreviations:** AIS, avian intestinal spirochaetosis; GI, gastrointestinal; HE, haematoxylin and eosin.
The prevalence of *Brachyspira* in free-range flocks in the UK has been estimated as high as 90% (Burch, 2010), with the incidence of AIS reported as increasing following the 2006 European Union ban on the subtherapeutic use of antibiotics in livestock (Castanon, 2007). With emerging resistance amongst *Brachyspira* to antimicrobials including tiamulin, a preferred treatment for AIS (Hampson et al., 2006b; Pringle et al., 2006), there is increased interest in alternative intervention strategies, such as probiotics (Collins et al., 2009).

Probiotics have been described as live micro-organisms that confer health benefits on the host when administered in adequate quantities (FAO/WHO, 2001), and include gastrointestinal (GI) commensals such as lactobacilli. Probiotics have been implicated in the competitive exclusion of pathogens via mechanisms including the secretion of antimicrobial compounds, competition for essential nutrients and host cell binding receptors, and immunomodulation (Vanderpool et al., 2008), and reduce colonization by enteric pathogens in the lower GI tract (Schoeni & Wong, 1994; La Ragione & Woodward, 2003; Stern et al., 2006; Vicente et al., 2008). Specifically, lactobacilli can exclude *Campylobacter* (Edens et al., 2001) against aggregation with the spirochaetes and eliciting a stress response (Bernardeau et al., 2007a; Higgins et al., 2007a; Higa et al., 2008) from poultry. Additionally, we and others have demonstrated that lactobacilli inhibit various aspects of the biology of *Brachyspira in vitro*. *Lactobacillus johnsonii* exhibited antimicrobial activity against *B. pilosicoli* and *Brachyspira hydysenteriae* through the production of hydrogen peroxide and a proteinaceous antimicrobial compound (Se et al., 2008). *Lactobacillus rhamnosus* and *Lactobacillus casei* have been implicated in inhibiting the motility of *Brachyspira* by co-aggregation with the spirochaetes and eliciting a stress response (Bernardeau et al., 2009). We demonstrated that *Lactobacillus reuteri* LM1 and *Lactobacillus salivarius* LM2 inhibited the motility, growth and adherence of *B. pilosicoli* B2904 *in vitro* (Mappley et al., 2011). We wished to test whether probiotics reduce colonization by *Brachyspira* and describe *in vivo* studies testing the hypothesis that *L. reuteri* LM1 competes against and reduces the pathogenic impact of *B. pilosicoli* in the chicken.

**METHODS**

**Bacterial strains and culture conditions.** *B. pilosicoli* B2904 was isolated from a chicken exhibiting clinical symptoms of AIS in the UK (Mappley et al., 2011), and was cultured from a stock maintained in fetal calf serum (FCS) (Sigma-Aldrich) + 30% (v/v) *Brachyspira* enrichment broth (BEB) (Rásbâck et al., 2005) at 80 °C. *B. pilosicoli* was cultured on fastidious anaerobe blood agar (FABA), *Brachyspira* selective agar and in BEB (Rásbâck et al., 2005), in an anaerobic cabinet (10% H2 and 10% CO2 in N2) (Don Whitley Scientific), statically at 37 °C for up to 5 days.

*L. reuteri* LM1 was isolated from the faeces of healthy conventional commercial poultry confirmed free from *Brachyspira* (Mappley et al., 2011), and was cultured from a stock maintained in heart infusion broth (HIB) + 30% (v/v) glycerol (Oxoid) at −80 °C. Lactobacilli were cultured on De Man–Rogosa–Sharpe (MRS) agar and in MRS broth (De Man et al., 1960), in an anaerobic jar (94% H2 and 6% CO2) using a GasPak plus system (BBL) at 37 °C for 16 h.

**Preparation of bacterial strains for the in vivo studies.** *B. pilosicoli* B2904 was harvested by centrifugation of a BEB broth culture (50 ml) at early exponential phase growth (approx. 4 days) determined from a growth curve produced using the FLUOstar Optima instrument (unpublished data) with enumeration of *B. pilosicoli* by Helber cell counting chamber. Bacterial cells were harvested by centrifugation (2447 g, 10 min) and the pellet was resuspended in 0.1 M PBS (pH 7.2) to yield 5 × 10^8 c.f.u. ml^-1; this was performed rapidly under aerobic conditions, to minimize exposure to oxygen.

**Experimental birds.** Thirty ISA Warren laying pullets were purchased from a commercial breeder at approximately 17 weeks of age. Upon arrival (day 1), the birds were randomly divided into two groups of 15 birds each and housed in separate, negative pressure rooms within a biosecure containment facility. Birds were housed with 12 h of artificial light per 24 h. Commercial unmedicated pelleted feed was provided *ad libitum*. Drinking water was provided *ad libitum*; one group received distilled water and the other group received distilled water supplemented with 2.5 × 10^8 c.f.u. ml^-1 L. reuteri LM1 throughout the study from day 1, and water replacements were provided daily. These studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, and were approved by the Animal Health and Veterinary Laboratories Agency ethics committee.

**Experimental challenge.** After a week of acclimatization with *L. reuteri* LM1 in the drinking water of one of the groups (18 weeks of age), the two groups each of 15 birds were pre-dosed with 2 ml 10% sodium bicarbonate solution to neutralize the crop acid and challenged by oral gavage on three alternate days (days 8, 10 and 12) with *B. pilosicoli*. Twenty minutes after crop neutralization, all birds were challenged by oral gavage with 5 × 10^8 c.f.u. ml^-1 B. pilosicoli B2904.

**Experimental monitoring.** All birds were weighed and cloacal swabs were taken upon arrival and throughout the study (days indicated in Fig. 1). Cloacal swabs were plated onto *Brachyspira* selective agar that was incubated anaerobically at 37 °C for 8 days. The cultures were examined at 24 h intervals for growth which, when detected visually, was picked aseptically from the plate using a sterile loop and used for PCR to verify the presence of *B. pilosicoli* (as described below). Biochemical tests for haemolysis on FABA agar, indole production, hippurate hydrolysis, and activities of x-galactosidase, x-glucosidase and β-glucosidase (Fellstrom et al., 1995, 1997), were performed on reisolated *Brachyspira* strains to ensure that they shared the same profile as the challenge strain.

Eggs were collected daily and weighed and scored for the extent of faecal eggshell staining without knowledge of which group they belonged to. Scoring was performed blind, with 0 denoting a clean eggshell and 5 a heavily stained eggshell, as previously described (Stephens & Hampson, 2002a).

Representative random samples of fresh faeces were taken from the floor of each of the rooms on a daily basis for the duration of the study, and PCR was applied to extracted faecal DNA to detect the...
Fig. 1. Experimental monitoring of birds throughout the study, demonstrating the percentage of cloacal swabs positive for *B. pilosicoli* by culture and PCR (a), the mean bird weight (b) and the mean faecal moisture content (c). The mean was based on 15 birds until day 17 and then 12 birds until the study end in both the untreated (closed circle, solid line) and *L. reuteri* LM1-treated (open square, dashed line) groups. The arrows on the x-axis indicate the days of challenge. Significance is shown in cases where the final data points of the challenged groups and the negative control group differed significantly; *P*<0.05; **P*<0.01.

Prior to challenge with *B. pilosicoli* B2904, all cultures from cloacal swabs from both groups of birds, and random faecal samples taken from the floor of both of the rooms in which the birds were housed, were negative by PCR and culture for the genus *Brachyspira* and species *B. pilosicoli*. Following challenge, the spirochaetes were readily reisolated from cloacal swabs of both groups. Fewer birds were positive for *B. pilosicoli* by culture in the *L. reuteri* LM1-treated group (75.0 %) than in the untreated group (83.3 %) (Fig. 1a), perhaps attributable to the protective

Identification and detection of bacteria by PCR. To identify and confirm the species of bacteria, when detected visually, colonies were picked aseptically using a sterile loop and used directly in PCR tests to verify the presence of the genus *Brachyspira* (Phillips et al., 2005) and species *B. pilosicoli* (Mikosza et al., 2001).

To detect the bacteria in faeces, DNA extracts were prepared from a mixed pool of faecal samples using the Maxwell 16 Tissue DNA Purification kit (Promega). The faecal DNA was subjected to PCR, using the *B. pilosicoli* genus- and species-specific primers described above and *L. reuteri*-specific primers (Kwon et al., 2004).

Examination at post mortem. At 5 and 21 days post final *B. pilosicoli* challenge, three birds per group were euthanized by cervical dislocation and subjected to post-mortem examination. At post-mortem examination, the caeca were removed and weighed. Sections (~1 g) of each of the duodenum, jejunum, ileum, caeca, colon, liver, spleen, ovary, infundibulum, magnum, isthmus, uterus and vagina were sampled aseptically and placed in 0.1 M PBS for bacterial enumeration. The tissue samples were homogenized in 9 ml 0.1 M PBS and diluted serially (10^0 to 10^-5), and samples (20 µl) were plated onto *Brachyspira* selective agar and incubated anaerobically for 3–5 days at 37 °C. This process was performed swiftly to minimize exposure to oxygen. *B. pilosicoli* colonies were enumerated subsequently and colonies confirmed as *B. pilosicoli* by PCR (Mikosza et al., 2001). In the absence of distinguishing features of *B. pilosicoli* B2904, it was assumed that these were the challenge strains, since the poultry were *Brachyspira*-free prior to challenge and were housed in a biosecure containment facility. At post-mortem, all birds were examined blind by a board-certified veterinary pathologist and any gross observations recorded. Tissue samples from each of the caecal tonsil, caeca, colon and liver were placed in 10 % neutral buffered formalin for histopathological examination. For histopathology, tissues were fixed for a minimum period of 24 h and then trimmed and processed to paraffin wax blocks. Tissues were then sectioned (4 µm sections), stained with haematoxylin and eosin (HE) and examined blind by a board-certified veterinary pathologist.

HE staining of tissue samples. Trimmed tissues fixed in 10 % neutral buffered formalin were processed in paraffin wax, and 4 µm sections were HE-stained. The samples were examined blind and photographed using a Leica DM4000B light microscope with a Leica DFC480 digital camera (Leica Microsystems). Two cross sections of caecal tonsil, caeca and colon, and one cross section of liver (right lobe), were examined per animal. Post-mortem examinations were performed aseptically, and deep tissue samples were taken where possible to exclude the possibility of surface contamination.

Statistical analysis. All results with quantitative data were presented as the mean and SD of the means. An unpaired *t* test was performed with commercially available software (GraphPad Prism), with a 95 % confidence interval.

RESULTS AND DISCUSSION

Colonization of chickens by *B. pilosicoli*

Prior to challenge with *B. pilosicoli* B2904, all cultures from cloacal swabs from both groups of birds, and random faecal samples taken from the floor of both of the rooms in which the birds were housed, were negative by PCR and culture for the genus *Brachyspira* and species *B. pilosicoli*. Following challenge, the spirochaetes were readily re-isolated from cloacal swabs of both groups. Fewer birds were positive for *B. pilosicoli* by culture in the *L. reuteri* LM1-treated group (75.0 %) than in the untreated group (83.3 %) (Fig. 1a), perhaps attributable to the protective
effect of _L. reuteri_ LM1 provided in the drinking water from a week prior to challenge and throughout the study. In the untreated group, ≥ 75.0 % remained culture-positive until 12 days after final challenge (day 24), whereas in the _L. reuteri_ LM1-treated group, the percentage of positive birds decreased to 50.0 % by this point. By 19 days after final challenge (day 33), the percentage of culture-positive birds decreased to 50.0 % in the untreated and 16.7 % in the _L. reuteri_ LM1-treated group. The number of _B. pilosicoli_-positive birds decreased at a greater rate in the _L. reuteri_ LM1-treated group, but the mechanism of this effect is unclear and may be due to competitive exclusion and/or a potential effect on the modulation of the intestinal microbiota. Given the _in vitro_ study data (Mappley et al., 2011), it is not unreasonable to argue that the negative impact on growth, motility and cellular invasion mediated by _L. reuteri_ LM1 contributed to the protective effects observed against _B. pilosicoli_. In future _in vivo_ studies, it would be interesting to interrogate these mechanisms further.

Faecal DNA isolated from representative random faecal samples taken from the floor of each of the rooms of birds tested positive for the genus _Brachyspira_ and species _B. pilosicoli_ by PCR from the day of the final challenge (day 12) and remained positive throughout the study in the untreated group. In the _L. reuteri_ LM1-treated group, the faecal samples tested positive for 16 days post final challenge (day 28) and then negative until the end of the study, perhaps reflecting an extensive reduction in the number of _B. pilosicoli_-positive birds. Detection of _B. pilosicoli_ by culture was mostly synonymous with direct PCR on faecal DNA. However, _B. pilosicoli_ was detected by PCR but not culture on five days from untreated group faeces (days 12, 14, 24, 30 and 32) and two days from _L. reuteri_ LM1-treated group faeces (days 13 and 27). Hence, the sensitivity of PCR was greater than that of culture, but as yet unquantified.

**Colonization of chickens by _L. reuteri_**

_L. reuteri_ LM1 was shown to survive in distilled water for 2.5 h without significant reduction in viability, and assuming each chicken consumed approximately 200 ml per day (20.8 ml in 2.5 h) (Grashorn & Simonovic, 2009), each chicken should have consumed about 5 × 10⁹ c.f.u. of viable lactobacilli. Assuming the majority of these remained viable within the GI tract of the birds, it is likely that active mechanisms of inhibition of _Brachyspira_ were involved in reducing the burden of this pathogen. _In vitro_ it has been demonstrated that LM1 inhibits _B. pilosicoli_ by both pH-dependent and -independent mechanisms, and since non-viable lactobacilli inhibit the motility of _B. pilosicoli_ (Mappley et al., 2011), ingestion of non-viable cells may also aid in the intervention against _B. pilosicoli_.

Representative faecal samples from the _L. reuteri_ LM1-treated group tested positive for _L. reuteri_ by PCR from day 1 and on each day of the study, whereas those from the untreated group were variably positive but tested negative on nine days of the study, indicating that the level of colonization was lower in this group due to its absence from the drinking water. The presence of _L. reuteri_ in the birds was therefore a probable contribution, both passive and active, to inhibition of _Brachyspira_. The numbers of LM1 were not enumerated, and whether inhibition was dose-dependent requires further investigation. The fact that sporadic samples from the control group were _L. reuteri_-positive by PCR during the study suggests that either the cell number or the specific _L. reuteri_ strain was important in inhibition.

The PCR used was not specific for _L. reuteri_ LM1, and therefore will have detected LM1 and other native strains. Thus, other _L. reuteri_ strains may have colonized the birds in both groups prior to the study. As this species is a common commensal of the GI tract (Abbas Hilmi et al., 2007) and the birds were 17 weeks of age on arrival, this is not unreasonable. We argue, however, that as groups were kept in biosecure containment, the effects seen were due to the additional _L. reuteri_ LM1 treatment. Without distinguishing features to enable specific detection of _L. reuteri_ LM1, detection of _L. reuteri_ primarily in the treated group strongly supports the argument that it was indeed _L. reuteri_ LM1, a strain of chicken origin that colonized the chickens to which it was administered. The data indicate elevated numbers of _L. reuteri_ in the treated group, which were probably strain LM1, plus native strains already present. The control group contained _L. reuteri_ but in lower numbers, and hence it is not unreasonable to argue that the impact on _B. pilosicoli_ infection may relate to the elevated numbers of all _L. reuteri_ strains or of LM1 specifically.

**Bird weights**

The mean bird weight increased at a similar rate for both groups up to challenge, after which the rate of weight gain in the _L. reuteri_ LM1-treated group was unaffected, whereas the rate decreased in the untreated group (Fig. 1b). By the end of the study (day 31), the mean weight of the birds in the _L. reuteri_ LM1-treated group was approximately 0.09 kg higher than that of the untreated group (P < 0.01). The administration of lactobacilli probiotics has demonstrated positive effects on weight gain in poultry before (Lan et al., 2003; Timmerman et al., 2006), and our findings suggest that strain LM1 acts similarly to those _Lactobacillus_ probiotics reported earlier.

**Faecal moisture content**

Following challenge with _B. pilosicoli_, the faecal moisture content of the untreated group increased significantly compared with the _L. reuteri_ LM1-treated group (P < 0.05) (Fig. 1c). The faecal moisture content of the untreated group continued to increase until 8 days post final
challenge (day 20), when it was significantly greater (~6.8 %) than that of the *L. reuteri* LM1-treated group (*P*<0.05). The faecal moisture content of both groups decreased after day 21. In the *L. reuteri* LM1-treated group, there was no significant difference in faecal moisture content between the first and final days of the study. However, there were significant differences between these time points for the untreated group (*P*<0.05). At the end of the study, the faecal moisture content of the *L. reuteri* LM1-treated group was significantly lower than the untreated group (*P*<0.05). Rather than a direct effect of the lactobacilli reducing faecal moisture content, it is probable that this effect resulted from the mitigation of *B. pilosicoli* colonization and infection, hence alleviating clinical symptoms including increased faecal moisture content (Jamshidi & Hampson, 2003).

**Egg production**

Egg production in both groups was similar up to challenge, after which there was an 8-day period when the *L. reuteri* LM1-treated group generally laid slightly greater numbers of eggs per day than the untreated group (Table 1). From 6 days post final challenge (day 18), the number of eggs laid per day appeared to stabilize, with both groups of birds laying between eight and 11 eggs per day. Previous studies have related probiotic supplementation to improved egg laying between eight and 11 eggs per day. Previous studies laid per day appeared to stabilize, with both groups of birds 6 days post final challenge (day 18), the number of eggs of eggs per day than the untreated group (Table 1). From LM1-treated group generally laid slightly greater numbers after which there was an 8-day period when the Egg production in both groups was similar up to challenge, but subsequently (except days 15, 17 and 32), the mean egg weight of the *L. reuteri* LM1-treated group was greater than that of the untreated group (Table 1). Mean egg weights of the *L. reuteri* LM1-treated group were significantly greater than those of the untreated group on days 18, 25–31 and 33 (*P*<0.05). Significant increases in egg weight have been associated with probiotic supplementation, and it was postulated that this resulted from increased digestion of nutrients by probiotic bacteria (Nahanshon *et al.*, 1992, 1994; Tortuero & Fernandez, 1995). The greater egg weights of the *L. reuteri* LM1-treated group may simply reflect the mitigation of *B. pilosicoli* infection aided by administration of *L. reuteri* LM1.

Eggs were scored on the extent of faecal staining without knowledge of the group to which they belonged. The mean score for the faecal staining of eggs from the *L. reuteri* LM1-treated group was generally lower than that of the untreated group after the challenge with *B. pilosicoli* (Table 1); on nine of the 21 days after the final challenge, there were significant differences between the mean scores of the two groups (*P*<0.05).

**Post-mortem examination at 5 days post final Brachyspira challenge**

At 5 days post final challenge, post-mortem examination revealed few macroscopic pathological findings. *Petchia* were detected in the duodenum and upper jejunal of two birds in both groups, but no *Brachyspira* were isolated from these tissues. Interestingly, the caeca from the untreated group were smaller, with a lower mean caecal weight (12.6±2.4 g), without significance, than that of the *L. reuteri* LM1-treated group (13.8±1.2 g). *B. pilosicoli* were recovered from the caeca and colon of all three birds in both groups, but at significantly greater numbers in the caeca (*P*<0.05) and colon (*P*<0.001) of birds from the untreated group (Fig. 2a). The mean numbers of *B. pilosicoli* recovered from the caeca and colon of the untreated group were nine- and 50-fold greater, respectively, than those recovered from birds of the *L. reuteri* LM1-treated group. This suggests that *L. reuteri* LM1 may have inhibited *B. pilosicoli* growth, motility and/or adherence, an effect previously observed in vitro (Mappley *et al.*, 2011). This greater colonization in the untreated birds may relate to the reduced mean weight of the caeca. *B. pilosicoli* were also isolated from the ileum, spleen and isthmus of one bird from the untreated group and two birds from the *L. reuteri* LM1-treated group, the vagina of two birds from the untreated group and one bird from the *L. reuteri* LM1-treated group, the liver of one bird from each group and the uterus of just one bird from the untreated group.

Microscopical examination of the tissues sampled at 5 days post final challenge revealed no pathology in the colon and caecal tissues in the untreated birds examined at post-mortem. However, crypt abscesses, secondary follicles and abundant diffuse gut-associated lymphoid tissue (GALT) were identified in the caecal tonsils of one of the three birds examined from the *L. reuteri* LM1-treated group. Interestingly, frequent multifocal lymphohistiocytic infiltration was noted in perportal spaces of the liver of two birds examined from the untreated group (Fig. 3a). Despite their recovery, this pathology was not observed in birds examined from the *L. reuteri* LM1-treated group. *B. pilosicoli* have previously been isolated from the liver (Kostman *et al.*, 1995) and bloodstream (Bait-Merabet *et al.*, 2008; Prim *et al.*, 2011) in humans, and *Brachyspira* infection was associated with hepatic and splenic amyloidosis in duck flocks (Glávit *et al.*, 2011). This is the first report of *Brachyspira* in the liver of chickens.

**Post-mortem examination at 21 days post final Brachyspira challenge**

Post-mortem examination at 21 days after the final challenge revealed that the caeca were again smaller in the birds of the untreated group, with their mean caecal weight (18.1±3.7 g) 1.6 g lower, without significance, than that of the *L. reuteri* LM1-treated group birds (19.7±4.4 g). *B. pilosicoli* were recovered from the caeca...
and colon of all three birds of the untreated group, but from the caeca of only two birds and colon of one bird from the L. reuteri LM1-treated group. The mean number of B. pilosicoli isolated from the caeca of birds from the untreated group was approximately 33-fold greater than that of the L. reuteri LM1-treated group ($P<0.05$) (Fig. 2b). Moreover, the mean number isolated from the colon of birds from the untreated group was approximately 24-fold greater than that of the L. reuteri LM1-treated group ($P<0.01$). The smaller caeca of birds from the untreated group at this time point may again be explained by the greater extent of B. pilosicoli colonization in untreated birds. Brachyspira were also recovered from the ileum, liver and uterus of just one bird from the untreated group, the isthmus of one bird of the untreated group and two birds of the L. reuteri LM1-treated group, and the vagina of two birds from the untreated group and one bird from the L. reuteri LM1-treated group.

Concordant with the bacteriological findings, more severe pathology was noted in birds from the untreated group, including secondary follicle proliferation, lymphoid hyperplasia

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<td>51.74 ± 2.72</td>
</tr>
<tr>
<td>24</td>
<td>11</td>
<td>52.24 ± 3.97</td>
</tr>
<tr>
<td>25</td>
<td>9</td>
<td>54.81 ± 3.49</td>
</tr>
<tr>
<td>26</td>
<td>11</td>
<td>52.54 ± 3.35</td>
</tr>
<tr>
<td>27</td>
<td>11</td>
<td>53.15 ± 2.86</td>
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<tr>
<td>28</td>
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<td>52.76 ± 2.78</td>
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<td>29</td>
<td>8</td>
<td>53.89 ± 2.61</td>
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<tr>
<td>30</td>
<td>12</td>
<td>52.45 ± 2.66</td>
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<tr>
<td>31</td>
<td>11</td>
<td>54.10 ± 2.65</td>
</tr>
<tr>
<td>32</td>
<td>10</td>
<td>54.90 ± 3.44</td>
</tr>
<tr>
<td>33</td>
<td>11</td>
<td>54.69 ± 3.14</td>
</tr>
</tbody>
</table>

*Faecal staining of egg score: 0, clean eggshell; 5, heavily stained eggshell.
†$P<0.05$.
‡$P<0.01$.
§$P<0.001$. 

Table 1. Monitoring of egg production

The number of eggs laid and mean egg weights were recorded alongside mean scores for the faecal staining of eggshells on a daily basis throughout the study. Fifteen birds were present in the study until day 17, and then 12 birds were present until the study end in both the untreated and L. reuteri LM1-treated groups. Significance is shown in cases where the mean score for the faecal staining differed significantly between the two groups.
(Fig. 3c), crypt abscesses, distended crypts and subepithelial haemorrhages (Fig. 3e) in the caecal tonsil, which have been described before in poultry colonized by \textit{B. pilosicoli} \cite{Feberwee et al., 2008}. Pathology is not necessarily correlated with numbers of \textit{B. pilosicoli}, since no pathology was noted in the colon and caeca at 5 days, despite \textit{B. pilosicoli} numbers differing to a similar extent between the two groups, although this may reflect the timing of induction of pathology. Less evident pathology was noted in the liver, with the liver of the bird from the

\textbf{Fig. 2.} Colonization levels of \textit{B. pilosicoli} in different tissues sampled at post mortem, both 5 days post final challenge (a) and 21 days post final challenge (b). The mean was taken from three birds examined at the two time points from birds of the untreated (closed bar) and \textit{L. reuteri} LM1-treated (open bar) groups. Significance is shown in cases where \textit{B. pilosicoli} colonization in the same tissue samples of the two groups differed significantly; *\textit{P} < 0.05; **\textit{P} < 0.01; ***\textit{P} < 0.001.

\textbf{Fig. 3.} HE-stained tissues demonstrating histopathological changes in birds experimentally challenged with \textit{B. pilosicoli} B2904 from the untreated (a, c and e) and \textit{L. reuteri} LM1-treated groups (b, d and f). The tissues were examined blind and the images were selected to display the pathology recorded. Pathology was noted in the liver at 5 days post \textit{Brachyspira} challenge (a, b) and the caecal tonsil (c–f) at 21 days post final \textit{Brachyspira} challenge. In tissues examined from birds in the untreated group, periportal lymphocytic infiltration was observed in the liver (i). Haemorrhages (ii), abundant secondary follicles (iii) and distended crypts containing bacterial colonies (iv) were observed in caecal tonsil tissues. Only mild pathology was noted in the tissues examined from the \textit{L. reuteri} LM1-treated group.
untreated group from which *B. pilosicoli* was isolated displaying few areas of lymphocytic infiltration, perhaps induced by *B. pilosicoli* infection. In birds from the *L. reuteri* LM1-treated group, active secondary follicles were noted in the caecal tonsil of one bird and crypt abscesses in the caecal tonsil of another, but in general, the tissues displayed less pathology than those of the untreated group (Fig. 3d, f). The reduction in *B. pilosicoli* colonization and the severity of the pathology observed in *L. reuteri* LM1-treated birds highlight a potential protective function of *L. reuteri* LM1 against AIS.

**Concluding remarks**

Previously, we demonstrated the ability of *L. reuteri* LM1 to mitigate the pathobiology induced by *Brachyspira* by inhibiting growth, motility and adherence *in vitro* (Mappley et al., 2011). Thus, the scope of this study focused on the potential competitive exclusion of *L. reuteri* LM1 against AIS in chickens experimentally challenged with *B. pilosicoli* B2904. Given the weight of data indicating a reduction in colonization and clinical symptoms, we argue that *L. reuteri* LM1 confers health benefits *in vivo* and can be truly described as a probiotic. Whilst some effects may relate to a direct effect from probiotic supplementation such as weight gain, it is possible that the antagonism of *B. pilosicoli* is largely responsible. The rapid growth and robust nature of lactobacilli compared with the slow-growing, fastidious *Brachyspira* make these species ideal probiotic candidates for intervention against AIS, possibly by niche competition, passive co-aggregation and/or acidification. Our *in vitro* studies demonstrated that heat-killed lactobacilli and cell-free supernatant inhibit aspects of *Brachyspira* biology (Mappley et al., 2011). Thus, even non-viable LM1 may also have a protective effect by purely passive mechanisms such as binding *Brachyspira*. Developing specific LM1 markers for detection would be valuable in tracing survival numbers *in vivo* and should be part of future studies that focus on elucidation of mechanisms behind the effects observed. Moreover, nutritional modification by prebiotics may enhance *L. reuteri* numbers, including those naturally present, to induce the same effect as supplementation of water with strain LM1, although further work is required to assess this. This study warrants further investigation into the development of *L. reuteri* LM1 as a prophylactic probiotic to protect against enteric infections such as *Brachyspira* and potentially as a therapeutic treatment for such infections.

**ACKNOWLEDGEMENTS**

The authors would like to thank members of the Animal Service Unit (ASU) and the Department of Histopathology at the Animal Health and Veterinary Laboratories Agency for their technical support with *in vivo* studies. We acknowledge receipt of funding for this study from the British Egg Marketing Board Research and Education trust.

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