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# Effects of Orally Administered *Bdellovibrio bacteriovorus* on the Well-Being and *Salmonella* Colonization of Young Chicks▽†

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***Bdellovibrio bacteriovorus* is a bacterium which preys upon and kills Gram-negative bacteria, including the zoonotic pathogens *Escherichia coli* and *Salmonella*. *Bdellovibrio* has potential as a biocontrol agent, but no reports of it being tested in living animals have been published, and no data on whether *Bdellovibrio* might spread between animals are available. In this study, we tried to fill this knowledge gap, using *B. bacteriovorus* HD100 doses in poultry with a normal gut microbiota or predosed with a colonizing *Salmonella* strain. In both cases, *Bdellovibrio* was dosed orally along with antacids. After dosing non-*Salmonella*-infected birds with *Bdellovibrio*, we measured the health and well-being of the birds and any changes in their gut pathology and culturable microbiota, finding that although a *Bdellovibrio* dose at 2 days of age altered the overall diversity of the natural gut microbiota in 28-day-old birds, there were no adverse effects on their growth and well-being. Drinking water and fecal matter from the pens in which the birds were housed as groups showed no contamination by *Bdellovibrio* after dosing. Predatory *Bdellovibrio* orally administered to birds that had been predosed with a gut-colonizing *Salmonella enterica* serovar Enteritidis phage type 4 strain (an important zoonotic pathogen) significantly reduced *Salmonella* numbers in bird gut cecal contents and reduced abnormal cecal morphology, indicating reduced cecal inflammation, compared to the ceca of the untreated controls or a nonpredatory Δ*pilA* strain, suggesting that these effects were due to predatory action. This work is a first step to applying *Bdellovibrio* therapeutically for other animal, and possibly human, infections.**

*Bdellovibrio bacteriovorus* is a small predatory deltaproteobacterium which invades and kills other Gram-negative bacteria, including a broad range of pathogens of vertebrates and humans (48, 57). In an age of increasingly problematic conventional antibiotic resistance in major human pathogens, such as *Escherichia coli* ST131 and others, *Bdellovibrio* could be of great use as a potential so-called living antibiotic (43, 52).

Since its discovery in the 1960s (55), there have been no reports of trials of *Bdellovibrio* use against such pathogens within warm-blooded animals, and there are only a few reports of it being used in environmental applications to plants and in fish farming (29, 38, 63). Some limited studies were carried out previously to try to test the safety of *Bdellovibrio* by feeding it to amphibians via an intragastric tube and by *ex vivo* tests in isolated rabbit ileal loops (63), and there are previous reports in the literature of *Bdellovibrio* being isolated from the intestinal contents of live animals and humans (47). Several *in vitro* studies recently have been published (14, 15, 60) looking at the susceptibility of human pathogens to predation by *Bdellovibrio*, and these have shown that *Bdellovibrio* is able to successfully reduce pathogen numbers under laboratory conditions.

With increasing antibiotic resistance being reported for Gram-negative pathogens, including the emergence of intestinal and uropathogenic *E. coli* ST131 (27), which are resistant to fluoroquinolone and extended-spectrum β-lactam antibiotics, we felt that it was important to begin assessing the potential of *Bdellovibrio* therapy experimentally by performing *in vivo* experiments using *Bdellovibrio* inside living, warm-blooded vertebrates.

In addition to assessing the beneficial aspects of *Bdellovibrio* therapy, we wished to monitor any potential health problems created by the application of *Bdellovibrio*, either by directly invading mammalian cells (although this is not thought to occur and has been tested in one previous report [35]) or due to the broad prey range of *Bdellovibrio* causing a harmful imbalance of the normal gut microbiota (dysbiosis). The idea of these studies was to see the effects upon normal gut microbiota of ingesting *Bdellovibrio*, so that whether they are to be applied in the future as topical agents for wound infections, or indeed any oral infection applications, the potential effects internally of even accidental ingestion would be known. Theoretically *Bdellovibrio* could be detrimental to Gram-negative gut microbiota and thus the eukaryotic host's well-being, intentionally or not, as it kills such Gram-negative cells and they contribute to animal nutrition.

We chose poultry as our model vertebrates, both to study whether *Bdellovibrio* had any effect on animal well-being and also because this is a well-studied model used in *Salmonella* infection and intestinal/cecal colonization experiments (22, 39, 62), which would allow us to determine any therapeutic effects of *Bdellovibrio* treatment. The model of *Salmonella* in poultry

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was chosen for several additional reasons: first, and importantly for animal welfare, *Salmonella enterica* serovar Enteritidis often infects poultry, particularly laying hens, without clinical symptoms (13), unlike serovars such as Gallinarum, the agent responsible for fowl typhoid (11).

Our experimental model used *S.* Enteritidis P125109, a genome-sequenced representative of phage type 4 (PT4), which was largely responsible for the significant increase in reported cases of human salmonellosis in England and Wales during the 1980s and 1990s (12) and continues to be the main *S.* Enteritidis phage type isolated from humans in the European Union (17). Elsewhere, *Salmonella* remains a major public health concern, as shown recently in a U.S. Food and Drug Administration report on the recall of more than half a billion eggs that were suspected to be contaminated with *Salmonella* Enteritidis (10, 19). Finally, the model has been studied previously with respect to another biological therapeutic agent, bacteriophage (4, 7, 62), so some comparative data exist on the *in vivo* reduction of pathogens by sampling and enumerating the *Salmonella* load in cecal contents of bird guts. At the outset of our studies we verified in the laboratory that the *Salmonella* strain was efficiently invaded by *B. bacteriovorus* HD100.

Predacious life in the gastrointestinal tract of poultry presents a variety of challenges to *Bdellovibrio*: the body temperature of a chicken is typically 42°C, whereas *Bdellovibrio* is routinely grown at an optimal temperature of 29°C (as found in early attachment studies [61]). In addition, the hypoxic or anoxic environment of the bird gut places additional pressure on *Bdellovibrio*, which is typically aerobically grown in the laboratory, although a single study has shown the survival of several *Bdellovibrio* strains in anaerobic conditions for up to 9 days (46). In our experiments, we used the genome-sequenced type strain *B. bacteriovorus* HD100, a strain that originally was isolated from soil and has been used in many laboratory-based predation studies (18, 25, 30, 44). Although it will be possible in the future to try to isolate *Bdellovibrio* from animal guts and study them, more is known physiologically and genetically about strain HD100 at this stage than about any other strain.

Our model system also allowed us to assay other potential side effects of *Bdellovibrio* therapy: by orally dosing the chicks, we were testing the effects of the ingestion of large numbers of *Bdellovibrio*, and this informed us of any possible adverse effects of accidental ingestions were *Bdellovibrio* to be used in future applications to surface wound dressings. In addition, we included an environmental monitoring element, where the birds were housed in groups in large normal pens (in contained rooms) with bedding on the floor and shared drink and food trays; thus, we were able to determine whether there was any spread of *Bdellovibrio* after dosing.

This study has shown that *B. bacteriovorus* HD100 is able to overcome many of the difficulties presented by the model of the *Salmonella* colonization of chicken intestine, including that, possibly contrary to expectations, aerobic *B. bacteriovorus* HD100 does survive anaerobic/microaerobic incubation at 42°C, whether inside prey bacteria or incubated alone, and although their reisolation from gut contents after administration was sporadic at best, suggesting short-lived survival, they have measurable and significant positive effects *in vivo*. Our work suggests that the reduction of cecal abnormalities, as well as the lowering of *Salmonella* numbers, were the result of the predatory action of *Bdellovibrio* and not just its live competitive metabolism or action as a dead inert source of organic nutrients in the gut environment.

This is the first report of a complete live-animal treatment with *Bdellovibrio*, and the lack of any negative side effects bodes well for future therapeutic trials.

## MATERIALS AND METHODS

**Bacterial strains, maintenance, and enumeration.** *E. coli* S17-1 was routinely used as prey to maintain predatory cultures of *Bdellovibrio* (31, 33). *E. coli* S17-1 was grown in YT (0.5% Difco Bacto yeast extract, 0.5% NaCl, 0.8% Difco Bacto tryptone, pH 7.5) broth (31, 34) at 37°C with shaking at 200 rpm for 16 h before it was used in a late-log-phase culture for addition to *Bdellovibrio*. *Salmonella* Enteritidis P125109 also was grown in YT broth under the same conditions as those for *E. coli*, also yielding a late-log-phase culture for use with *Bdellovibrio*.

Host-dependent (HD) *Bdellovibrio bacteriovorus* HD100 (43, 55) was grown in predatory cultures consisting of Ca-HEPES buffer (25 mM HEPES, 2 mM $CaCl_2$, pH 7.6), late-log-phase prey (either *E. coli* S17-1 or *S.* Enteritidis P125109, produced as described above), and a previously grown *Bdellovibrio* culture in a ratio of 50:3:1 (vol/vol/vol). The complete lysis of prey typically was achieved within 24 h when grown on *E. coli* S17-1, and almost complete lysis (see the next section) occurred in 48 h when grown on *Salmonella* Enteritidis P125109 as prey. Host-independent (HI) derivatives were grown in PY broth (18, 49) and derived as described by Evans et al. (18). *Bdellovibrio* (both HD and HI) was grown aerobically at 29°C with shaking at 200 rpm.

HD *Bdellovibrio* viable counts were made using double-layer YPSC (0.1% Difco yeast extract, 0.1% Difco Bacto peptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.025% $CaCl_2 \cdot 2H_2O$, pH 7.6) (31) agar plates as described elsewhere (25, 34). *E. coli* was enumerated on YT plates (25), and *S.* Enteritis was enumerated on YT (for *in vitro* experiments) or on modified brilliant green agar (CM0329; Oxoid) plates.

**Testing *Bdellovibrio in vitro*: predation of *Salmonella* Enteritidis versus that of *E. coli*.** *Bdellovibrio bacteriovorus* HD100 (43, 55) is routinely used in laboratory conditions to prey upon *E. coli* strains, such as ML35 and S17-1 (50), but in later *in vivo* experiments the prey was *Salmonella* Enteritidis strain P125109 (58). Thus, *Bdellovibrio* predation efficiency on this strain of *Salmonella* and its entry into this prey was verified.

*Bdellovibrio* entry into prey was visualized using electron microscopy. Cultures consisting of 2 ml of a *Bdellovibrio* predatory culture (which had completed prey lysis and typically contained $2.5 \times 10^8$ PFU $ml^{-1}$) and 300 μl of a 16-h *Salmonella* culture (grown to late log phase in YT broth aerobically at 37°C for 16 h) were incubated for 15 min at 29°C before grids were prepared. Cells were stained with 0.5% uranyl acetate (URA), pH 4.0, and imaged using a JEOL JEM 1010 electron microscope as described previously (18, 20).

The comparison of *Bdellovibrio* predation rate and efficiency on different prey was achieved by measurements of the optical density at 600 nm ($OD_{600}$) using a Fluostar Optima plate reader (BMG Labtech). Predatory cultures of *B. bacteriovorus* HD100 which had completed prey lysis were split, with half being heat killed by incubation at 105°C for 5 min and then cooled to room temperature. Sixty-four μl of either live predatory *Bdellovibrio* or heat-killed *Bdellovibrio* cells was added to wells of a 96-well Optiplate (Porvair Sciences Ltd.). To this, 200 μl of a prey and buffer mix was added, which consisted of 10 ml of Ca-HEPES buffer and 400 μl of a 16-h culture of either *E. coli* S17-1 or *S.* Enteritidis P125109. The plate then was sealed with a Breathe-Easy (Web Scientific) gas-permeable sealing membrane and incubated in the Fluostar plate-reader at 29°C with shaking at 200 rpm for 24 h, with an $OD_{600}$ reading every 1 h. The initial enumerations of both *Bdellovibrio* and prey were made as described above, and prey again were enumerated (from wells containing live *Bdellovibrio*) upon the completion of the incubation time.

***In vitro Bdellovibrio* survival at 42°C microaerobically and anaerobically.** To verify that *Bdellovibrio* would survive conditions in the bird gut, *in vitro* tests were set up. *B. bacteriovorus* HD100 was pregrown for 24 h in a typical predatory culture consisting of 150 ml Ca-HEPES buffer, 9 ml of a *E. coli* S17-1 culture grown for 16 h, and 3 ml of a *Bdellovibrio* predatory culture. Experimental cultures were set up initially in 500-ml conical flasks containing *Bdellovibrio*-only controls (50 ml *Bdellovibrio* starter culture and 68 ml Ca-HEPES), *Bdellovibrio* with *E. coli* cultures (50 ml *Bdellovibrio* starter culture, 18 ml of a 16-h *E. coli* S17-1 culture, and 50 ml Ca-HEPES), and *Bdellovibrio* with *Salmonella* cultures (50 ml *Bdellovibrio* starter culture, 18 ml of a 16-h *S.* Enteritidis P125109 culture, and 50 ml Ca-HEPES). These starter cultures were incubated aerobically at 29°C with shaking at 200 rpm for 1 h, allowing the *Bdellovibrio* organisms time to

attach to and enter prey cells in the mixed cultures, forming stable bdelloplast structures.

After the 1-h incubation, the cultures were placed in 5-ml aliquots in tissue culture flasks with vented lids (25-$cm^2$ polystyrene tissue culture flasks with PE vented cap; Sarstedt) and then incubated under the following conditions (all without shaking): at 29°C aerobically, at 42°C aerobically, at 42°C microaerobically (within a 7-liter gas-sealed box [Mitsubishi, bioMérieux Corporation] containing CampyGen gas packs [CN0035A, Oxoid]), and at 42°C anaerobically (again within a 7-liter gas-sealed box containing AnaeroGen gas packs [AN0035A; Oxoid]). One sealed box was used for each survival sampling point, with each box containing a flask of each experimental culture, and a flask was set up for each time point for aerobic incubations. Each flask was used once.

At each time point tested, 1, 4, 24, 48, and 72 h after incubation at 42°C (or 29°C for the control samples), 100 μl of each sample was spotted onto a YPSC double-layered prey lawn overlay plate (25, 34) on which the top layer agar had been inoculated with *E. coli* S17-1. The plates then were incubated agar side down at 29°C until areas of clearing appeared on the plates inoculated with samples from *Bdellovibrio* flasks incubated at 29°C aerobically. Zones of clearing for the different conditions were compared to those of the control (see Fig. S1 in the supplemental material).

**Construction of a silent-deletion Δ*pilA Bdellovibrio* mutant strain.** In previous work, a kanamycin resistance cassette disruption of the *pilA* gene in *B. bacteriovorus* was shown to result in the complete loss of predatory ability (18). For our experiments to show whether predatory *Bdellovibrio* activity or merely the presence of live metabolizing *Bdellovibrio*, or dead *Bdellovibrio* biomass, was affecting the outcomes, we required a nonpredatory *Bdellovibrio* strain which did not contain any antibiotic resistance cassettes or engineered foreign DNA. Thus, we produced a silent-deletion version of the Δ*pilA* mutant in *B. bacteriovorus* HD100, using an adaptation of the method described by Steyert and Pineiro (54). One kb of flanking DNA from either side of the *pilA* gene was amplified and joined together to give an in-frame deletion of the *pilA* open reading frame (ORF) (retaining the start codon, the final three codons, and the stop codon). This then was ligated into the kanamycin-resistant suicide vector pK18*mobsacB* (23, 45) and conjugated into *B. bacteriovorus* HD100 (as described previously [18]). pK18*mobsacB* previously has been shown to recombine into the *B. bacteriovorus* HD100 chromosome and to be suitable for making gene knockouts (20, 44). The resulting merodiploid exconjugants were grown with kanamycin selection in predatory cultures before being turned HI by culture on complete peptone yeast extract (PY) medium without prey (18, 34). At the time of being turned HI the antibiotic selection was removed; the resulting HI cultures were screened by both PCR and Southern blotting (53) to verify the *pilA* gene deletion and the absence of the suicide plasmid. The resulting Δ*pilA* strain was confirmed to be nonpredatory by the fluorescent prey assay described in detail previously (18).

**Preparation of *Salmonella* and *Bdellovibrio* for orally dosing birds.** *Salmonella* Enteritidis P125109 was grown in 50 ml of YT broth containing nalidixic acid (which is selective for the strain) at 25 μg $ml^{-1}$ under standard conditions. This culture then was washed twice. The cells were centrifuged at 5,100 × *g* for 10 min and then resuspended in 50 ml of maximum recovery diluent (MRD; CM0733; Oxoid); this step was repeated. The suspension then was diluted to an $OD_{600}$ of 0.34 in MRD, 100 μl of which then was given by oral gavage to each 2-day-old chick. Subsequent enumeration of the *Salmonella* in these suspensions, by viable counting on modified brilliant green agar plates with nalidixic acid (CM0329; Oxoid), showed that each chick received an average *Salmonella* count of $3.16 \times 10^7$ CFU (per 100 μl dose). This had been shown in our earlier experiments (data not shown) to give consistent colonization of the chicks' gastrointestinal tract by the *Salmonella* organisms.

Host-dependent (HD) *B. bacteriovorus* HD100 was grown in a predatory culture containing 500 ml Ca-HEPES buffer, 30 ml of a 16-h prey culture, and 50 ml of a 24-h predatory culture of *Bdellovibrio*, and then it was incubated at 29°C with shaking at 200 rpm for 48 h. The prey used in these cultures depended upon the trial in which they were used. For the bird well-being trials, *Bdellovibrio* was pregrown on *E. coli* S17-1 to eliminate any possibility of introducing any surviving *S.* Enteritidis into the chicks along with the *Bdellovibrio* organisms, which could have caused significant changes to the birds' health and well-being. For the therapeutic trials, where the birds were precolonized with *S.* Enteritidis, the introduction of a small number of *Salmonella* cells with *Bdellovibrio* did not pose such a problem, so for these trials the *Bdellovibrio* organisms were grown using *S.* Enteritidis P125109 as prey.

After the 48-h incubation period, the cultures were checked microscopically for prey lysis and filtered once through a 0.45-μm-pore-size filter to remove any remaining prey cells, and then the *Bdellovibrio* organisms were pelleted (in 50-ml Falcon tubes) by centrifugation at 5,100 × *g* at 29°C for 30 min. The supernatants were decanted, and the *Bdellovibrio* organisms were resuspended in the residual liquid. The cells then were diluted 10-fold in fresh Ca-HEPES buffer, giving an average *Bdellovibrio* count of $9.8 \times 10^7$ PFU per 100-μl dose (as determined after the experiments due to *Bdellovibrio* forming plaques only after at least 5 days on an overlay plate).

The host-independent (HI) *B. bacteriovorus* Δ*pilA* mutant was grown as described above in 50 ml PY broth for 24 h. Forty ml of HI cells was pelleted by centrifugation at 5,100 × *g* at 29°C for 20 min, resuspended in 20 ml Ca-HEPES buffer, and centrifuged again before being resuspended in 40 ml of fresh Ca-HEPES buffer. HI doses were matched to their comparable HD dose by protein content using a Lowry assay (37). The protein assay was used because it is rapid (not requiring several days of growth to enumerate HD plaques or HI colonies on plates) and matches cell biomass for *Bdellovibrio* organisms, which are too small to give a reliable optical density reading and thus cannot be enumerated by the $OD_{600}$.

HD *Bdellovibrio* doses were enumerated (after use) as viable counts by plaque formation on YPSC prey overlay plates (25, 34); any remaining *Salmonella* survivors in the inocula (which were very few) were enumerated on modified brilliant green agar plates (CM0329; Oxoid), and HI *Bdellovibrio* organisms were enumerated on PY agar plates (18, 34, 49). The inocula were transported to the animal facilities within 1 h. Previous experiments (data not shown) showed that the viability of the *Bdellovibrio* strains under these conditions did not significantly alter. Immediately prior to dosing, 10 ml of each dose (or Ca-HEPES buffer-only control) was added to 1.43 g $CaCO_3$ to act as an antacid protectant for the inoculum as it passed through the birds' crops and mixed to give a homogenous suspension; 100 μl of this suspension then was given by oral gavage to each chick.

***Bdellovibrio* and bird well-being experimental setup.** Day-old Hy-line brown male chicks from a layer hen line (Hy-line Hatcheries, Warwickshire, United Kingdom) were separated at random into two groups of 12 (control and treated). Each group of 12 chicks was housed inside a single-floor pen in a separate room and individually identified using colored markings. The birds were provided with food and water *ad libitum*. Cage temperature was 32°C for the first 3 days and 30°C after that. At 2 days of age, the birds in the treated group each received 100 μl of a $1.9 \times 10^7$ PFU $ml^{-1}$ suspension of *Bdellovibrio bacteriovorus* HD100 in Ca-HEPES buffer (pregrown on *E. coli* S17-1 as prey so as to eliminate any potential of *Salmonella* being introduced into the chicks with the *Bdellovibrio*) containing 14% (wt/vol) $CaCO_3$ by oral inoculation; the birds in the control group received 100 μl of Ca-HEPES buffer with $CaCO_3$ only. Following inoculation, the birds were observed for any signs of ill health (e.g., lethargy, hunched posture, ruffled feathers, drooping wings, weight loss, abnormal excreta, and pasty vent) twice daily using the score sheet proposed by the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement (24). The birds in each group were weighed every day following (and including) the day of *Bdellovibrio* challenge, and these data were compared to industry standards for these birds (26). A pool of three freshly voided fecal samples and a separate 10-ml sample of water from the drinking trough were collected from each group at regular intervals throughout the trial (48- to 72-h intervals) for the enumeration of bacterial populations (see below). At 4 weeks of age, the birds were sacrificed and populations of various bacterial genera enumerated from the cecal contents (see below). Ceca are the conventional site of choice for the enumeration of *Salmonella* in the guts of poultry (rather than the whole gut), as they are an anatomically discrete compartment where *Salmonella* colonizes. Ceca can be readily tied off and isolated without the loss of contents and are less subject to differences to which the whole gut is susceptible, such as differing recent inputs or outputs due to episodes of eating food or defecation (22).

**Examination of drinking water and fecal and cecal samples.** Fecal and cecal samples were decimally diluted in Ca-HEPES buffer (for *Bdellovibrio* enumeration) or MRD (CM0733; Oxoid) for all other bacterial populations. For *Bdellovibrio* enumeration, fecal/cecal suspensions first were filtered through a 0.45-μm-pore-size filter, and then 100 μl of filtrate was added to the top layer of a double-layer YPSC plate (as described above) with *E. coli* S17-1 as prey. Plates were incubated at 29°C aerobically for a minimum of 5 days.

Other bacterial populations were enumerated by spread plating 100 μl of $10^{-1}$ to $10^{-8}$ dilutions (dilution ranges varied according to the target bacterial population) in duplicate onto selective agar. The selective agars used (and target bacterial populations) were modified brilliant green agar (*Salmonella* spp.; CM0329 and sulfamendalate supplement [SR0087]); mitis salivarius agar (fecal streptococci 229810 plus tellurite supplement 211917; Becton Dickinson, Oxford, United Kingdom); modified charcoal cefoperazone deoxycholate agar (mCCDA; *Campylobacter* spp.; CM0739 plus *Campylobacter* selective supplement SR0155); Rogosa agar (*Lactobacillus* spp.; CM0627; Oxoid); bifidus selective medium (*Bifidobacterium* sp.; 88517 plus BSM supplement 83055; Sigma); Columbia

blood agar with 100 μg $ml^{-1}$ neomycin trisulfate (anaerobic bacteria; CM0331; Oxoid); and chromogenic (brilliance) *E. coli*/coliform agar (CM0956). The plates were incubated under the following conditions: brilliant green and chromogenic, 37°C for 24 h; and mCCDA and Rogosa, 42°C for 48 h under microaerobic conditions (5% $H_2$, 5% $O_2$, 10% $CO_2$, 80% $N_2$). The remaining plates were incubated at 37°C for 48 h under anaerobic conditions (5% $CO_2$, 5% $H_2$, 90% $N_2$).

***Bdellovibrio* therapeutic trial experimental setup.** For each biological repeat, day-old male Hy-line brown chicks were separated at random into two (trial 1) or three (trial 2) groups of 18 birds. Each group of 18 chicks was placed in a separate room inside a single-floor pen and provided with food and water *ad libitum*. For both trial 1 and trial 2, all birds received a 100-μl dose of *Salmonella* Enteritidis P125109 (prepared as described above) by oral gavage, containing an average *Salmonella* count of $3.16 \times 10^7$ CFU per dose.

In trial 1, 10 days after the *Salmonella* challenge, the birds in the first group received 100 μl of a $9.8 \times 10^7$ PFU $ml^{-1}$ suspension of predatory *Bdellovibrio bacteriovorus* HD100 (pregrown as described above with *S.* Enteritidis as prey) containing 14% (wt/vol) $CaCO_3$ as an antacid; the second group received 100 μl of Ca-HEPES buffer containing 14% (wt/vol) $CaCO_3$. For trial 2, the first two groups were treated identically to those in trial 1. The remaining group was given a nonpredatory *B. bacteriovorus* HI Δ*pilA* mutant in Ca-HEPES buffer containing 14% (wt/vol) $CaCO_3$. This was matched, by protein content, to the host-dependent *Bdellovibrio* dose as explained above.

Bird numbers were chosen to allow for a minimum of 80% power to detect a 1-$\log_{10}$ difference between the group mean values based on a group size of 10. To minimize the use of live birds in our research in this, we sought advice from pathology statistician Alan J. Hedges (University of Bristol). Two biological repeats were performed in each trial (i.e., four groups for trial 1 and six groups for trial 2 in total). This gave 24 birds per day in total for the control and predatory *Bdellovibrio* treatments and 12 birds per day for the HI Δ*pilA* nonpredatory *Bdellovibrio* treatment.

***Bdellovibrio* therapeutic trials: examination and scoring of bird guts postmortem.** During the postmortem examination of the birds, very obvious differences were seen in the appearance and contents of the ceca of the intestinal tract. These differences meant that in abnormal ceca there were almost no dark free-flowing gut contents to sample for bacterial counts, but that the ceca were impacted with white/cream mucoid matter. This has been reported previously as an inflammatory response with white blood cell infiltration caused by the *Salmonella* infection (1, 2, 16, 36, 42). Thus, we defined ceca as abnormal if they were very pale and/or their contents were mucoid, solid, white, very watery yet full and white, or weighed ≤0.1 g. We photographed and recorded examples of these and scored each pair of ceca by these criteria.

**Statistical treatment of results.** The data from four independent biological repeats for each control and *Bdellovibrio*-treated group were pooled prior to statistical analysis (data from two biological repeats were pooled for nonpredatory HI Δ*pilA* mutant *Bdellovibrio*-treated groups). All bacterial counts were transformed to $\log_{10}$ CFU $g^{-1}$ cecal contents. Statistically significant differences in *Salmonella* counts from cecal contents were determined using a *t* test with Welch's correction (as recommended by Skovlund and Fenstad [51]). Cecal content weights were analyzed for normality by the Kolmogorov-Smirnov test, and subsequently they were analyzed for statistical differences by the nonparametric Kruskal-Wallis test with Dunn's post test. The statistical significance of differences in the proportion of abnormal ceca between *Bdellovibrio*-treated and control groups was determined using Fisher's exact test. Statistical analyses were performed using either GraphPad Prism (version 4.00 for Windows) or GraphPad Instat (version 3.10 for Windows).

**Ethical statement on bird experiments.** All experiments involving the use of animals were subjected to a National United Kingdom Government approval process by the United Kingdom Home Office, which grants licenses for specific work to specific individuals. Work on this project was approved under United Kingdom Government Home Office Project Licensing ASPA86. All project licenses are reviewed internally by the University Ethics Committee prior to submission to the Home Office. This includes the scrutiny of animal welfare, ethics, and handling. All of our individual experiments were reviewed and approved by a member of the senior management committee within the School of Veterinary Science at University of Nottingham, which was responsible for the work. They also are scrutinized by the Named Veterinary Surgeon (NVS) and the Named Animal Care and Welfare Officer (NACWO) before each experiment is allowed to proceed. This ensures minimal numbers of animals used in procedures and the highest standards of welfare and ethics.

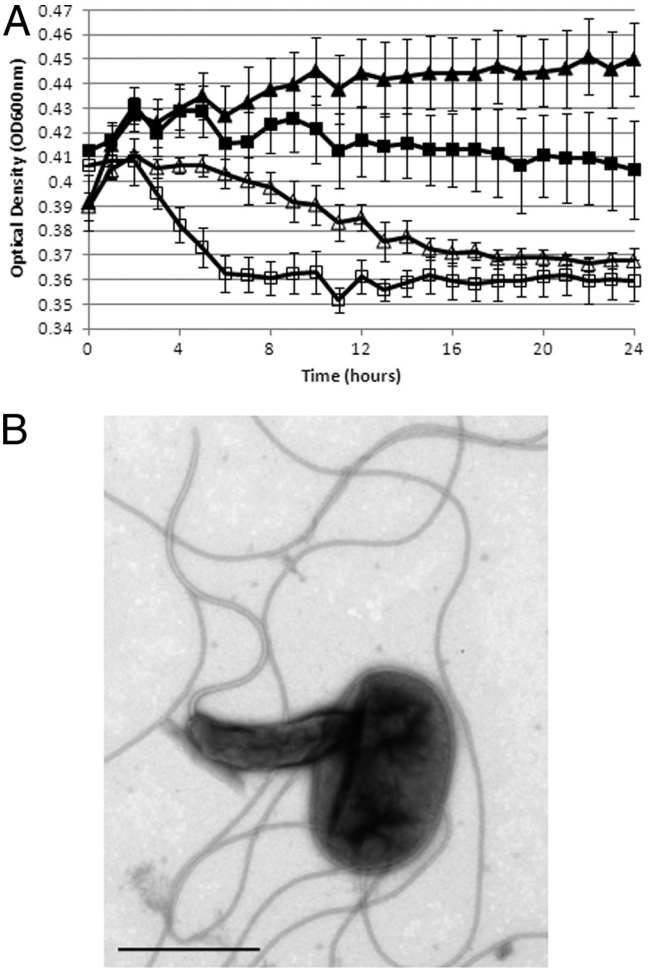


FIG. 1. (A) Predatory kill curves of *E. coli* S17-1 and *S.* Enteritidis P125109 by *B. bacteriovorus* HD100. Curves show the optical density (at 600 nm) of *E. coli* with heat-killed *Bdellovibrio* (solid squares), *E. coli* with live predatory *Bdellovibrio* (open squares), *Salmonella* with heat-killed *Bdellovibrio* (solid triangles), and *Salmonella* with live predatory *Bdellovibrio* (open triangles). Predatory *Bdellovibrio* was added to late-log-phase prey (either *E. coli* S17-1 or *S.* Enteritidis P125109), diluted in Ca-HEPES buffer, and incubated aerobically at 29°C with shaking at 200 rpm. A small amount of prey replication occurred due to the carryover of remaining nutrients within the prey cultures, but this growth was limited due to the dilution of the prey into the nonnutrient Ca-HEPES buffer. The drop in $OD_{600}$ reflects the successful predation on, and lysis of, the prey cells both early (during the small amount of prey growth) and later during the incubation period when the prey cells entered stationary phase. Each point represents the means from three biological repeats, and error bars show the standard errors above and below the means. (B) A *B. bacteriovorus* HD100 cell entering an *S.* Enteritidis P125109 prey cell. Cells were stained with 0.5% uranyl acetate (URA), pH 4.0. The scale bar represents 1 μm.

## RESULTS

***In vitro* predation by *Bdellovibrio* on *Salmonella* Enteritidis.** *Bdellovibrio bacteriovorus* HD100 is routinely grown in the laboratory on *E. coli* (typically S17-1 or ML35) prey, and it is known to be predatory against a wide range of Gram-negative bacteria, but not all of them. Thus, before use against *Salmonella* Enteritidis *in vivo*, its efficacy in *in vitro* experiments was determined. When the predation efficiency of *B. bacteriovorus* HD100 on *S.* Enteritidis P125109 was compared to predation

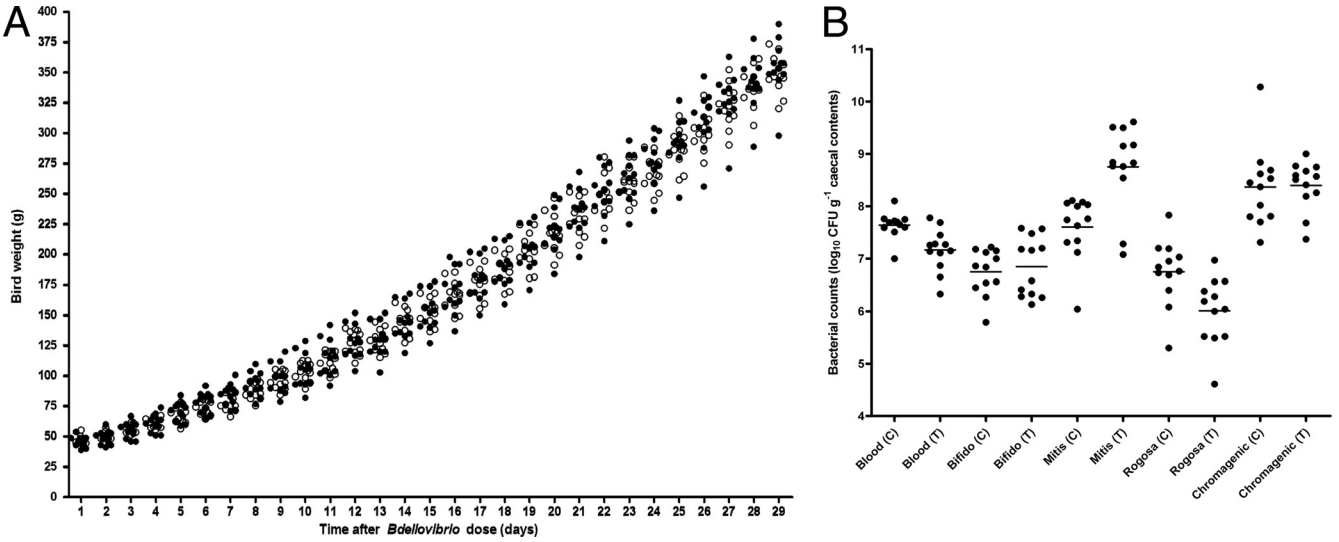


FIG. 2. (A) Weight gain by control and *Bdellovibrio*-treated chickens. Birds were dosed with *Bdellovibrio* (treated) or buffer (control) at 2 days of age and then weighed daily for the next 28 days. ●, control; ○, *Bdellovibrio* treated. (B) Scatter plot showing counts of bacterial populations from the cecal contents of control (C) and *Bdellovibrio*-treated (T) chickens. A group of 12 Hy-line brown birds each were challenged orally with 100 μl of approximately $1.9 \times 10^7$ PFU/ml of *B. bacteriovorus* HD100 at 2 days of age. An identical control group was challenged with Ca-HEPES buffer. At 28 days postdose, the birds were sacrificed and targeted bacterial populations were enumerated on selective agar. Abbreviations: blood, total anaerobic count on blood agar incubated under anaerobic conditions; bifido, bifidus selective agar; mitis, mitis salivarius agar; Rogosa, Rogosa agar; chromogenic, chromogenic agar. Each data point represents counts of bacterial populations from the cecum of a single bird. The horizontal lines represent the means for each group.

on *E. coli* S17-1 (as shown by a drop in the optical density of the prey cells in Fig. 1A), *E. coli* was preyed upon slightly faster than the *Salmonella* prey, although both reached a minimal optical density within the 24-h study period. Endpoint enumerations showed that although the *E. coli* population was reduced to 0.02% of its starting population size, the *Salmonella* population was reduced to 3.03%, thus showing that a considerable reduction in the *Salmonella* population was achieved, albeit not quite to the same extent as that of the *E. coli* population. During the same time period, the cultures containing *Salmonella* incubated with heat-killed *Bdellovibrio* were seen to increase in optical density, whereas the optical density of *E. coli* with heat-killed *Bdellovibrio* did not significantly alter during the course of the experiment. Therefore, the lesser reduction of the *Salmonella* populations by *Bdellovibrio* may be due in part to the ability of *Salmonella* to act as a more efficient scavenger and liberator of amino acids from the remnants of both dead prey and dead *Bdellovibrio*. *Bdellovibrio* cells were seen to efficiently enter *Salmonella* prey cells (Fig. 1B); entry was observed approximately 15 min after addition to prey, which is similar to the time previously noted for *E. coli* S17-1 prey (30, 32). Thus, while *B. bacteriovorus* HD100 does not prey upon *Salmonella* Enteritidis as efficiently as the routinely used *E. coli*, it is still an effective predator against *Salmonella*, causing significant reductions of 97% of the original numbers within 24 h within a buffered *in vitro* environment. As there are always survivors reported from *Bdellovibrio* predation, this predatory result was typical and suitable for the study of a reduction in numbers *in vivo*.

***Bdellovibrio* survival at 42°C and in microaerobic and anaerobic conditions relative to that in bird gut conditions.** *Bdellovibrio* is routinely cultured aerobically at 29°C, and while there has been a previous study showing survival in anoxic environments for up to 9 days (46), we assayed the survival of *B. bacteriovorus* HD100 both as attack-phase cells and inside bdelloplasts made from either *E. coli* S17-1 prey cells or *S.* Enteritidis P125109 cells at temperatures and gas conditions that would be found within the ceca of the birds. After incubation at 42°C, with and without oxygen or in a reduced-oxygen microaerobic environment, *Bdellovibrio* survival was assayed by the successful production of areas of clearing on lawns of prey (see Fig. S1 in the supplemental material). *B. bacteriovorus* HD100 was seen to survive both as attack-phase cells and within bdelloplasts for up to 48 h of incubation at 42°C aerobically, microaerobically, and anaerobically. This shows that, in buffered environments at least, *Bdellovibrio* should be able to survive the temperature and reduced-oxygen environments found within the gut. While these tests do not replicate the gut conditions precisely, it was important to establish *in vitro* that there was the expectation of survival of *Bdellovibrio* in the *in vivo* bird guts prior to the *in vivo* tests.

***Bdellovibrio* and bird well-being: testing for any effects of *Bdellovibrio* on bird health and behavior.** The effect of *Bdellovibrio* treatment on the health of Hy-line brown layer chicks was assessed for 28 days after inoculation by daily measurements of weight and twice-daily assessments of any health or behavioral abnormalities (according to the framework proposed by the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement [24]). *Bdellovibrio* was given to the birds at 2 days of age. Each bird received either $1.9 \times 10^6$ PFU *Bdellovibrio* (per dose) resuspended in Ca-HEPES buffer with 14% (wt/vol) $CaCO_3$ or a buffer with $CaCO_3$ control. None of the birds in either the control or *Bdellovibrio*-treated groups exhibited any signs of ill health or behavioral abnormality during the 4-week trial. Both groups gained weight (Fig. 2A) at or above the industry guideline rate for this strain of bird when

housed in industry-standard conditions (http://www.hyline.com/userdocs/Hy-LineBrown.pdf). Throughout the trial, only on day 6 were the bird weights for the control group significantly greater than those of the *Bdellovibrio*-treated group, although this difference was marginal ($P = 0.0404$ by Student's *t* test with Welch's correction for unequal variances); otherwise, the weights of the birds in both groups were indistinguishable from each other and met or exceeded industry standards (26).

**Experimental *Bdellovibrio* was not detected in the cage environment after dosing.** As the oral gavage of *Bdellovibrio* was simply administered to the birds from plastic syringes without a needle or cannulum (the birds then were returned to their floor pens), it was possible that *Bdellovibrio* organisms in the birds' mouths would find their way into the shared water dispenser and feed bowl of that group of birds, or that *Bdellovibrio* which survived passage through the intestinal tract would enter the environment in the birds' fecal deposits. As each treatment group of birds in our experiment was housed in pens within contained, biosecure rooms, cross-contamination was not an issue. However, we wished to see whether *Bdellovibrio* could be detected in the food and water of the birds to inform bird-housing considerations were *Bdellovibrio* to be practically applied as a therapy. *Bdellovibrio* is isolated from environmental water and soil sources (28, 29, 55), so it was possible that they could survive our dosing, and also it was possible that naturally occurring *Bdellovibrio* was present in the water and bedding and on the birds themselves (none of which were germfree). Our sampling was aimed to detect any excess of *Bdellovibrio* in the dosed birds versus that in controls, but no *Bdellovibrio* organisms were isolated from any sample in this experiment. The bacterial populations recorded in the fecal and water samples did not reveal any consistent differences between the control and *Bdellovibrio*-treated groups (data not shown).

**Alterations to the native cecal bacterial populations by *Bdellovibrio*.** Testing the fecal pellets did not reveal *Bdellovibrio* transiting the bird guts; however, in cecal contents taken from dissected birds at the end of the trial (28 days), blood agar and Rogosa plate counts (indicative of total culturable anaerobic and *Lactobacillus* populations, respectively) (Fig. 2B) were significantly lower ($P = 0.0029$ and 0.0095, respectively) in *Bdellovibrio*-treated birds than in the untreated control group (Student's *t* test with Welch's correction for unequal variances). Conversely, counts on mitis salivarius agar (indicative of culturable fecal streptococcal populations) were significantly higher in the cecal contents of *Bdellovibrio*-treated birds than in those of the controls ($P = 0.0008$). The counts of both the culturable bifidobacteria and coliform populations showed no differences between the two groups (Fig. 2B). However, *Bdellovibrio* was not recovered from the *Bdellovibrio*-dosed fecal samples throughout the trial or the samples of cecal content at the end of the trial. This suggests that *Bdellovibrio* is, at most, a short-lived population in the chicken intestine and does not readily contaminate the immediate environment of the birds. The significant changes in the culturable bird gut microbiota were taken as evidence of *Bdellovibrio* activity in the ceca just after inoculation, having effects on gut flora which persisted long enough to be measured at 28 days. A fuller DNA-based study of the whole microbiotal contents (including viable nonculturable species) is warranted on the basis of these detected changes in culturable populations, but this was beyond the scope of the study here.

***Bdellovibrio* reduces *Salmonella* colonization in chicks.** A scatter plot (Fig. 3) showing the distribution of *Salmonella* counts (in $\log_{10}$ CFU $g^{-1}$) in the cecal contents of control and predatory HD *Bdellovibrio*-treated birds shows that in all three days of the trial, the control groups harbored significantly higher numbers of *Salmonella* in their cecal contents than predatory HD *Bdellovibrio*-treated birds ($P < 0.004$). The mean reduction in *Salmonella* numbers in the ceca of *Bdellovibrio*-treated animals compared to treatment with the buffer controls was 0.76, 1.09, and 0.64 $\log_{10}$ CFU $g^{-1}$ for days 1, 2, and 3 of the trial, respectively. In contrast, there were no statistically significant differences between *Salmonella* counts in the nonpredatory HI Δ*pilA* *Bdellovibrio*-treated birds and those given a buffer control (Fig. 3). However, on days 1 and 2 there was a significant difference ($P < 0.05$) between the mean *Salmonella* numbers for the HD *Bdellovibrio*-treated birds and the nonpredatory HI Δ*pilA* *Bdellovibrio*-treated birds, with the HD *Bdellovibrio*-treated birds showing a mean reduction of 0.47 and 0.78 $\log_{10}$ CFU $g^{-1}$ for days 1 and 2 compared to levels for the HI Δ*pilA* *Bdellovibrio*-treated birds. This confirmed a predatory effect on those 2 days.

***Bdellovibrio* reduces cecal abnormalities and results in increased weight of cecal contents.** It has been published previously by others that the *Salmonella* Enteritidis PT4 infection of chicks causes full white ceca due to infiltration by white blood cells (1, 2, 16, 36, 42). We found these cecal abnormalities in our studies. Ceca were classified as abnormal if they were small (weighing less than 0.1 g) or were solid and white or white and mucoid, with almost no dark, free-flowing contents. The proportion of cecal abnormalities from control chicks and predatory HD and nonpredatory HI *Bdellovibrio*-treated chicks were compared for statistically significant differences using Fisher's exact test (Table 1), and typical examples of abnormal and normal ceca were photographed (Fig. 4). No significant differences in the proportion of cecal abnormalities were seen between the control group and the nonpredatory Δ*pilA* mutant HI *Bdellovibrio*-treated groups on any of the three days following treatment. However, birds treated with predatory HD *Bdellovibrio* harbored a significantly smaller proportion of abnormal ceca than the control on two of the three days (Table 1).

The weight of cecal contents from control and HI Δ*pilA* nonpredatory *Bdellovibrio*-treated birds as well as HD predatory *Bdellovibrio*-treated birds were analyzed using the nonparametric Kruskal-Wallis test with Dunn's post test (51). A significant ($P < 0.05$) increase in the weight of cecal contents, accompanying the observation of free-flowing normal dark cecal contents, was seen in the HD predatory *Bdellovibrio*-treated birds on the first 2 days following treatment, compared to that with the buffer control (Table 2).

## DISCUSSION

*Bdellovibrio* strains have long been proposed as a future alternative for antimicrobial therapy, and it has been suggested that they would be suitable for external use (such as in infected skin wounds [52]); however, in such applications the accidental ingestion of *Bdellovibrio* may be an issue. *Bdellovibrio* species are reported to be unable to prey on eukaryotic cells, and as

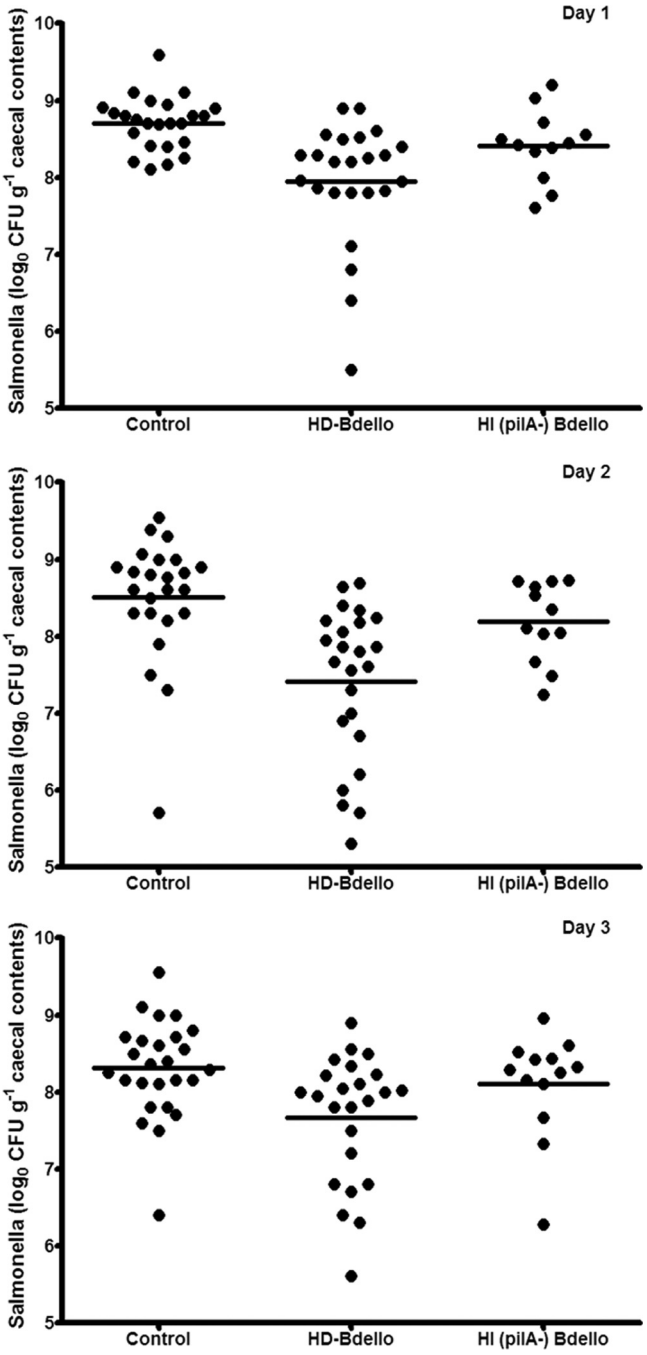


FIG. 3. Scatter plot showing the effect of *Bdellovibrio* treatment on colonization of chicken ceca by *Salmonella* Enteritidis. Ten groups of 18 Hy-line brown chicks each were challenged orally with approximately $3 \times 10^7$ CFU of *S.* Enteritidis P125109 at 2 days of age. Four of these groups subsequently were dosed orally with approximately $9.8 \times 10^7$ PFU of *Bdellovibrio bacteriovorus* HD100 at 6 days of age. Two further groups were dosed with nonpredatory HI Δ*pilA Bdellovibrio*, matched to the predatory *Bdellovibrio* number by total protein content. During each of the 3 days following *Bdellovibrio* treatment, six birds from each group were sacrificed, and the number of *Salmonella* organisms in the cecal contents of each bird was determined by spread-plating serial dilutions of cecal suspensions onto brilliant green agar. The results from four independent biological repeats were pooled from each day. Each data point represents the number of *Salmonella* organisms in the cecal contents of a single bird. The horizontal line represents the means for each group.

TABLE 1. Changes to cecal morphology and pigmentation in treated and control birds[a]

| Treatment | No. of cecal abnormalities/total no. of birds (*P*) on day: | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Control buffer | 9/24 | 9/24 | 8/26 |
| HD *Bdellovibrio* | 1/24 (0.0102) | 5/24 (NS) | 0/24 (0.0043) |
| HI *Bdellovibrio* nonpredatory Δ*pilA* | 4/12 (NS) | 2/12 (NS) | 2/13 (NS) |

[a] Shown are the number of cecal abnormalities in each treatment group on each day following treatment. Statistical analysis of each group compared to the control was performed using Fisher's exact test, and *P* values less than 0.05 are given. NS, nonsignificant ($P > 0.05$).

such they pose no direct risk to human or animal health, but the indirect consequences of their predation on the natural microbiota of a treated animal or human may be detrimental to health. Our study is the first in which *Bdellovibrio* strains have been used internally in a live-animal model to reduce pathogen numbers *in vivo*, and it provides the first insights into their potential effects on the native gut flora should they be accidentally ingested.

We have shown that *Bdellovibrio bacteriovorus* HD100 is a good predator in *in vitro* assays on *Salmonella* Enteritis P125109 prey, effectively reducing *Salmonella* numbers by 97% during a 24-h incubation period (Fig. 1). *S.* Enteritidis P125109 is a phage type 4 (PT4) strain that was isolated from a food-

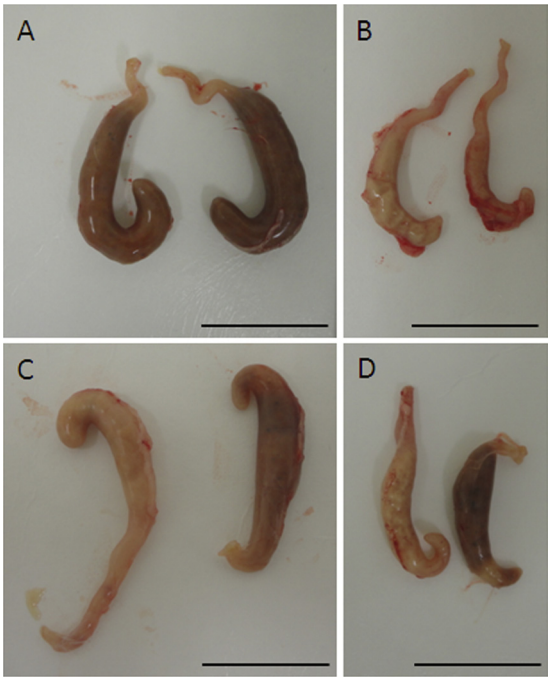


FIG. 4. Representative ceca from treated birds. (A) Predatory HD *Bdellovibrio*-treated bird; (B) nonpredatory HI Δ*pilA Bdellovibrio*-treated bird; (C and D) control birds. Scale bars, 5 cm. Normal ceca contain dark free-flowing fecal material, but *Salmonella* infection is widely reported (1, 2, 16, 36, 42) to cause pale ceca with dense non-free-flowing contents due to inflammation and white blood cell infiltration.

TABLE 2. Weights of cecal contents from each treatment group following *Bdellovibrio* treatment[a]

| Treatment | Cecal content wt (means ± SD) on day: | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Control buffer | 0.49 ± 0.28 | 0.48 ± 0.37 | 0.77 ± 0.46 |
| HD *Bdellovibrio* | 0.75 ± 0.30 ($P < 0.05$) | 0.87 ± 0.51 ($P < 0.01$) | 0.94 ± 0.49 (NS) |
| HI *Bdellovibrio* nonpredatory Δ*pilA* | 0.84 ± 0.48 (NS) | 0.72 ± 0.50 (NS) | 1.08 ± 0.54 (NS) |

[a] A nonparametric Kruskal-Wallis with Dunn's post test was performed on each group versus the control group, and *P* values versus the control for statistically significantly different groups are shown. NS, nonsignificant ($P > 0.05$).

poisoning outbreak linked to a poultry farm, and it has been shown to be highly virulent in young chicks and to be invasive in laying hens, resulting in contaminated eggs (5, 6, 58). Many strains of *Salmonella* Enteritidis are known to be capable of passing between host species, increasing the potential for outbreaks as they pass from animals to humans, and those belonging to the PT4 groups are primarily responsible for salmonellosis outbreaks in Europe (40). Thus, the *Salmonella* strain used in this study is of relevance to both human and animal disease, and it has been responsible for real outbreaks of disease.

Dosing 2-day-old (*Salmonella*-free) chicks with wild-type predatory *B. bacteriovorus* HD100 did not cause any observable negative health effects on the birds, which continued to eat, drink, and behave normally; they grew (Fig. 2A) at a rate comparable to that of birds of the same strain reared under standard poultry industry conditions (26).

We were able to show *in vitro* that at least a portion of the *B. bacteriovorus* HD100 population survived anaerobic and microaerobic incubations at 42°C for 48 h, and it retained predatory capability; thus, the atmospheric and temperature conditions that they would experience in the cecum would not rapidly kill them, although they were clearly not active within the gut flora after 26 days in our well-being experiment. That there were long-lasting changes in the culturable gut flora of birds following a single *Bdellovibrio* dose at 2 days was somewhat unexpected, but it was clear that the changes were associated with only positive or neutral impacts on bird well-being (Fig. 2B). The unexpected increase in counts on the mitis agar (taken to be streptococcal, Gram-positive bacteria) for the *Bdellovibrio*-treated birds in the well-being experiment may have been due to a niche becoming available due to a predatory reduction in Gram-negative bacteria, which was recorded as a reduction in total anaerobic counts (Fig. 2B). Previous *in vitro* studies (25) of *Bdellovibrio* predation in mixed cultures containing both susceptible prey (*E. coli*) and live decoys of nonsusceptible Gram-positive bacteria (in that case, nonsporulating *Bacillus subtilis*) showed that in cultures containing all three species of bacteria, a rise in both prey and live decoy numbers occurred due to the recycling of nutrients released from the remnants of burst prey and dead *Bdellovibrio* cells, showing that *Bdellovibrio* predation can indirectly affect other bacterial populations which are not susceptible to predation (25). To reduce our use of birds to the minimum number possible, we decided not to investigate this change further, as we were confident from the growth data and well-being of the birds that any effects of this *Bdellovibrio*-induced change in the culturable microbiota which we studied here were not detrimental. Future DNA-based studies examining the total gut microbiota and the effect of *Bdellovibrio* predation upon it would be interesting and important to fully measure the effects (56).

Our results have shown that orally dosing chicks with predatory wild-type *Bdellovibrio bacteriovorus* HD100, but not a nonpredatory Δ*pilA* strain (18) (reconfirmed here by silent deletion), is associated with a significant reduction in the numbers of *Salmonella* organisms in the cecal contents of live-infected chickens compared to results for the untreated controls (Fig. 3). In addition, only birds treated with predatory wild-type *Bdellovibrio* had a significantly smaller proportion of abnormal ceca (which showed inflammatory characteristics described previously [1, 2, 16, 36, 42]) than that of untreated controls (Table 1 and Fig. 4).

Birds treated with the nonpredatory Δ*pilA* strain of *Bdellovibrio* did not show any significant reduction in *Salmonella* numbers compared to the control group, nor were the numbers of abnormal ceca reduced compared to those given for the buffer control. It was noted that the birds in the Δ*pilA*-treated group showed a small (but nonsignificant) improvement in cecal mass and appearance; thus some, but not all, of the therapeutic effects seen by predatory *Bdellovibrio* treatment may be due to competition for resources, including nutrients and the limited available oxygen.

That many of the *Salmonella*-treated birds which did not receive predatory *Bdellovibrio* had pale ceca with abnormal dense white mucoid gut material within suggested that the untreated *Salmonella* had caused the inflammation of the ceca and recruitment of heterophils to that area of the gut, as others have reported (1, 2, 16, 36, 41, 42). It was marked how treatment with predatory *Bdellovibrio*, but not the Δ*pilA* strain, had ameliorated this effect (Table 1 and Fig. 4). This observation should be followed up by further histological studies. In addition, the cecal contents of predatory *Bdellovibrio*-treated birds were of a normal color and consistency compared to the pale, solid material often contained within the ceca of untreated control birds. This may have resulted in an underrepresentation of *Salmonella* numbers in the latter, as access to the cecal lumen (from which the material for counting was sampled) was restricted by the viscosity of the pale solid material in the cecal cavity.

In these poultry trials we were not able to readily culture *Bdellovibrio* from the cecal contents of birds, although we did recover some live *Bdellovibrio* sporadically in a pilot stage of the trial in younger 6- to 9-day-old birds (see Fig. S2 in the supplemental material). However, we were able to record their effects on culturable gut microbiota in the well-being experiment (Fig. 2B) and on the reduction of abnormal cecal numbers and *Salmonella* colonization in the therapeutic trial (Table

1). It was clear that by day 3 of our trial the effects of the HD *Bdellovibrio* were waning, presumably because they were short lived.

The most characteristic postmortem finding was abnormal ceca (i.e., typhlitis), seen in approximately one-third of birds dying of nontyphoidal salmonellosis, with the ceca distended by hard, white, necrotic cores (1, 2, 16, 36, 42), and it is clear (Table 1) that predatory HD *Bdellovibrio* ameliorated this effect. Comparisons to nonpredatory *Bdellovibrio* and the buffer control showed that live predatory *Bdellovibrio* bacteria were producing a reduction in *Salmonella* numbers, albeit modestly, by 1 $\log_{10}$. This is the first time that the reduction of pathogens by *Bdellovibrio* has been shown in live animals.

For an ideal treatment, multiple rounds of *Bdellovibrio* replication on the *Salmonella* prey in the gut would be required to eradicate the *Salmonella* rather than to simply reduce their numbers, but our study is a good start in that direction and has used the minimum number of birds possible to show a statistically significant effect. In future studies, the isolation of novel gut-associated *Bdellovibrio* strains may give increased predation efficiency in these hot, anoxic environments against *Salmonella*. We used the type strain of *Bdellovibrio*, HD100, as it showed good *in vitro* predation efficiency on our chosen *Salmonella* strain and as its biology has been the subject of most recent studies. *Bdellovibrio* species have been isolated from the feces of both humans and animals, including chickens (47), but their predatory activity on a range of gut pathogens and commensals has not been extensively analyzed. The further investigation of such strains may reveal that there are *Bdellovibrio* species which are more suitable for use in the gut setting, and they may improve on the reduction in pathogen numbers seen here.

Nevertheless, our mild therapeutic effect can be compared to those of previous studies of bacteriophage treatment of *Salmonella* in live birds. *Salmonella*-specific bacterial viruses (bacteriophages) have been used both prophylactically (8, 9) and therapeutically (3, 7, 59) to prevent/reduce the colonization of the chicken gastrointestinal tract. The efficacy of bacteriophage treatment in young birds (less than 30 days of age) varied from modest (21) to moderate reductions of 1 to 2 $\log_{10}$ CFU $g^{-1}$ cecal contents (7). A treatment using solely phage P22 tail spike protein against *Salmonella enterica* serovar Typhimurium colonization of 2-day-old chicks showed a 2-log reduction in *Salmonella* numbers if the treatment was administered 1 h after *Salmonella* gavage and a 1-log reduction if administered 18 h after *Salmonella* dosing (62). Thus, the reduction achieved in our first *Bdellovibrio* trial is not out of line with the reductions seen using other biocontrol agents in similar models and may be improved by further multiple dosings.

Thus, while we acknowledge that more studies on the level and number of doses of *Bdellovibrio* required to effect a greater reduction or the possible eradication of *Salmonella*, we are glad to have made this start with *Bdellovibrio* being tested for its *in vivo* effects. Our study has shown that *Bdellovibrio* species do survive gut conditions for a short while and for long enough to have a beneficial therapeutic effect, and this can be built upon. We have also shown that, in this model at least, *Bdellovibrio* species do not have a detrimental effect on the health and well-being of noninfected birds, although some changes to the commensal gut flora are seen. The way ahead for improving *Bdellovibrio* for therapeutic applications is clear, and our study of birds suggests that there is no need to fear negative consequences on the gut microbiota if, during use as an external treatment, *Bdellovibrio* species were to be accidentally ingested.

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R.J.A., L.H., and R.E.S. conceived and designed the experiments. L.H., R.T., R.J.A., R.E.S., C.L., M.J.C., T.R.L., and A.K.F. performed the experiments. P.B. hosted and facilitated the animal work. L.H. and R.J.A. statistically analyzed the data. R.E.S., L.H., and R.J.A. wrote the paper.

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