

The potential for probiotics to prevent reproductive tract lesions in free-range laying hens

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Abstract. A study was undertaken to investigate the ability of two commercial probiotics applied in free-range laying hens (from 18 to 22 weeks of age) in reducing the occurrence of reproductive tract pathologies, and improving hen health and performance. In all, 630 17-week-old brown layers were transferred to a freshly cleaned free-range laying facility, and randomly divided into three groups, with three replicates of 70 birds each. Both probiotics were administered in the drinking water (Groups 1 and 2) on a daily basis for 4 weeks, while Group 3 was left untreated. At 38 weeks of age, the results demonstrated that treatment with either probiotic significantly reduced the occurrence of reproductive tract pathologies (control vs probiotics, 33% vs 22% and 11%; $P < 0.01$), mortalities (control vs probiotics; 3.8% vs 1.5 and 1.9%; $P < 0.01$), and increased the performance of hens, for another 20 weeks post-treatments (hen day production for control vs probiotics 75% vs 90% and 94%; $P < 0.01$). Birds treated with probiotics maintained their bodyweight and egg weights at standard ranges, while untreated birds did not perform at this level. Although we were unable to show any effect on cloacal bacterial colonisation, the results of the present study provided some initial evidence that reproductive pathologies that often cause drops in egg production and sudden deaths of birds, can be reduced if free range hens are treated with a commercial probiotic before or during the onset of lay. The use of a probiotic benefits the health and performance status of hens, resulting in better hen welfare and significant economic gains to egg producers.

Additional keywords: egg production, good bacteria, laying hens, oviduct pathology.

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Introduction

Free range accounts for ~26% of Australia's commercial egg production (AECL 2010). Although this system allows hens to express full behavioural repertoires, hens held under free-range conditions can be exposed to pathogenic bacteria that may disturb the normal flora of their body, leading to a range of diseases. From an early age, the chicken's intestinal tract is colonised by a large number of microbial species that play an important role in some physiological processes of the host in the gastrointestinal tract and other body systems, such as respiratory and reproductive system (Apajalahti *et al.* 2004). Several husbandry factors (in earlier stages and during laying period) alter the composition of the intestinal microflora and cause increased susceptibility of hens to pathogens and food safety-related bacteria, especially *Clostridium perfringens*, avian pathogenic *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp. (Durant *et al.* 1999; Yamauchi *et al.* 2006; Burkholder *et al.* 2008). Other predisposing conditions such as mucosal damage (of gut or reproductive tract) and immunosuppression caused by stress can contribute to

increased morbidity of gastrointestinal and reproductive tract and decreased performance or mortality of birds (Yamauchi *et al.* 2006; Burkholder *et al.* 2008).

In laying hens, the oviduct is the site for egg formation, and defence against pathogens in this organ is essential not only for the health of birds, but also for the production of safe eggs. Pathologies such as oophoritis, salpingitis, peritonitis and metritis are frequently encountered at onset and during the laying period, causing losses in egg production (Jordan *et al.* 2005). Most bacteria commonly associated with reproductive tract infections originate from both the ascending and descending route (Gast and Beard 1990; Shivaprasad *et al.* 1990; Hoop and Pospischil 1993; Keller *et al.* 1995). Consequently, before or after eggshell formation, developing eggs can be exposed to bacteria. Eggs from free-range systems were typically more contaminated than those from cage systems (De Reu *et al.* 2008). New data from extensive surveys in Europe and Australia have shown that reproductive tract infections such as salpingitis and peritonitis are more common in layers in non-cage or litter-based (Tauson *et al.* 1999) and free-range

systems (Nagle and Shini 2008; Fossum *et al.* 2009; Neubauer *et al.* 2009), providing a strong link between a contaminated environment and reproductive tract diseases.

One key problem facing the egg industry is that there are virtually no suitable agents to prevent, or treat, reproductive tract infections. In recent years, probiotics or direct-fed microbials have been proposed as a natural and useful choice for providing protection against enteric diseases, improved immunity and performance of broiler chicks and laying hens (Nahashon *et al.* 1996; Balevi *et al.* 2001; Willis and Reid 2008). It has been proposed that after oral administration, probiotics can change the bacterial community structure in the avian gastrointestinal tract (Mountzouris *et al.* 2007; Willis and Reid 2008).

The presence of lactobacilli in the cloaca and uterus of hens has been seen as an important factor in maintaining the microbial ecosystem and preventing the growth of pathogens, such as *Salmonella* (Van Coillie *et al.* 2007). Studies in humans have demonstrated that the use of orally delivered probiotics containing lactobacilli restores commensal vaginal flora. Reid and Bocking (2003) showed that oral or vaginal administration of certain lactobacilli strains can safely colonise the vagina, displace and kill pathogens including *Escherichia coli*, and modulate the immune responses. Oral formulations of lactobacilli intended for use in genitourinary infections have been shown to be capable of maintaining their structural integrity during passage through the gut, or when delivered to the rectal area can colonise the vaginal tract (Shalev *et al.* 1996; Reid *et al.* 2003). As another example, normalisation of the urogenital tract in females was observed 14 days after oral administration of lactobacilli (Morelli *et al.* 2004).

Poultry producers, and especially organic producers, are interested in the use of probiotics in laying hens to improve egg production and health of hens kept in free range. Evidence is required of the efficacy of commercial probiotic use. Randomised controlled trials can provide evidence of probiotic efficacy in the prevention of diseases and improvement of hen liveability and profitability. The objective of the present study was to explore the ability of two commercially available probiotics when applied in drinking water before and during the onset of lay in reducing the occurrence of reproductive tract pathologies and improving general health and performance of hens kept in free range.

Materials and methods

Birds, housing and treatments

In total, 630 17-week-old Hy-Sex Brown layers were transferred to a freshly cleaned free-range laying facility and randomly divided into three groups, with three replicates of 70 birds each. Sheds contained automated chain feeders and drinkers for supplying feed and water, respectively. Birds were kept at a density of two birds/m² floor space (inside the shed) and had access to an outdoor area (separated area for each replicate) of 15 birds/100 m². Birds were vaccinated for avian encephalomyelitis, egg drop syndrome, fowl pox, infectious bronchitis, Marek's disease, Newcastle disease, fowl cholera, infectious coryza, *Mycoplasma gallisepticum* and *M. synoviae* and had regular worming. Other bird husbandry and management (feeding regime, lighting, and indoor and outdoor conditions) were as

recommended by breeding company and in accordance with industry standards, the Free Range Egg and Poultry Association of Australia, and Australian regulations (Primary Industries Standing Committee 2002).

All groups (two treatments and one control) received the same diet, a corn-based organic diet (containing an average of 11.6 MJ/kg ME, 19% crude protein, 4.3% fibre, 3.82% Ca, and 0.83% P) with no probiotics or other antimicrobial agents. Two commercially available probiotics approved for use in poultry in Australia were employed in the present study. The probiotics were in a powder form and were dissolved in the drinking water on a daily basis for 4 weeks (from 18 to 22 weeks of age). Probiotic 1, a multi-strain probiotic product, contained a source of live viable naturally occurring microorganisms isolated from the crop (*Lactobacillus reuteri*), jejunum (*Enterococcus faecium*), ileum (*Bifidobacterium animalis*), and caecum (*Pediococcus acidilactici* and *Lactobacillus salivarius*) of healthy adult chickens and had a total bacterial count of 2×10^{12} colony-forming units (cfu)/kg of product. The fructo-oligosaccharides used in Probiotic 1 are derived from a natural plant source and are selected for their ability to stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli in the intestine. Following recommendations of the manufacturer, Probiotic 1 was administered in the drinking water at a level to supply 10^8 bacteria/hen.day for 4 weeks. Probiotic 2 was a highly concentrated pre-mix containing seven strains of bacteria and two yeasts (*Lactobacillus plantarum* 1.89×10^{10} cfu/kg, *L. delbrueckii* subsp. *bulgaricus* 3.09×10^{10} cfu/kg, *L. acidophilus* 3.09×10^{10} cfu/kg, *L. rhamnosus* 3.09×10^{10} cfu/kg, *Bifidobacterium bifidum* 3.00×10^{10} cfu/kg, *Streptococcus salivarius* subsp. *thermophilus* 6.15×10^{10} cfu/kg, *Enterococcus faecium* 8.85×10^{10} cfu/kg, *Aspergillus oryza* 7.98×10^9 cfu/kg, *Candida pintolopesii* 7.98×10^9 cfu/kg), with all microorganisms having been isolated from a wide range of feed, plant, animal, bird and human sources. Probiotic 2 was also administered for 4 weeks at a dose recommended by manufacturer (1 g/L in the drinking water). Control hens received water with no probiotics. All procedures conducted in the study were approved by the Animal Ethics Committee of the University of Queensland (approval number SVS/248/09/POULTRY CRC).

Sampling and data collection

The health status of birds was checked daily and birds found dead were recorded and necropsied to assess for reproductive tract pathologies. Samples (for microbiological and histology tests) were taken before the treatment started (at 18 weeks of age), 4 weeks after first treatment (at 22 weeks of age), 4 weeks after last treatment (at 26 weeks of age) and at the point of lay period (at 38 weeks of age).

Microbiological testing

Microbiological samples from cloaca and oviduct were collected only from live or freshly killed and necropsied birds. At 18, 22, 26, and 38 weeks of age, samples from cloaca of nine control hens and nine hens per each treatment were collected aseptically by swabs, and streaked onto 5% sheep blood agar, MacConkey agar and xylose–lysine decarboxylase agar. The plates were incubated

aerobically at 37°C overnight. The following day, bacteria considered significant were single-colony picked onto a fresh sheep-blood agar plate. All primary sheep-blood plates were re-incubated for a further 24 h and re-examined. Normal bacterial growth (e.g. coliforms, *Pseudomonas* spp., *Bacillus* spp.) were identified purely on colony morphology. An abbreviated identification scheme based on conventional phenotypic tests was used to identify *Escherichia coli* (only if present as pure culture with no other coliforms evident on the MacConkey agar) and *Gallibacterium anatis* biovar *haemolytica*. The phenotypic tests used were Gram stain, oxidase reaction, catalase reaction, indole reaction and colony type on MacConkey agar. Isolates were presumptively identified as *E. coli* if they were Gram negative, oxidase negative, catalase and indole positive and produced typical *E. coli* colonies on MacConkey agar. Isolates were presumptively identified as *Gallibacterium anatis* biovar *haemolytica* if they were Gram negative, oxidase and catalase positive and indole negative and produced only weak or no growth on MacConkey agar. The control stains used for the phenotypic tests were *E. coli* ATCC 25922 and *Gallibacterium anatis* biovar *haemolytica* CCUG 15563.

On the same day, the birds used for cloacal swabs were humanely killed and then necropsied. Swab samples were aseptically collected from oviduct (in the region between the magnum and isthmus) of each hen for microbiological testing as above. Birds were subsequently examined to determine the prevalence of the subclinical and chronic forms of the reproductive tract lesions.

Post-mortem examination

All birds that died during the experimental period (from 18 to 38 weeks of age) and birds that were killed at each sampling point (at 22, 26 and 38 weeks of age) were subjected to a post-mortem examination to identify the cause of death or evaluate the reproductive tract, respectively. The necropsy included the bodyweight (BW) of the bird, an examination of the overall condition, and external and internal observations. Where required, a tentative diagnosis was based on the presence of macroscopic lesions in organs. A scoring system was used for an effective recognition of macroscopic changes observed in dead or killed hens. Birds were inspected for pathological changes, including inflammatory signs such as, for example, hyperaemia and oedema of the mucosal membranes and the presence of serous, fibrinous or caseous exudates in the peritoneal cavity, the ovary, the oviduct or all of them. The area of the vent and cloaca was examined for signs of infections and prolapse.

Histology

Samples for histology evaluation (six from each treatment) were taken from intestinal tract (ileum segments at the mid-point) and the oviducal magnum of killed birds at 38 weeks of age. Oviducal magnum samples were taken from hens that had an egg in the mid-magnum. All samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin stain before microscopic examination. For each section, 10 randomly located areas were assessed using light microscopy at $\times 4$, $\times 10$, $\times 40$ and $\times 100$

magnification. Structural integrity of the villus was assessed from the ratio of villus height: crypt depth, which were measured under a light microscope ($\times 100$ magnification) by using a calibrated ocular micrometer. The values of the villus height and crypt depth in each bird were obtained from the mean of measurements made from four sections for each segment per bird. All slides were evaluated in a blind manner by two observers.

Performance records

The BW of hens was recorded on a monthly basis (before treatments started at 18 weeks of age and then at 22, 26, 30, 34 and 38 weeks of age). Fourteen hens per replicate (or 20% of hens) were weighed individually at each time and the average BW (expressed as g per bird) was calculated. Feed intake was recorded daily per each replicate (i.e. 70 birds), and the average feed consumption was calculated as g/bird.day. The feed conversion ratio (FCR) was calculated weekly for the whole duration of the experiment. Egg production was recorded daily for each replicate, and the percentage hen-day egg production (%HDP) was expressed on a weekly basis from 18 to 38 weeks of age. Egg weight was recorded on a monthly basis at 22, 26, 30, 34, and 38 weeks of age. In total, 50% of eggs per replicate were individually weighed at each sampling point and the average weight (g/egg) was calculated.

Statistical analyses

Statistical analyses were performed using the general linear modelling procedure of SAS (SAS Institute 1996). Data on performance parameters (BW, %HDP and egg weight) were on a replicate basis. To test for the treatment effect at each sampling point, recorded values were subjected to one-way ANOVA. Statistically significant effects were further analysed, and means were compared using Duncan's multiple-range test. Statistical significance was determined at $P \leq 0.05$. To evaluate whether significant differences existed for pathological findings, an unpaired *t*-test was used for comparing two means (control and Probiotic 1 or 2 treated) and determine the *P*-value. A 99% confidence interval for the true difference between the means was set, and in this case, the values were considered significant at $P \leq 0.01$.

Results

Bacterial evaluation of the cloaca

Table 1 presents data on the type and the frequency (%) of bacteria isolated from cloacal swabs before and after treatments with the respective probiotic. There were no major differences in the colonisation of the cloacal microflora between probiotic-treated and control hens before the treatment started and at each sampling point (4 weeks after the first treatment started, and 8 and 16 weeks after the last treatment with the probiotic). It is to be noted that all isolates of *Gallibacterium anatis* were the biovar *haemolytica* form of this species.

Evaluation of reproductive tract (pathological and bacteriological findings)

In total, 81 birds (of ~630 birds) were killed and the reproductive tracts were examined. Table 2 summarises pathological and

Table 1. Type and the frequency (%) of bacteria isolated from cloaca in different treatments (Control, Probiotic 1 and Probiotic 2)

Each value is the mean of nine replicate samples per treatment. Probiotic treatments were carried out for 4 weeks (from 18 to 22 weeks of age)

Type of bacterium	Before treatment started (at 18 week of age)	4 weeks after treatment started (at 22 week of age)	4 weeks after the last treatment (at 26 week of age)	16 weeks after the last treatment (at 38 week of age)
<i>Control</i>				
Coliforms	100	100	100	100
Coryneforms	33.3	55.5	77.7	33.3
<i>Gallibacterium anatis</i>	55.5	66.6	77.7	100
α -Streptococci	44.4	55.5	44.4	55.5
β -Streptococci	11.1	–	–	22.2
<i>Proteus</i> spp.	11.1	11.1	11.1	–
Micrococci	22.2	11.1	–	55.5
<i>Bacillus</i> spp.	11.1	22.2	–	22.2
<i>Probiotic 1</i>				
Coliforms	100	100	100	100
Coryneforms	–	100	77.7	22.2
<i>Gallibacterium anatis</i>	44.4	66.6	88.8	66.6
α -Streptococci	44.4	33.3	55.5	66.6
<i>Bacillus</i> spp.	–	–	44.4	11.1
<i>Proteus</i> spp.	11.1	–	22.2	11.1
<i>Probiotic 2</i>				
Coliforms	100	100	100	100
Coryneforms	22.2	77.7	88.8	55.5
<i>Gallibacterium anatis</i>	–	100	88.8	88.8
<i>Proteus</i> spp.	11.1	–	–	44.4
α -Streptococci	77.7	44.4	22.2	55.5
Micrococci	11.1	–	–	22.2
<i>Staphylococcus</i> spp.	11.1	–	–	–
<i>Bacillus</i> spp.	22.2	–	–	–
<i>Pseudomonas</i> spp.	–	–	11.1	–

bacteriological findings of the control and probiotic-treated hens. At 22 weeks of age, treated hens had a significantly ($P < 0.01$) lower occurrence of the reproductive tract pathologies than did control hens (22% vs 44% of birds necropsied). At 38 weeks of age, the incidence of reproductive tract pathologies in probiotic-treated hens had decreased to 11%, whereas control hens had an incidence of 33% ($P < 0.001$).

Gross examination of oviducts, ovaries and peritoneum of control and probiotic-treated hens (Table 2) showed the presence of inflammation in some birds. In affected birds, oviducal mucosa was found to be hyperaemic and sometimes covered with greyish-white or fibrinous exudate. The peritoneum was in many cases congested, especially the part covering the ovary having hyperaemic, deformed or atrophied follicles. In the case of chronic infections, the mesentery was also congested or covered with white fibrinous or caseous exudate. In three birds, caseous yolk material was found in the oviduct (between magnum and uterus) mixed with inflammatory debris and affecting the oviduct. There were some cases of abdominal cavity containing egg material, thickened yolk and caseous material associated with inflamed ovary or follicular atresia. In the case of chronic pathologies, birds were commonly in good condition, but the ova and occasionally the oviduct were inactive atrophied and/or covered with

thickened exudates). Fig. 1a, b shows pathological reproductive tract lesions that were found in hens after the post-mortem of clinically normal birds killed during sampling, while Fig. 1c, d shows pathologies from control birds that died during the trial.

In general, there was a low contamination of the oviduct with aerobic bacteria in all birds (Table 2). A total of 60% of the oviduct samples (from control and probiotic-treated hens) had no bacterial growth. The most prevalent bacteria recovered from control hens were micrococci, while probiotic-treated birds had a mixed bacterial growth, with *Gallibacterium anatis*, α -streptococci and coryneforms being common. No bacteria were recovered from the oviducts of hens with pathological mesentery, ovaries and oviducts. Only two samples from oviducts with pathology (one from control hens and one from probiotic-treated) showed bacterial growth. A correlation between clinical symptoms of reproductive pathologies and specific bacteria could not be established. No correlation was also noticed between bacteria isolated from the cloaca and oviduct. At 38 weeks of age, a large number of hens necropsied showed infestation with parasites (roundworms and tapeworms), but surprisingly with a lower frequency in probiotic-treated hens (control vs probiotics, 66% vs 33%).

Table 2. Pathological, parasitological and bacterial findings of oviducts from euthanised hens in different treatments (Control, Probiotic 1 and Probiotic 2)

Normal = reproduction tract appears normal. Parasite includes parasites, nematodes and cestodes. Each value is the mean of nine replicate samples per treatment

Condition	4 week after first treatments (at 22 week of age)	4 week after last treatment (at 26 week of age)	16 week after last treatments (at 38 week of age)
<i>Control</i>			
Normal (%)	56	56	67
Peritonitis (oophoritis, salpingitis) (%)	44	44	33
Micrococci ^A (%)	22	11	33
Parasite ^C (%)	0	11	66
<i>Probiotic 1</i>			
Normal (%)	89	100	89
Peritonitis/oophoritis/salpingitis (%)	11	0	11
<i>G. anatis</i> ^A (%)	0	11	0
α -Streptococci ^A (%)	11	0	11
Parasite (%)	0	0	33
<i>Probiotic 2</i>			
Normal (%)	78	89	78
Peritonitis/oophoritis/salpingitis (%)	22	11	22
<i>G. anatis</i> ^A (%)	11	0	0
<i>Bacillus</i> spp ^A (%)	11	0	0
Coryneform ^A (%)	0	0	33
Parasite (%)	0	11	33

^ABacteria isolated from oviduct swabs.

Histology

The histological examination of sections from intestine of control and probiotic-treated hens (Fig. 2*a–f*) did not show any significant changes in the ileal villus height, crypt depth and villus height: crypt depth ratio for any of the treatment and control birds (data not shown). However, birds treated with probiotic showed a thicker ($P < 0.05$) mucosa layer than did controls (Fig. 2*a, d*). Further histological examination showed a more uniform epithelial thickness for probiotic-treated hens (Fig. 2*b, e*) and an increased ($P < 0.05$) density of intraepithelial leukocytes (Fig. 2*c, f*). Histological slides from the oviducal magnum of control and probiotic-treated hens (Fig. 3*a–f*) showed differences ($P < 0.01$) in the density of mucosal folds, the thickness of epithelia (Fig. 3*a, d*), and density of hair-like cilia (Fig. 3*c, f*). Areas of granular cells were more evident between ciliated cells of the magnum in the hens from probiotic-treated groups.

Cumulative mortality

The percentage of cumulative mortality in control and probiotic-treated hens in the period from 18 to 38 weeks of age is presented in Fig. 4*a*. Starting at 26 weeks of age, cumulative mortality in control hens was significantly ($P < 0.01$) higher than in both of the probiotic-treated groups (3.5% vs 1.5% and 1.9%, respectively). The necropsy examination of the total of eight chickens that died

in the control group indicated that the death in four chickens was caused by acute reproductive pathologies. Two hens had a combination of pericarditis, perihepatitis and airsacculitis (polyserositis) and one of the hens was cannibalised. In one bird, the cause of death could not be identified. In the Probiotic 1 treatment group, only three birds died during the experimental period (two were cannibalised, and one of the hens had salpingoperitonitis). In the Probiotic 2 treatment group, four birds died in total (two were cannibalised, one had acute peritonitis, and one had neck injury).

Bodyweight

Hen weight increased as the flock aged (Fig. 4*b*). At 18 weeks of age, there were no significant differences in BWs among all experimental groups. At all other points of measurement, probiotic-treated hens were heavier ($P < 0.01$) than non-treated (control) hens. Furthermore, at 30, 34, and 38 weeks of age, the BW of the Probiotic 2-treated hens was significantly higher ($P < 0.05$) than that of Probiotic 1-treated hens.

Egg production and egg weights

From 18 to 23 weeks of age, the HDP% increased at a similar rate in all experimental groups (Fig. 4*c*). There were two significant ($P < 0.01$) drops in %HDP of controlled hens (at Weeks 23 and 24, and at Weeks 34 and 35). Overall, the birds in the probiotic-treated groups performed better than those in the control group (control vs probiotics, 75% vs 90% and 94%). Average egg weights from onset until peak of lay (i.e. from 18 to 38 weeks of age) are presented in Fig. 4*d*. The egg weights increased as the flock aged. However, at 34 and 38 weeks of age, the average egg weights of both probiotic-treated groups were significantly ($P < 0.05$) higher than that of control birds. At 26 weeks of age, hens in the Probiotic 2 treatment group had a significantly ($P < 0.05$) higher egg weight than did those in the control and the Probiotic 1 group, respectively.

Feed consumption and FCR

There were no significant ($P > 0.05$) differences between control and probiotic-treated groups for feed consumption and FCR.

Discussion

The effects of two commercial probiotics administered in the drinking water for 4 weeks (from 18 to 22 weeks of age) on the prevention of the occurrence of reproductive tract pathologies in laying birds were investigated. The experiments performed in the present study demonstrated that treatment with probiotics significantly improved the reproductive tract health, reduced mortality and increased performance (BW, egg production and egg weight) of hens at 4 weeks post-first treatment and in the subsequent period, for further 20 weeks post-treatments. A decrease of reproductive pathologies together with an improvement of overall health (i.e. decreased mortality) and performance of probiotic-treated hens was demonstrated in the present study. To the best of our knowledge, the present study is the first demonstration of the commercial probiotic use for preventing reproductive tract pathologies in free-range laying hens.

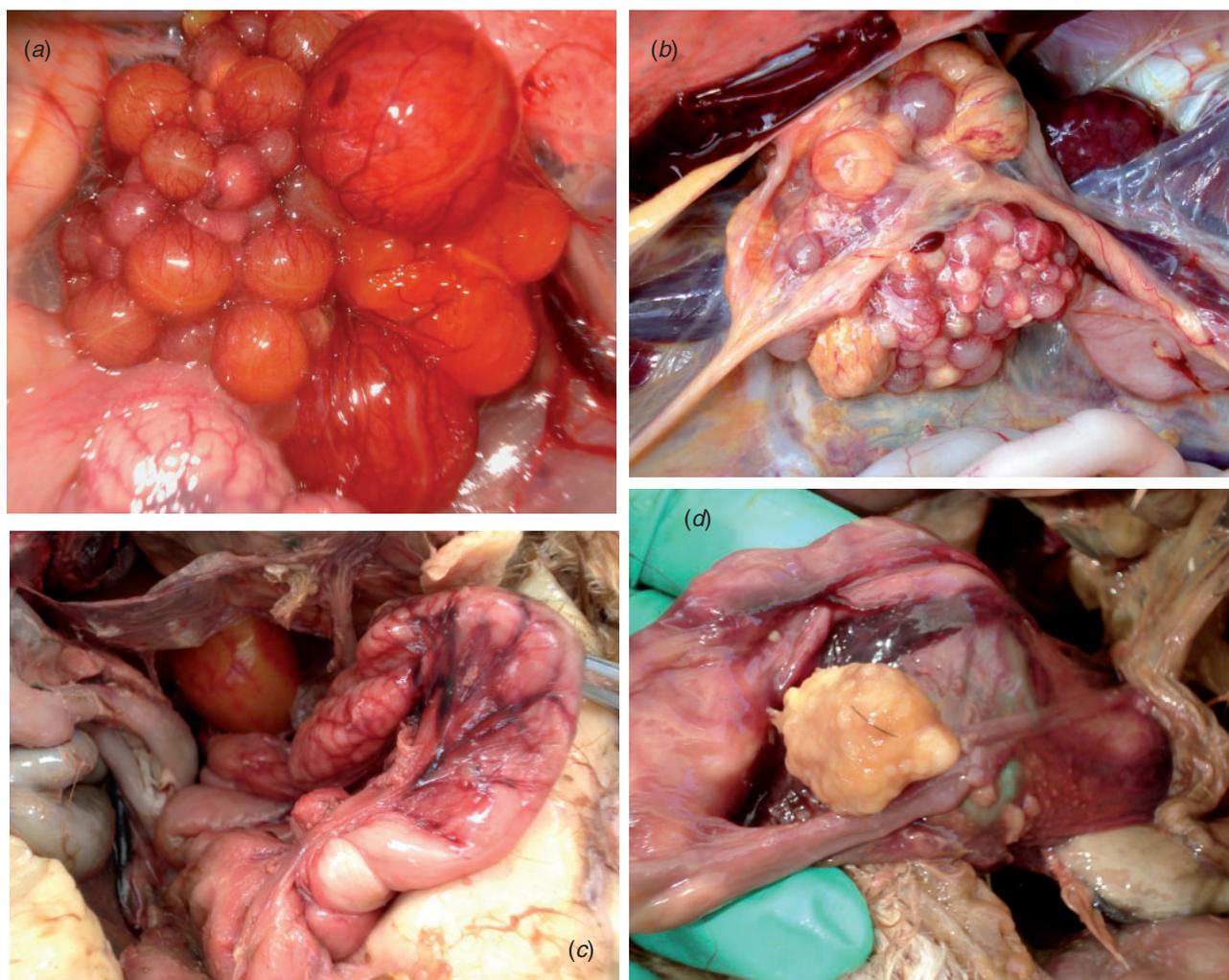


Fig. 1. Reproductive tract pathologies from clinically normal birds (*a, b*) killed during sampling (acute oophoritis) and (*c, d*) birds that died during the trial and were necropsied (acute and chronic salpingo-peritonitis).

Reproductive tract pathologies such as peritonitis and salpingoperitonitis are conditions often referred to in laying hens as egg peritonitis (Jordan *et al.* 2005). There are many factors that can initiate such pathologies. In particular, in alternative production systems, birds live in an open environment and they can become contaminated from several different sources. Thus, bacterial infections become a major contributory factor that should be taken into consideration if the frequency of reproductive lesions increases in a flock. In some cases, hens may look healthy, but due to chronic pathologies of the ovary or oviduct, they stop laying eggs. Mortalities from reproductive pathologies are rare, and in most of cases, are caused by other complications, such as acute and chronic peritonitis (Jones and Owen 1981; Kinde *et al.* 2000; Jordan *et al.* 2005). Various bacteria have been reported to cause primary or secondary reproductive tract infections in free-range birds (Shini *et al.* 2008; Neubauer *et al.* 2009). Although the route of infections is not clearly known, contamination of vent, cloaca and oviduct with faecal material has been seen as an important source of such infections (Keller *et al.* 1995). Many

investigators have previously isolated pathogenic bacteria (e.g. *Mycoplasma gallisepticum*, *E. coli*, *Salmonella* spp., *Pasteurella multocida* and *Staphylococcus aureus*) from lesions in the peritoneum and reproductive tract of hens (Gross and Siegel 1959; Jones and Owen 1981; Riddell 1996; Trampel *et al.* 2007). Mirle *et al.* (1991) examined 496 hens with reproductive tract lesions and isolated *Gallibacterium* in pure culture from 23% of the diseased organs. Haemolytic *G. anatis* was associated with infection in birds kept in alternative husbandry systems and suffering from reproductive disorders (Neubauer *et al.* 2009).

In the current study, the bacterial evaluation of the intestinal and reproductive tract of hens did not demonstrate particular changes of the microbial populations in control or probiotic-treated birds, with or without pathological changes. It should be noted that the microbiological analysis was limited in nature. A more extensive examination, including the use of molecular profiling methods could have helped detect bacterial changes and potentially established correlations between bacteria in the cloaca and oviduct of normal birds and birds with reproductive

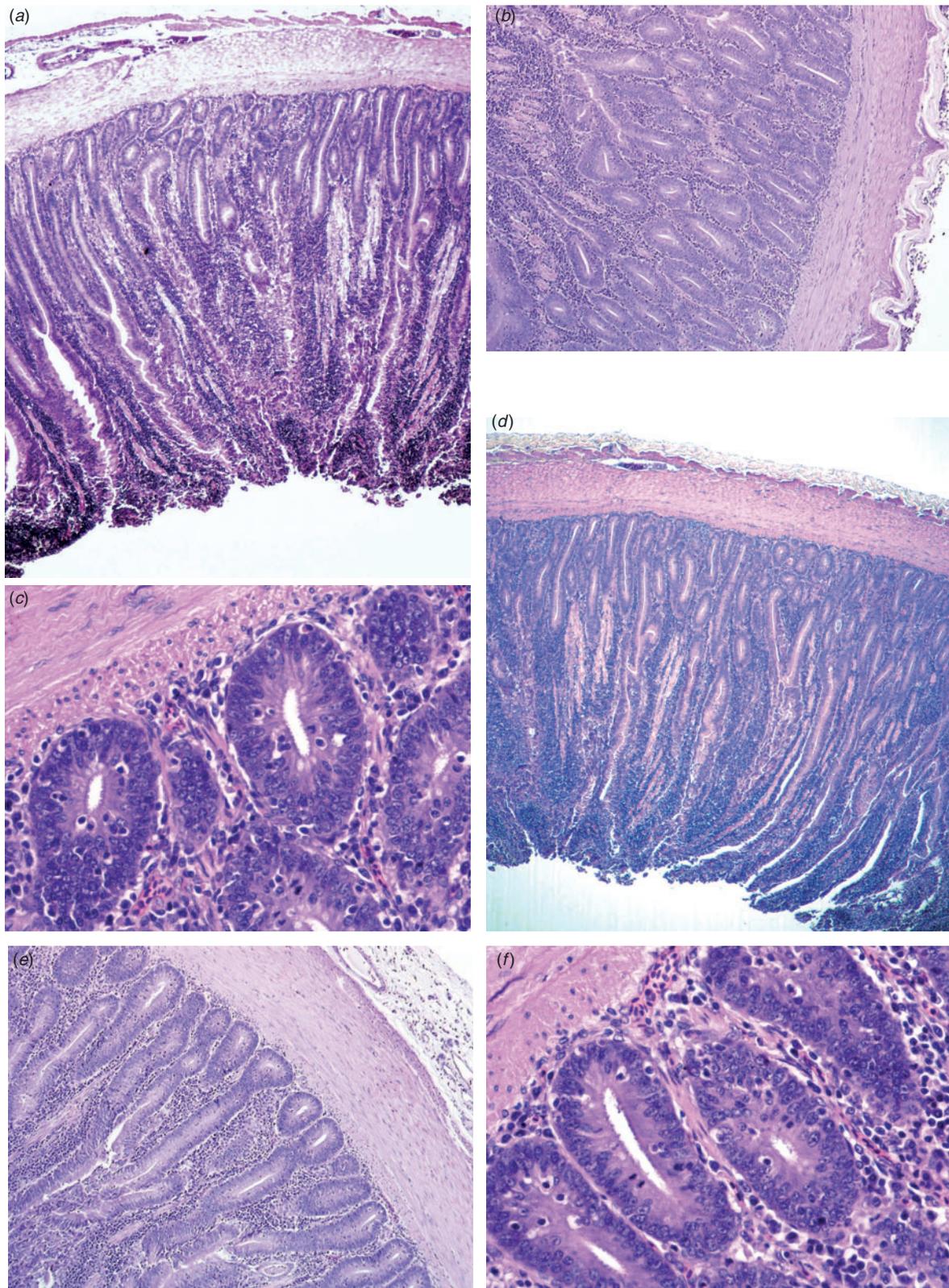


Fig. 2. Light microscopy (using $\times 4$ (a, d) $\times 10$ (b, e) and $\times 40$ (c, f) magnification) of hen intestinal villi (ileum) in (a–c) the control and (d–f) probiotic groups. Muscle thickness in the probiotic treatment was increased in comparison with that observed in control chickens. Treated chickens had a more uniform mucosa, with larger crypts of Lieberkühn and with more mononuclear cells migrating to the lamina propria. No changes in the ileal villus height and crypt depth for any of treatments and control birds were seen.

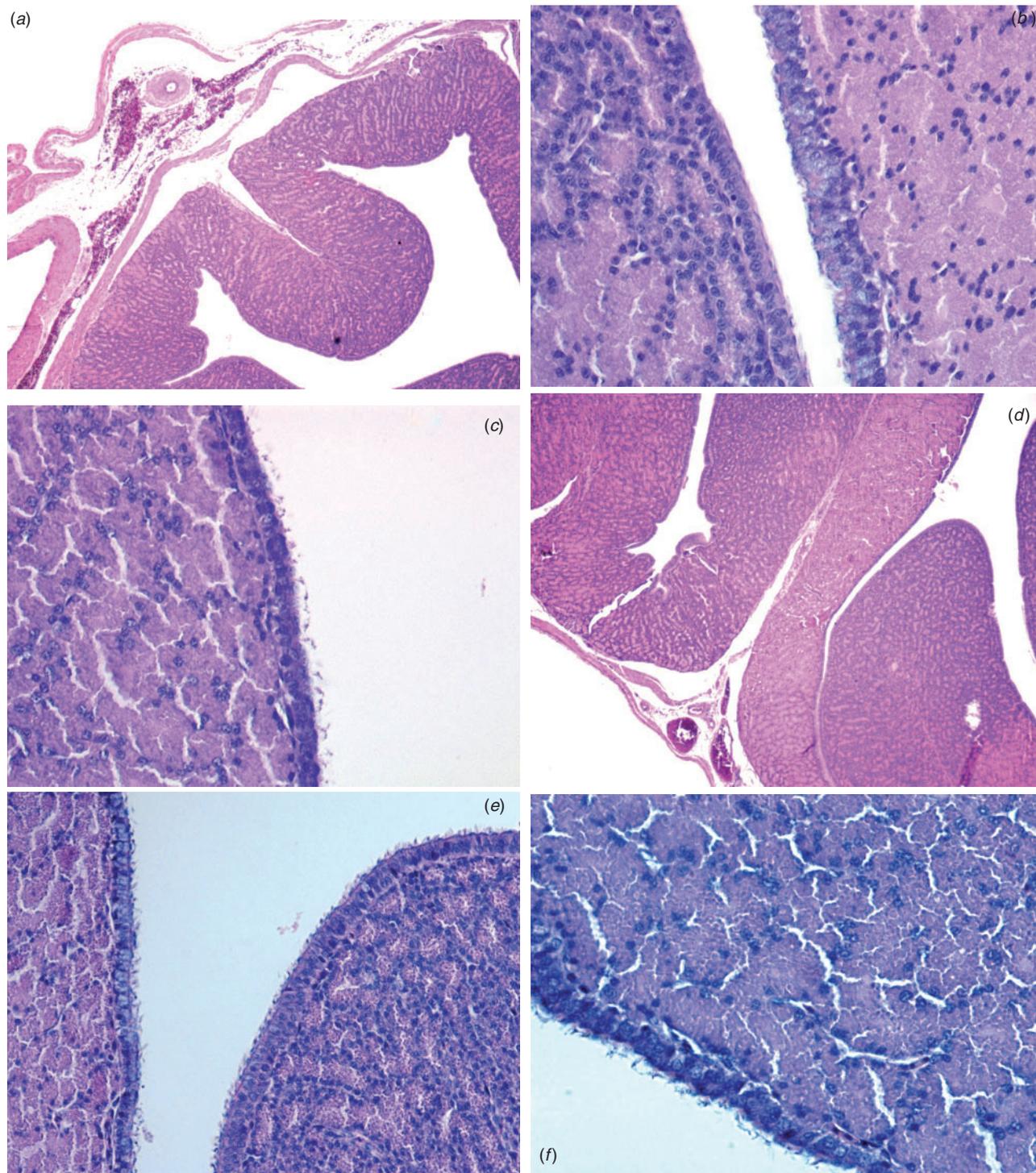


Fig. 3. Light microscopy ($\times 4$, $\times 20$ and $\times 40$ magnification) of hen oviducal magnum in (a–c) the control and (d–f) probiotic groups. Thickness of epithelia and density of mucosal folds and density of hair-like cilia were increased in probiotic-treated hens.

pathologies, respectively. It has been shown that, in a healthy state, the chicken gut contains a diverse bacterial population. The relative proportions of major groups of bacteria may change, but, are mainly dominated by bacteria classified as lactobacilli, *Enterobacteriaceae*, clostridia, *Bacteroides*, and enterococci and

many other groupings, both anaerobic and facultative (Ewing 2008). As stated above, most of the bacteria that were isolated in the present study are endemic to hens and are commonly found in the environment of chickens (Reiber *et al.* 1995). As expected, coliforms were found present in the cloaca of all sampled hens,

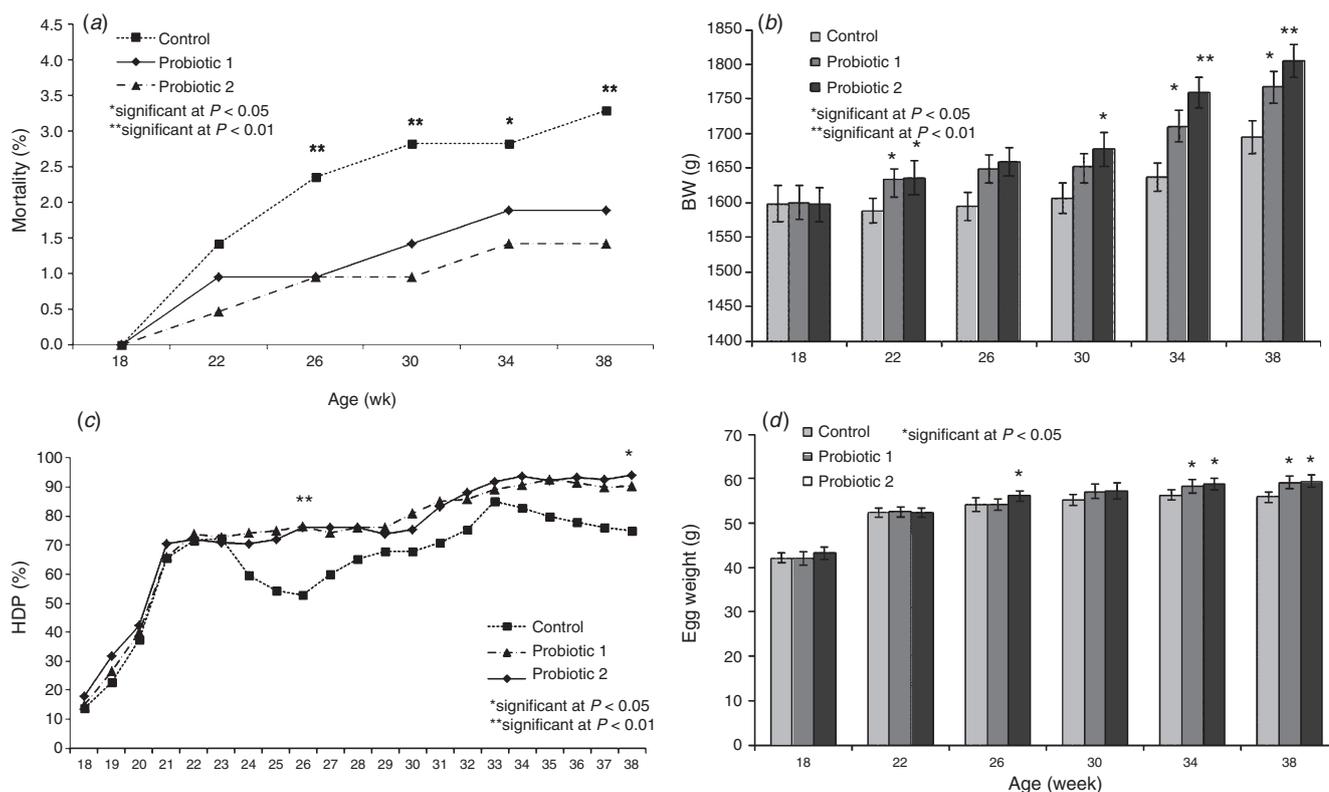


Fig. 4. The effects of probiotics on (a) mortality, (b) bodyweight (BW) and production parameters, (c) hen day production (HDP) and (d) egg weight of hens from 18 to 38 weeks of age.

in all sampling points. While, α -streptococci were detected at all sampling points, the frequency of detection was lower than for the coliforms, averaging 50% of sampled birds. *Gallibacterium anatis* was consistently found in most cloacal samples. At 38 weeks of age, micrococci were found to be significantly ($P < 0.001$) higher in the cloaca of control hens than were both probiotic groups (control vs probiotics, 55% vs 0% and 22%, respectively). Surprisingly, micrococci were never found in the oviduct of probiotic-treated hens, while control hens showed some presence of micrococci in the oviduct (11–33% of samples were positive). Another interesting observation from the present study was an increased presence of coryneforms in samples of probiotic-treated hens in both cloaca and oviduct. Coryneforms are a group of Gram-positive rod-shaped bacteria (a common genus being *Corynebacterium*) that are environmental residents of normal flora often isolated from poultry litter (Schefferle 1966; Chinivasagam *et al.* 2010). Thus, it is often difficult to determine their significance. There is some evidence on the probiotic activities of coryneform bacteria in fish. It has been reported that strains of *Pseudomonas* sp., *Vibrio* sp., *Aeromonas* sp. and groups of coryneforms isolated from salmonid hatcheries showed antiviral activity against infectious haematopoietic necrosis virus, with more than 50% plaque reduction (Kamei *et al.* 1988). This observation could be of interest for further identification of coryneform organisms from laying hens, and their potential probiotic properties.

Increased parasitic incidence is an important problem, and an additional risk to free-range flocks. Heavy worm burdens can

predispose birds to develop secondary bacterial infections. In some cases, *Ascaridia galli* eggs may act as mechanical vectors of reproductive tract bacterial infections such as *Salmonella* (Chadfield *et al.* 2001). In the present study, at 38 weeks of age, necropsied birds from the control group had higher ($P < 0.05$) infestation with round- and band-worms (Table 2), potentially contributing to a lower performance and higher incidence of reproductive tract infections and mortality.

In the current study, probiotic-treated hens showed a significant reduction in reproductive tract pathologies, potentially due to an improvement of general immunity and an enhancement of the local (oviduct) immunity. An increased resistance to the diseases, resulting in a significantly decreased mortality and significantly higher performance of probiotic-treated hens was also seen. The mechanism, by which probiotics could have enhanced mucosal immunity and reduced pathologies of the reproductive tract of treated birds is unclear, and was not thoroughly investigated in the present study. However, it is known that cellular and molecular events in the local mucosa can contribute to an improved mucosal defence against pathogens (Patterson and Burkholder 2003). From the small number of histology samples that were analysed in the present study, it was shown that there was an improvement of structural integrity of mucosal ileum and magnum of probiotic treated hens, as evidenced by an increased thickness of epithelial layer and a higher density of the intraepithelial cellular components. Other investigators have shown longer villi in the broiler birds treated with probiotic (Awad *et al.* 2009; Lee

et al. 2010). Longer villi provide an increased surface area that allows greater absorption of available nutrients, consequently promoting growth (Yamauchi *et al.* 2006). In the current study, some beneficial changes in the architecture of intestinal histology were seen, but these changes were not associated with significant changes of the villus height, and villus height : crypt depth ratio.

Hen performance (egg production, egg weight and BW) increased in all treatment groups as the flock aged. Significant differences were found between probiotic-treated and control birds. Both probiotics significantly improved the performance of birds, with Probiotic 2 showing a greater effect on all production parameters. Previous investigators have revealed that addition of probiotics to the feed of poultry (broilers and laying hens) has beneficial effects on growth performance and egg production. In laying hens, Gallazzi *et al.* (2008) indicated that egg production and FCR were significantly improved when hens were treated with probiotic strain *Lactobacillus acidophilus* D2/CSL. Similar results were found by Li *et al.* (2005) when a *Bacillus subtilis* culture was used. It has been proposed that these effects are achieved by different mechanisms, including competitive exclusion of pathogens (Morishita *et al.* 1997; Nisbet 1998) and improved digestion and absorption of nutrients (Thomke and Elwinger 1998). There have been some previous attempts to correlate gut microbiota with higher levels of performance (Apajalahti *et al.* 2004; Torok *et al.* 2008). However, because of the varying nature of such investigations, this area would need to be investigated further.

Overall, the results of the present study have provided promising initial data for the use of probiotics as a tool to reduce reproductive pathologies in free-range laying hens.

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