Lactobacilli Antagonize the Growth, Motility, and Adherence of *Brachyspira pilosicoli*: a Potential Intervention against Avian Intestinal Spirochetosis[▽]

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Avian intestinal spirochetosis (AIS) results from the colonization of the ceca and colorectum of poultry by pathogenic *Brachyspira* species. The number of cases of AIS has increased since the 2006 European Union ban on the use of antibiotic growth promoters, which, together with emerging antimicrobial resistance in *Brachyspira*, has driven renewed interest in alternative intervention strategies. Probiotics have been reported as protecting livestock against infection with common enteric pathogens, and here we investigate which aspects of the biology of *Brachyspira* they antagonize in order to identify possible interventions against AIS. The cell-free supernatants (CFS) of two *Lactobacillus* strains, *Lactobacillus reuteri* LM1 and *Lactobacillus salivarius* LM2, suppressed the growth of *Brachyspira pilosicoli* B2904 in a pH-dependent manner. In *in vitro* adherence and invasion assays with HT29-16E three-dimensional (3D) cells and in a novel avian cecal *in vitro* organ culture (IVOC) model, the adherence and invasion of *B. pilosicoli* in epithelial cells were reduced significantly by the presence of lactobacilli ($P < 0.001$). In addition, live and heat-inactivated lactobacilli inhibited the motility of *B. pilosicoli*, and electron microscopic observations indicated that contact between the lactobacilli and *Brachyspira* was crucial in inhibiting both adherence and motility. These data suggest that motility is essential for *B. pilosicoli* to adhere to and invade the gut epithelium and that any interference of motility may be a useful tool for the development of control strategies.

Avian intestinal spirochetosis (AIS) is recognized worldwide as an enteric disease that affects layer and broiler breeder chickens, leading to clinical enteritis and reduced performance (52). The disease results from the colonization of the ceca and colorectum by the fastidious anaerobic spirochete *Brachyspira*. Clinical symptoms of AIS include reduced egg production with delayed onset of laying, chronic diarrhea with fecal staining of eggs, weight loss, and increased flock morbidity rates (7, 49). Currently, *Brachyspira alvinipulli*, *B. intermedia*, and *B. pilosicoli* are considered pathogenic to poultry (20, 50, 51). However, other *Brachyspira* species have been isolated from poultry with decreased egg production (8). Although the mechanisms of pathogenesis are unclear, colonization of poultry, swine, and humans by *B. pilosicoli* is characterized by its ability to form end-on attachments to the intestinal epithelial surface and to invade the surface epithelium (18, 24, 25).

In the United Kingdom, the incidence of *Brachyspira* in commercial and free-range flocks has been estimated at 74% and 90%, respectively (8). AIS disease associated with *Brachyspira* infection is reported to be increasing, which may be attributed at least partially to the 2006 European Union (EU) ban on the use of antibiotics as growth promoters in

livestock (9). In 2006, the annual cost of the disease to the United Kingdom laying industry was estimated at £14 million, and this figure continues to rise (7). Additionally, antibiotic resistance appears to be increasing among *Brachyspira* organisms, including an emerging resistance to the most commonly used antibiotic for AIS treatment, tiamulin. Resistance has been reported for porcine *Brachyspira* strains (27, 34, 42), and elevated MICs have been demonstrated for strains of poultry origin (21). The rise of endemic diseases since the ban and of antimicrobial resistance has increased interest in developing alternative intervention strategies; one such alternative therapy which is being researched extensively is the use of probiotics (12).

Probiotics, which include genera such as *Bifidobacterium* and *Lactobacillus* (12), are described as live microorganisms that confer health benefits on the host when administered in adequate quantities (17). Multiple mechanisms have been proposed for the competitive exclusion (CE) of pathogenic microorganisms by probiotics, and they include secretion of antimicrobial compounds, competition for essential nutrients, competition for host cell binding receptors, and immunomodulation of the gut mucosa (54). Probiotics have demonstrated promise, *in vitro* and *in vivo*, as CE agents against *Escherichia coli*, *Salmonella*, *Clostridium*, and *Campylobacter* infection in poultry (31, 32, 46, 53, 55) and have been shown to colonize the ceca (41), the host niche of many of these pathogens, including *Brachyspira*.

Recently, a patent application was published (46a) describ-

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ing the use of *Lactobacillus johnsonii* D115 as a probiotic against *Brachyspira* species, based on its ability to inhibit *B. pilosicoli* and *B. hyodysenteriae* through the production of hydrogen peroxide and a proteinaceous antimicrobial compound. *Lactobacillus rhamnosus* and *Lactobacillus farciminius* strains have also been implicated in inhibiting the motility of *Brachyspira* by coaggregation with spirochetes and by eliciting a stress response (6). However, to date, no studies have investigated the adherence and invasion dynamics of avian *B. pilosicoli* with epithelial cells in relation to treatment with probiotics. Here we have employed motility, growth inhibition, adhesion, and invasion assays to investigate the *in vitro* antagonistic effects of *Lactobacillus reuteri* LM1 and *Lactobacillus salivarius* LM2 on *B. pilosicoli* B2904.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. pilosicoli* B2904 was originally isolated in the United Kingdom from the feces of a chicken exhibiting clinical signs of AIS. *B. innocens* B2960 was isolated in the United Kingdom from the feces of a healthy chicken. *L. reuteri* LM1 and *L. salivarius* LM2 were isolated from conventional commercial poultry feces proven free from *Brachyspira* by culture and multiplex PCR (M-PCR) (1). *Escherichia coli* DH5 α -K-12 was obtained from Gibco. Stock cultures of lactobacilli and *E. coli* were maintained in heart infusion broth (HIB) plus 30% (vol/vol) glycerol (Oxoid), and *Brachyspira* stock cultures were maintained in fetal calf serum (FCS) (Sigma-Aldrich) plus 30% (vol/vol) *Brachyspira* enrichment broth (BEB) (43), at -80°C .

Lactobacilli were cultured in de Mann-Rogosa-Sharpe (MRS) broth in an anaerobic jar (94% H₂ and 6% CO₂) in a GasPak Plus system (BBL) at 37°C for 16 h. *L. reuteri* LM1 tested positive for hydrogen peroxide production by a previously described method (36). *E. coli* cells were cultured aerobically in Luria-Bertani (LB) broth for 16 h at 37°C , with gentle agitation (225 rpm). *Brachyspira* organisms were cultured on fastidious anaerobe blood agar (FABA) or in BEB in an anaerobic cabinet (10% H₂ and 10% CO₂ in N₂) (Don Whitley Scientific) at 37°C for 3 to 5 days.

Heat-inactivated lactobacilli were prepared by heating aliquots of viable bacterial suspensions for 20 min at 80°C . Spent *Lactobacillus* growth medium was obtained by centrifuging *Lactobacillus* broth cultures (10^9 CFU/ml) at $2,500 \times g$ for 10 min at ambient temperature and then filtering the supernatant through a 0.2- μm filter (Sartorius Stedim) to yield the cell-free supernatant (CFS). The pH value of the CFS was adjusted using 10 M sodium hydroxide (Sigma-Aldrich).

Growth and inhibition assays. Heat-inactivated lactobacilli (10^6 CFU/ml) and their CFS (10% [vol/vol]), at an original (3.8) or adjusted pH value (4.5 or 7.2), were added to BEB inoculated with either *B. pilosicoli* B2904 or *B. innocens* B2960 (10^6 CFU/ml) and incubated anaerobically at 37°C . Control broths were prepared with MRS (10% [vol/vol]) at pH 3.8, 4.5, and 7.2. *Brachyspira* organisms were enumerated at 24-h intervals over a 120-h period by using a Helber counting chamber (Hawksley) under dark-field microscopy (Olympus CX21; magnification, $\times 400$). Additionally, 100 μl of each broth mixture was inoculated into wells of a microtiter plate (Iwaki) and incubated in a FLUOstar Optima instrument containing an anaerobic atmosphere at 37°C . Optical densities at 600 nm (OD₆₀₀ values) were taken every 2.77 h for 125 h. Assays were performed in triplicate on three separate occasions.

Agar motility inhibition assay. Agar motility inhibition assays were performed using the spot test as previously described (6). Each *Lactobacillus* strain, either viable or heat inactivated and resuspended in 0.1 M phosphate-buffered saline (PBS) (1×10^9 CFU/ml), was preincubated anaerobically with either a *B. pilosicoli* B2904 or *B. innocens* B2960 cell suspension in 0.1 M PBS (1×10^9 CFU/ml) (1/1 [vol/vol]) for 4 or 24 h in a microcentrifuge tube (Eppendorf) at 37°C . Following preincubation, 5 μl of each mixed suspension was spotted in triplicate onto *Brachyspira* selective agar (43) and incubated anaerobically at 37°C for 8 days. The extents of motility and hemolysis were examined visually at 24-h intervals and compared to the growth of *B. pilosicoli* B2904 and *B. innocens* B2960 cell suspension controls. Following the monitoring period, growth from each assay was subcultured onto FABA to assess viability. Assays were performed in triplicate on three separate occasions.

Culture of mucus-secreting, colonic HT29-16E 3D cells. HT29-16E cells were cultured in a three-dimensional (3D) cell model as previously described (22). HT29-16E bead stock cultures were stored in liquid nitrogen (-196°C). Cells were thawed at 37°C in a water bath, reconstituted in Dulbecco's modified

Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% FCS, 1% nonessential amino acids ($100\times$), 2 mM L-glutamine, and gentamicin (50 $\mu\text{g/ml}$) (Sigma-Aldrich) in a 250-ml tissue culture flask, and incubated in the presence of 5% CO₂ at 37°C until a confluent monolayer was present. Trypsinized cells were resuspended in GTSF-2 medium (33) to 2×10^5 cells/ml, combined with 5 mg/ml Cytodex microcarrier beads (Cytodex 3; 133 to 215 μm) (Sigma-Aldrich) and 500 μl sterile glucose solution (1 g/liter), and then dispensed into a 50-ml rotating wall vessel (RWV) (Synthecon). The RWV was incubated in the presence of 5% CO₂ at 37°C for 21 to 23 days; to allow cell adherence to the beads, the culture medium was not changed for the first 48 h, but subsequently, 90% of the culture medium was changed every 24 h. Over 21 to 23 days, the rotation speed was gradually increased from 13.0 to 30.0 rpm to ensure that cell-bead aggregates remained in suspension.

Adhesion and invasion assays using HT29-16E 3D cells. Human HT29-16E mucus-secreting colonic cells were selected for use in adhesion and invasion assays because this cell line is well established for studies of the interaction of enteroinvasive bacteria, such as *Salmonella* and *E. coli*, with the intestinal epithelium (28, 37) and has demonstrated the ability to differentiate (10, 26). Furthermore, preliminary studies (not described here) confirmed that *B. pilosicoli* B2904 adhered to and invaded this cell line.

Adhesion and invasion assays were performed essentially as described previously (14, 47). Briefly, *B. pilosicoli* and *Lactobacillus* inocula were prepared by centrifugation ($2,447 \times g$, 10 min) of 5-day and 24-h broth cultures, respectively, after which the pellets were resuspended in tissue culture medium to yield 5×10^7 CFU/ml. Following 21 to 23 days of incubation, cell aggregates were removed from the RWV, resuspended to yield 5×10^5 cells/ml, and seeded into 1.5-ml microcentrifuge tubes (Eppendorf). Cells were inoculated by different experimental strategies (Table 1) with a total of 1 ml bacterial inoculum prepared as described above and then were incubated at 37°C in an anaerobic cabinet with gentle agitation.

To enumerate the *B. pilosicoli* organisms associated with infection, the cells were washed three times with Hanks' balanced salt solution (HBSS) (Sigma-Aldrich), and a homogenous cell suspension was achieved by gentle pipetting. To differentiate the intracellular (invaded) *B. pilosicoli* organisms, 100 $\mu\text{g/ml}$ gentamicin solution (Sigma-Aldrich) was added to incomplete DMEM, delivered to each microcentrifuge tube, and incubated for another 2 h. Cells were subsequently washed three times with HBSS, and a homogenous cell suspension was prepared as described above. Cell suspensions were diluted serially (10^0 to 10^{-7}), plated onto *Brachyspira* selective agar, and incubated anaerobically for 3 to 5 days at 37°C . The number of adherent *Brachyspira* organisms was determined by subtracting the number of invaded from the number of associated *Brachyspira* organisms. All HT29-16E 3D cell experiments were conducted in triplicate on three separate occasions.

Avian cecal IVOC association assays. Immediately prior to *in vitro* organ culture (IVOC) studies, 36 commercial 20-week-old ISA brown laying hens (confirmed free of *Brachyspira* by pooled fecal culture and M-PCR) were euthanized by cervical dislocation, and at postmortem examination, the ceca were sampled aseptically and stored in precooled complete RPMI 1640 medium containing 10% FCS, 0.25% lactalbumin hydrolysate, 75 mM mercaptoethanol, 0.2 $\mu\text{g/ml}$ hydrocortisone (in chloroform-ethanol [1:1]), 0.1 $\mu\text{g/ml}$ insulin, and 2 mM (each) L-glutamine and L-aspartate (Sigma-Aldrich) (19). Tissues were prepared as described previously (11). Briefly, tissues were washed in complete medium and trimmed aseptically to remove excess mesenteric adipose tissue. Tissue sections were immobilized in CellCrown inserts (Scaffdex) so that the mucosal side was immobilized between the insert and its base, providing a polarized IVOC system with a fixed surface area where the bacterial inoculum was limited to the mucosal side of the explant tissue. Immobilized tissues were placed into a 24-well plate (BD Biosciences) with the mucosal side facing upwards and then submerged in 500 μl complete medium.

For IVOC association assays, *B. pilosicoli* and both viable and heat-inactivated *Lactobacillus* inocula were prepared by centrifugation ($2,447 \times g$, 10 min) of 5-day (*B. pilosicoli*) and 24-h (lactobacilli) broth cultures, after which the pellets were resuspended in complete medium to yield 10^8 CFU/ml. Tissues were inoculated by a number of different experimental strategies (Table 1) with a total of 1 ml bacterial inoculum and then were incubated at 37°C in an anaerobic cabinet. Uninfected control tissues were used to confirm the absence of preexisting *Brachyspira* infection. Following infection, tissues were washed thoroughly using HBSS, homogenized in 0.1 M PBS, and serially diluted to facilitate enumeration. Dilutions (10^0 to 10^{-7}) were plated onto *Brachyspira* selective agar and incubated anaerobically for 3 to 5 days at 37°C to determine the numbers of associated *Brachyspira* cells. All IVOC experiments enumerating viable bacteria were repeated in quadruplicate on two separate occasions.

TABLE 1. Experimental strategies for HT29-16E 3D cell and avian cecal IVOC infection studies

Assay	Experimental strategy or step	Bacterial inoculum (CFU/ml) (incubation time)	
		HT29-16E 3D cells	Avian cecal IVOC ^b
Protection assay ^a	<i>L. reuteri</i> or <i>L. salivarius</i> preincubation	5×10^7 (30 min)	10^8 (30 min)
Competition assay	<i>B. pilosicoli</i> inoculation	5×10^7 (5 h)	10^8 (2 h)
	Simultaneous inoculation of <i>B. pilosicoli</i> with <i>L. reuteri</i> or <i>L. salivarius</i>	5×10^7 (5 h) ^c	10^8 (2 h) ^c
Displacement assay ^a	<i>B. pilosicoli</i> inoculation	5×10^7 (5 h)	10^8 (2 h)
CFS assay	<i>L. reuteri</i> or <i>L. salivarius</i> administered postinoculation	5×10^7 (30 min)	10^8 (30 min)
	<i>B. pilosicoli</i> inoculation with 10% <i>L. reuteri</i> or <i>L. salivarius</i> CFS	5×10^7 (5 h)	10^8 (2 h)
Controls			
<i>B. pilosicoli</i> B2904	<i>B. pilosicoli</i> inoculation with medium in place of lactobacillus inoculation (containing 10% MRS for CFS assay control)	5×10^7 (5 h)	10^8 (2 h)
<i>L. reuteri</i> LM1/ <i>L. salivarius</i> LM2	<i>L. reuteri</i> or <i>L. salivarius</i> inoculation with medium in place of <i>B. pilosicoli</i> inoculation	5×10^7 (30 min in protection and displacement assays; 5 h in competition assay)	10^8 (30 min in protection and displacement assays; 2 h in competition assay)
Uninfected	Complete medium only added (supplemented with 10% MRS, pH 5.8 or 3.8, for CFS assay control)	No bacterial inoculum added	No bacterial inoculum added

^a Between delivery of different bacterial inocula, HT29-16E cells or IVOC tissues were washed twice with HBSS.
^b Both viable and heat-inactivated lactobacilli were administered under separate conditions for avian cecal IVOC assays.
^c The values refer to the concentrations and incubation times of both *B. pilosicoli* and the *Lactobacillus* spp.

SEM and TEM. Samples from 3D cell and IVOC studies were fixed in 3% (vol/vol) glutaraldehyde (Sigma-Aldrich) for at least 24 h prior to processing by the Electron Microscopy Unit of the Animal Health and Veterinary Laboratories Agency (AHVLA), Weybridge, United Kingdom. Scanning (SEM) and transmission (TEM) electron microscopy was carried out as previously described (30). Duplicate samples for each condition were examined blind.

Statistical analysis. All results are presented as means and standard deviations of the means. A one-way analysis of variance (ANOVA) was performed with commercially available software (GraphPad Prism), using the Bonferroni test with a 95% confidence interval.

RESULTS

pH-dependent inhibition of *B. pilosicoli* growth by *Lactobacillus* CFS. *Lactobacillus* CFS obtained from spent MRS was at pH 3.8, whereas MRS was at pH 5.8 prior to the growth of lactobacilli. The effects of 10% (vol/vol) *L. reuteri* LM1 and *L. salivarius* LM2 CFS at pH 3.8, 4.5, and 7.2 on *B. pilosicoli* B2904 growth were monitored using a FLUOstar Optima system to measure the OD (Fig. 1) and by bacterial cell counts using a Helber counting chamber. In comparison with MRS controls at the respective pH values, significant inhibition of growth of *B. pilosicoli* was observed with CFS from both *L. reuteri* ($P < 0.05$) and *L. salivarius* ($P < 0.001$) at pH 3.8 and with the CFS of *L. reuteri* only at pH 4.5 ($P < 0.001$). At pH 7.2, neither CFS had an effect on the growth of *B. pilosicoli*. Neither of the two heat-inactivated *Lactobacillus* strains had a significant impact on *B. pilosicoli* growth. Furthermore, the growth of *B. innocens* under each of the conditions was similar to that of *B. pilosicoli*.

Inhibitory effect of *Lactobacillus* whole cells on *B. pilosicoli* motility. Agar motility inhibition assays were performed using the motile strain *B. pilosicoli* B2904 and viable and heat-inactivated lactobacilli; heat-inactivated lactobacilli were used to eliminate the potential effects of competition for nutrients and synthesis of antimicrobial substances. The motility of *B. pilosicoli* was inhibited by both viable and heat-inactivated *L. reuteri* and *L. salivarius* following 4-h and 24-h contact times, respectively. Also, viable lactobacilli inhibited hemolysis by *B. pilosicoli* in these tests. *B. pilosicoli* recovered by subculture from all assays displayed motility and hemolytic growth, indicating viability. The effects of each of the conditions on motility were similar for both *B. pilosicoli* and *B. innocens*.

Inhibitory effects of lactobacilli on *B. pilosicoli* adherence to and invasion of HT29-16E 3D cells. HT29-16E 3D cells exhibited excellent cellular differentiation (brush borders, confluence across the cell surface, and tight junctions), giving multi-layered cell aggregates (see Fig. 3) measuring up to 350 μ m in diameter.

To determine whether viable *L. reuteri* LM1 or *L. salivarius* cells were able to reduce the adherence to and invasion of HT29-16E 3D cells by *B. pilosicoli*, protection, competition, and displacement assays were performed (Table 1). The adherence and invasion of *B. pilosicoli* were reduced significantly by both organisms in protection and competition assays ($P < 0.001$) but not in displacement assays (Fig. 2A, B, and C). Specifically, *L. reuteri* reduced invasion by *B. pilosicoli* 13.6-fold in protection assays and 30.0-fold in competition assays. *L. reuteri* was associated with significantly greater reductions of adherence and invasion by *B. pilosicoli* than those induced by *L. salivarius* ($P < 0.05$). The assays described above were performed with CFS rather than lactobacilli (Table 1) to de-

FIG. 1. Growth of *B. pilosicoli* B2904 in BEB supplemented with 10% *L. reuteri* LM1 (A) or *L. salivarius* LM2 (B) CFS (open shapes) adjusted to pH 3.8 (circles), 4.5 (squares), or 7.2 (triangles) or with heat-inactivated lactobacilli (diamonds), measured using OD₆₀₀ as a reporter of growth. Controls with pH-adjusted MRS (closed shapes) and with no additive to *B. pilosicoli* culture (x's) are also shown. *B. pilosicoli* cells were also enumerated using a Helber counting chamber: an OD₆₀₀ of 0.05 represents $\sim 1 \times 10^7$ CFU/ml, and an OD₆₀₀ of 0.25 represents $\sim 3.5 \times 10^8$ CFU/ml. Means $\pm$ standard errors for 9 repeats are presented. Significance, if any, is shown for differences between the final growth point readings for *B. pilosicoli* with *Lactobacillus* CFS and those for the respective control (+10% MRS). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

termine whether the inhibitory effects on the adherence and invasion of *B. pilosicoli* were a result of compounds secreted by the lactobacilli or a result of the bacteria themselves. No significant reduction of *B. pilosicoli* adherence was observed with *Lactobacillus* CFS (Fig. 2D).

Electron microscopic analysis of HT29-16E 3D cells following adhesion and invasion assays. Samples from competition assays were examined by SEM and TEM (Fig. 3). Qualitatively, larger numbers of adherent *B. pilosicoli* cells were observed in the absence of lactobacilli, with dense populations invading at tight junctions (Fig. 3), supporting the data derived from bacteriological counts. Of particular note were direct interactions between *B. pilosicoli* and both *L. reuteri* LM1 (Fig. 3E and F) and *L. salivarius* LM2.

EM of cell aggregates that were infected with *Brachyspira* alone indicated apoptosis (blebbing, loss of microvilli, disintegrated cytoplasm with vacuolation, chromatin condensation

and fragmentation, and cell sloughing) (Fig. 3C and D). With coadministration of *L. reuteri* LM1 (Fig. 3E and F) or *L. salivarius* LM2, end-on attachment of *B. pilosicoli* was observed less frequently, with apparent interactions between the two bacterial species and colocalization at the cell surface. Minimal pathology was apparent in the presence of lactobacilli, and the integrity of the brush border was maintained (Fig. 3E and F).

Inhibitory effect of lactobacilli on association of *B. pilosicoli* with avian cecal IVOC tissues. Avian cecal IVOC association assays were performed to determine whether *L. reuteri* LM1 or *L. salivarius* LM2, either viable or heat inactivated, was able to reduce the association of *B. pilosicoli* with IVOC tissues. This model generated reproducible bacterial association values and was used alongside the data generated in 3D cell assays to compare and validate the findings of each method. As found in the HT29-16E cell assays, viable *L. reuteri* and *L. salivarius* significantly reduced the association of *B. pilosicoli* with cecal

FIG. 2. Effects of probiotic treatment on *B. pilosicoli* B2904 adherence to (white bars) and invasion of (hatched bars) HT29-16E cells grown in a 3D cell culture model. *L. reuteri* LM1 and *L. salivarius* LM2 were used in protection (A), competition (B), and displacement (C) assays, and their CFS were used at 10% in CFS studies (D). *B. pilosicoli*-only controls are shown, where tissue culture medium was added in place of lactobacilli and 10% MRS (pH 5.8 and 3.8) was added as a control in CFS assays. *E. coli* K-12 was used as a negative control for invasion (black bars). The values presented are means with standard errors for 9 repeats. Significance is shown in cases where adhered or invaded *B. pilosicoli* cell numbers differed significantly between probiotic treatment and the no-probiotic control and, for the CFS studies, in cases where CFS differed significantly from the MRS (pH 3.8) control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

IVOC tissue in protection and competition assays (Fig. 4A and B) ($P < 0.001$). Heat-inactivated lactobacilli shared a similar effect ($P < 0.01$), with the level of reduction in association observed in protection and competition assays decreased up to 6.5-fold. A greater reduction of *B. pilosicoli* association resulted when viable or heat-inactivated lactobacilli were administered in competition, as previously noted in the 3D cell assays, and *L. reuteri* reduced the association of *B. pilosicoli* to a greater degree than did *L. salivarius*. In displacement assays, neither of the *Lactobacillus* strains displayed any significant ability to reduce *B. pilosicoli* association (Fig. 4C).

CFS assays were also performed with the IVOC model (Table 1) to assess the effects of secreted compounds from the lactobacilli on *B. pilosicoli* association. The *L. salivarius* CFS exerted no effect on *B. pilosicoli* association; however, the association was decreased significantly in the presence of the CFS from *L. reuteri* ($P < 0.001$), although numerically, the difference was small (Fig. 4D).

Samples from competition assays were processed by SEM (Fig. 5). In a qualitative analysis, adherent *B. pilosicoli* cells

were observed in greater abundance in the absence of *L. reuteri* or *L. salivarius* coadministration (Fig. 5A), confirming bacteriological counts. Furthermore, direct interactions between both *Lactobacillus* species and *B. pilosicoli* were apparent.

DISCUSSION

Growth inhibition studies indicated that the CFS of *L. reuteri* LM1 and *L. salivarius* LM2 suppressed the growth of *B. pilosicoli* B2904 in a pH-dependent manner. *L. salivarius* CFS elicited a greater suppressive effect on the growth of *B. pilosicoli* at pH 3.8 than did *L. reuteri* CFS, although the *L. reuteri* CFS also induced significant suppression of *B. pilosicoli* growth at pH 4.5 (Fig. 1). The suppression induced by CFS at pH 3.8 was significantly greater than that by MRS at the same pH, suggesting that the suppressive effect was not attributed solely to acidity. A possible explanation may be the strain-dependent production of a pH-dependent active compound(s), such as hydrogen peroxide, and/or other antimicrobial compounds, such as reuterin or bacteriocins (29). *Lactobacillus* CFS has been shown to

FIG. 3. SEM and TEM of HT29-16E 3D cells left uninfected (A), infected with *B. pilosicoli* B2904 (B to D), and following coinubation with *L. reuteri* LM1 (E and F). Uninfected control cell aggregates exhibited a prominent brush border that was confluent across the cell surface (i) and showed superiorly differentiated tight junctions (ii). When administered, *B. pilosicoli* was observed adhering at the epithelial cell surface (iii), and when lactobacilli were coadministered, adherent lactobacilli were observed (iv), in addition to their interactions and coaggregation with *B. pilosicoli* (v). Cells treated with *B. pilosicoli* only exhibited signs of blebbing (vi), loss of microvilli (vii), disintegrated cytoplasm with vacuolation (viii), chromatin condensation and fragmentation (ix), and cell sloughing (x). Overall minimal pathology was apparent in the presence of lactobacilli, and specifically, the integrity of the brush border was maintained (xi).

induce a stress response in *Brachyspira*, often with a lethal effect attributable to lactic acid (6). Heat-inactivated lactobacilli had no effect on *B. pilosicoli* growth, supporting the role of secreted compounds in inhibiting growth. Since heat-inactivated lactobacilli and CFS had similar effects on both *B. innocens* and *B. pilosicoli* growth, it appears that CFS has a universal effect on both pathogenic and nonpathogenic *Brachyspira* growth.

Incubation of *B. pilosicoli* B2904 or *B. innocens* B2960 with

both *Lactobacillus* strains, whether viable or heat inactivated, resulted in a loss of motility. The similarity of results for the assays with viable and heat-inactivated lactobacilli suggests that this is a passive process and is not physiological. Since the motility of *B. pilosicoli* and *B. innocens* was inhibited under similar conditions, the factor which results in this inhibition most likely does not relate to the pathogenicity of *Brachyspira*. Our EM observations showed coaggregation between the two bacterial species (Fig. 3C and D and Fig. 5B), supporting

FIG. 4. Effect of probiotic treatment on association of *B. pilosicoli* B2904 with avian cecal IVOC tissues. Viable (white bars) and heat-inactivated (hatched bars) *L. reuteri* LM1 and *L. salivarius* LM2 were used in protection (A), competition (B), and displacement (C) assays, and their CFS were used at 10% in CFS studies (D). *B. pilosicoli*-only controls (gray bars) are shown, where tissue culture medium was added in place of lactobacilli and 10% MRS (pH 5.8 and 3.8) was added as a control in CFS assays. The values presented are means with standard errors for 8 repeats. Significance is shown in cases where associated *B. pilosicoli* cell numbers differed significantly between probiotic treatment and the no-probiotic control and, for the CFS studies, where CFS differed significantly from the MRS (pH 3.8) control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

similar observations described previously (6). Coaggregation may prove detrimental to *Brachyspira* by rendering it incapable of escaping the eliminating effect of mucus, for which motility and chemotaxis are considered key virulence features (35, 38).

Interestingly, the motility of *B. pilosicoli* and *B. innocens* was inhibited after 4 h of incubation with *L. reuteri* but only after 24 h of incubation with *L. salivarius*. The biochemical basis of adherence and the avidity of binding (3, 44, 45) is worthy of

FIG. 5. SEM of avian cecal IVOC tissues infected with *B. pilosicoli* B2904 (A) and following coincubation with *L. reuteri* LM1 (B). When administered, *B. pilosicoli* was observed adhering to the mucosal surface of the tissue (i), and when lactobacilli were coadministered, adherent lactobacilli were also observed (ii), in addition to their interactions and coaggregation with *B. pilosicoli* (iii).

further investigation, as this may identify lectins for further development and exploitation.

Prior to adhesion and invasion studies, preliminary studies (not described here) confirmed the adhesive and invasive properties of *B. pilosicoli* B2904 with HT29-16E cells; this tropism may be attributed to the mucus-secreting properties of this cell line, since *B. pilosicoli* previously exhibited a chemoattraction to mucin (39). A 3D cell model which maintains a differentiated 3D architecture of the parental tissue, creating a more physiologically relevant platform, was adopted for adhesion and invasion assays. Findings from 3D cell assays encouraged the continuation of studies using IVOC cecal tissue explants from laying hens, a novel and possibly more physiologically relevant *in vitro* platform with which to study the potential use of probiotics to protect against AIS. In IVOC studies, tissues were maintained as physiologically active tissues, with their natural architecture and mucin layers; SEM showed that the tissues remained well preserved throughout the study (Fig. 5). However, the nature of the IVOC study did not allow separate enumeration of adherent and invaded *B. pilosicoli* cells, so the total association was assessed. *L. reuteri* LM1 and *L. salivarius* LM2 significantly reduced the adherence and invasion of *B. pilosicoli* with HT29-16E cells and with the mucosal surface of avian cecal IVOC tissues in protection and competition assays (Fig. 2A and B and 4A and B). This is most probably explained by coaggregation between the lactobacilli and *B. pilosicoli* (Fig. 3E and F and 5B). High levels of exopolysaccharide (EPS) production have been associated with the coaggregative properties of lactobacilli with enteric pathogens such as *E. coli* (3). EPSs are produced by other probiotic members of the normal gut microflora, including bifidobacteria (44), and thus may provide additional protection against *Brachyspira* infection. Surface proteins such as (co)aggregation-promoting factor (Cpf) have also been implicated in the coaggregative phenotype of lactobacilli with pathogens (45). Whether there was any physiological, pH-dependent impact on adherence and invasion is unclear. However, this is less likely to have occurred than passive coaggregation, because there was no evidence of pH-associated cellular tissue damage of the HT29-16E cells or the mucosal surface of avian cecal IVOC tissues.

When coadministered, viable lactobacilli were distributed evenly across the cell surface of the HT29-16E 3D cells (Fig. 3E and F) and the mucosal surface of the avian cecal IVOC tissue (Fig. 5), potentially occupying specific receptor sites and thus limiting the number of adherent *Brachyspira* cells by niche competition. Larger numbers of lactobacilli would have been present in competition assays than in protection assays, where the washes following the 30-min pretreatment would have removed nonadhered lactobacilli; hence, this may explain the more profound reduction of the adherence and invasion of *B. pilosicoli* in competition assays, as more lactobacilli were available to interact with *Brachyspira*. *L. reuteri* induced a greater reduction of *B. pilosicoli* adherence and invasion than did *L. salivarius*, regardless of delivery. This trait may be attributed to an ability of *L. reuteri* to compete for a wider range of receptor binding sites, produce additional antimicrobial compounds, or coaggregate more efficiently.

The inability of either *Lactobacillus* strain to reduce the adherence or invasion of *B. pilosicoli* in displacement assays (Fig. 2C and 4C) may have been due to the absence of lacto-

bacilli to interact with *B. pilosicoli* during the 5-h incubation and the inability of the *Lactobacillus* posttreatment to reverse adherence and invasion. These results suggest that lactobacilli must be present prior to or with *Brachyspira* in order to interact with the spirochete and prevent association with epithelial cells. These data suggest that probiotic treatment may have little effect in birds that are already colonized with *B. pilosicoli*.

The inability of *Lactobacillus* CFS to reduce the adherence and invasion of *B. pilosicoli* in the 3D cell model (Fig. 2D) further supports the notion that direct interactions with lactobacilli are crucial in reducing *B. pilosicoli* association. In spite of this, CFS studies with the IVOC model revealed a small but statistically significant ability of *L. reuteri* CFS, but not *L. salivarius* CFS, to reduce the association of *B. pilosicoli* with the tissues. The effect of *L. reuteri* CFS on the association of *B. pilosicoli* may be due to the production and release of one or more metabolic by-products capable of limiting *B. pilosicoli* association or of a bioactive component(s) which may block adhesion molecules on *B. pilosicoli* or host cells. Interestingly, the CFS of *Lactobacillus delbrueckii* subsp. *bulgaricus* inhibits the cytotoxic effects of *Clostridium difficile* and its adhesion to Caco-2 cells, and these effects are attributable to the production of bioactive compounds that inhibit the toxin, its receptors, or bacterial adhesion molecules (4).

Further supporting the concept of passive coaggregation between the lactobacilli and *Brachyspira* was the ability of heat-inactivated lactobacilli to elicit a significant reduction in *Brachyspira* association in protection and competition assays with the IVOC model (Fig. 4A and B). However, heat-inactivated lactobacilli reduced *B. pilosicoli* association to a lesser degree than did viable lactobacilli; this may have been due to the increasing numbers of viable lactobacilli throughout the assays or, perhaps, to the active production and secretion of inhibitory compounds. A probable mechanism by which lactobacilli induce a significant reduction in *B. pilosicoli* association when delivered prior to or with *Brachyspira* is by passive coaggregation between the different species, which inhibits *Brachyspira* motility, hence trapping the spirochete and mitigating its ability to adhere to and invade host cells.

In 3D cell assays, HT29-16E cells that had been infected with *B. pilosicoli* showed signs of membrane blebbing (Fig. 4C and D), which is indicative of apoptosis due to physical or chemical stresses (13, 16). *B. pilosicoli* may induce bleb formation via a type 3 secretion system (T3SS)-dependent invasion mechanism, as has been observed with *Pseudomonas aeruginosa* (2), or it may adopt a mechanism similar to that of *Bacteroides fragilis*, which produces an enterotoxin that acts on the cytoskeleton (15). Genes encoding components of a T3SS and putative cytotoxin genes have been reported for *Brachyspira* (5, 56). Effacement of microvilli was observed in addition to shrunken cytoplasm, intracellular vacuolation, and cell sloughing, which are indicative of apoptosis; these findings are consistent with histopathological studies of tissues from infected birds (23, 48). Moreover, chromatin condensation was noted, which has been observed in infected avian tissues and human colorectal cell (Caco-2) monolayers (40), and genes encoding ankyrin proteins, which bind host cell chromatin, have been identified in *Brachyspira* (5). Further supporting the ability of lactobacilli to intervene in *Brachyspira* infection in

vitro was the apparent protection against this cellular pathology that they conferred in competition assays (Fig. 3E and F).

The effects of lactobacilli on the growth, motility, and host cell association of *B. pilosicoli* encourage *in vivo* studies to assess the efficacy of these strains to protect against AIS. The rapid growth and robust nature of lactobacilli compared with the slow-growing, fastidious *Brachyspira* cells make these species ideal probiotic candidates for intervention against *Brachyspira* infection by niche competition. Our results indicate that acidification will inhibit *B. pilosicoli*; however, this may be detrimental to the host, and therefore a key effector in control may be the passive coaggregation we observed. Supplementing the diet of poultry with coaggregative lactobacilli may therefore be a useful control strategy for AIS.

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