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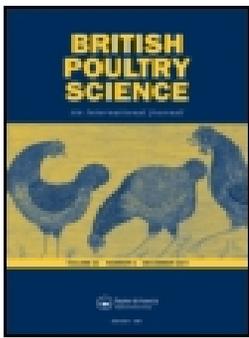
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**Administration of *Lactobacillus johnsonii* FI9785 to chickens affects colonisation by *Campylobacter jejuni* and the intestinal microbiota**

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**Running Head: *Lactobacillus johnsonii* exclusion of *Campylobacter***

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ACCEPTED MANUSCRIPT

**Abstract.** 1. *Campylobacter jejuni* is the most common bacterial cause of human foodborne gastroenteritis in the world largely from contaminated poultry meat. New control measures to reduce or eliminate this pathogen from the animal gastrointestinal tract are urgently required, and the use of probiotics as competitive exclusion agents is a promising biocontrol measure to reduce *C. jejuni* in the food chain.

2. In this study, we assessed the potential of *Lactobacillus johnsonii* FI9785, which has shown efficacy against *Clostridium perfringens*, to combat *C. jejuni*. The effect of prophylactic administration of *L. johnsonii* on the ability of *C. jejuni* to colonise chickens was determined.

3. Two doses of *L. johnsonii* given a week apart led to a reduction in *C. jejuni* colonisation in the caecal contents, but this biocontrol seemed reliant upon a high level of initial colonisation by the probiotic.

4. The microbial composition in the chicken gut was significantly altered by the probiotic treatment, as shown by denaturing gradient gel electrophoresis of 16S rRNA gene amplicons.

5. Together these results demonstrate the potential of this probiotic strain to be tested further as a competitive exclusion agent in poultry against *C. jejuni*.

Keywords: *Campylobacter*, broilers, probiotics, *Lactobacillus*, competitive exclusion, microbiota

## INTRODUCTION

*Campylobacter jejuni* is a leading cause of *Campylobacter* infections in humans. The European Food Safety Authority (EFSA) found that campylobacteriosis remains the most reported zoonotic infection in humans in the EU since 2005, with the number of cases

increasing until 2012, possibly as a consequence of a ban on use of antimicrobial growth promoters in poultry (EFSA, 2015). EFSA estimated costs of campylobacteriosis to public health systems and lost productivity in the EU to be around EUR 2.4 billion per year.

Globally, *Campylobacter* is one of the most frequent causes of foodborne illness, with 96 million cases in 2010 (Havelaar *et al.*, 2015). Poultry represents an important source of infection and increases in production will continue to add to this reservoir (Skarp *et al.*, 2016). A UK-wide survey showed that 73% of retail fresh whole chilled chickens were contaminated with *Campylobacter* (FSA, 2015), and a pressing need exists for strategies to control the problem during broiler production.

Because the intestine of living poultry is the main amplification site for *Campylobacter* in the food chain, reducing the caecal *Campylobacter* load in poultry is expected to significantly reduce the incidence of human campylobacteriosis (Hermans *et al.*, 2011a; Meunier *et al.*, 2016). The basis of persistent colonisation of the chicken GI tract by *C. jejuni* is still poorly understood, but is a multifactorial process (Hermans *et al.*, 2011b). Just a few viable *C. jejuni* cells can lead to colonisation, and once some birds become infected *C. jejuni* can spread rapidly through a flock, which remains colonised up to the time of slaughter (van Gerwe *et al.*, 2009; Conlan *et al.*, 2011). The highest levels of colonisation are found in the colon and caeca of infected birds (Beery *et al.*, 1988). Although *C. jejuni* has been commonly viewed as a harmless commensal in poultry, recent work has demonstrated negative effects on growth and weight gain and even gut pathology in some broiler lines (Awad *et al.*, 2014; Humphrey *et al.*, 2014; Awad *et al.*, 2015).

For over 25 years, probiotics have been suggested as an alternative to reduce the presence of enteropathogenic bacteria in poultry (Gaggia *et al.*, 2010; Mohan, 2015). Probiotics are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. The bacterial strains characterised as probiotic are frequently

*Lactobacillus* species. Different mechanisms of action of probiotics have been proposed, such as competitive exclusion, including competition for limiting nutrients or attachment sites, production of antimicrobial factors such as volatile fatty acids or bacteriocins, alteration of the intestinal microbial communities, and effects on the host such as enhancement of host barrier defences and modification of host signalling (Mead, 2000; Servin, 2004; Schneitz, 2005; Campana *et al.*, 2012; Mohan, 2015).

*Lactobacillus johnsonii* FI9785 is a poultry-derived isolate that adhered well to tissue culture and chick gut explant tissues, out-competing challenge bacteria in previous studies (La Ragione *et al.*, 2002). Several papers have been published on the effect of *L. johnsonii* strains on the exclusion of enteropathogens in chickens, including *C. perfringens* (strain FI9785 (La Ragione *et al.*, 2004) and *Salmonella* Enteritidis (strain R-17504 (Van Coillie *et al.*, 2007). In this study, we describe the effect of predosing poultry with *L. johnsonii* on the colonisation ability of *C. jejuni*, and on the chicken intestinal microbiota.

## MATERIALS AND METHODS

### **Bacterial strains, culture conditions and media**

*L. johnsonii* strain FI10058 is derived from strain FI9785 and contains plasmid pFI2431, produced by the insertion of a chloramphenicol resistance gene into the highly stable native small plasmid p9785 (Horn *et al.*, 2005). It was routinely grown at 37°C on de Man, Rogosa, Sharpe agar or broth (MRS, Oxoid) supplemented with 10 µg/ml neomycin and 7.5 µg/ml chloramphenicol. Overnight cultures used for inoculation were harvested by centrifugation at 3000 g for 15 min at 20°C, washed twice and resuspended in phosphate-buffered saline (PBS) at approximately  $1 \times 10^9$  colony forming units (cfu)/ml. *C. jejuni* strain 81-176 was

isolated from an outbreak associated with unpasteurised milk (Korlath *et al.*, 1985). It is known that this strain readily colonises chickens (Guccione *et al.*, 2008). It was routinely cultured on sheep blood agar (Oxoid) under standard microaerobic conditions (10% O<sub>2</sub>, 5% CO<sub>2</sub> and 85% N<sub>2</sub>) at 37°C in a MACS-VA500 incubator (Don Whitley Scientific). Cultures used for inoculation were incubated for 24 h in 10 ml Mueller-Hinton broth (MH, Oxoid). Long-term storage of *Campylobacter* was at -80°C in Microbank vials (Prolab Diagnostics). *Campylobacter* blood-free selective agar (CBF) plates were prepared according to the manufacturer's instructions from *Campylobacter* blood-free selective agar (CCDA, Oxoid) and CCDA selective supplement (Oxoid).

### **Experimental animals and challenge model**

Animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 under PPL 30/2462 with the approval of the local Ethical Review Committee.

Specific pathogen-free outbred Light Sussex chickens were hatched at the Institute for Animal Health and housed in group cages in high-biosecure accommodation. Birds were reared in wire cages at 30°C from 1 d of age, decreasing to 20°C at 3 weeks of age and given *ad libitum* access to water and a vegetable-based protein diet (Special Diet Services, Witham, UK). Birds were confirmed to be culture-negative for *Campylobacter* prior to inoculation. Birds were wing-banded to allow identification of individuals.

The birds were randomly allocated to one of 8 cages within a 2×2 factorial arrangement of treatments (2 cages/treatment, 6 chicks per cage). On the day of hatch (d 0), 24 chicks (treatment groups C and D) were separately dosed by oral gavage with 0.1 ml of a washed *L. johnsonii* suspension containing c. 1×10<sup>9</sup> cfu/ml in PBS, the other 2 groups (treatment groups A and B) received PBS alone. This inoculation was repeated a week later with a fresh

probiotic suspension. At two weeks of age (d 14), birds in treatment groups B and D were orally inoculated with 0.3 ml of MH broth cultures containing c.  $1 \times 10^8$  cfu *C. jejuni*, while the others were mock challenged with broth only (Table 1).

**Table 1 near here**

*Post-mortem* samples to detect the presence of *L. johnsonii* were taken from different parts of the chicken GI tract (crop, duodenum, ileum, caeca and colon) on d 14 (before challenge with *C. jejuni*, 1 chick/cage), d 15 (1 d post challenge, 2 or 3 chickens/cage) and d 20 (6 d post challenge, remaining chickens) after dislocation of the neck. Viable *C. jejuni* in caecal contents and spleen were enumerated (cfu/g content) on days 15 and 20. Tissues were homogenised in 10 ml PBS/g and viable *L. johnsonii* and *C. jejuni* were enumerated by direct plating of 10-fold serial dilutions in triplicate on MRS-neomycin/chloramphenicol and CBF agar plates, respectively. The sensitivity of detection was taken to be 167 cfu/g.

#### **DNA extraction from caecal samples**

Genomic DNA was extracted from chicken caecal samples, which had been flash frozen and stored at  $-80^{\circ}\text{C}$ , using the QIAamp Stool Mini Kit (Qiagen) following the general guidelines of the manufacturer but using 500 mg of caecal material with a proportionally larger volume of lysis buffer, as recommended by others (Tourlomousis *et al.*, 2010). The purity, concentration and quality of the extracted DNA were measured by spectrophotometry and agarose gel electrophoresis.

#### **PCR amplification and DGGE analysis**

The highly variable V3 region of the 16S rRNA gene was amplified using a protocol described previously (Muyzer *et al.*, 1993), which amplifies a region of approximately 193

nucleotides. PCR amplification was performed using the HotMaster Taq Polymerase kit (5 Prime) in 50 µl reactions using the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 20 s, 58°C for 10 s, 65°C for 20 s, and a final extension of 10 min at 65°C. PCR products were visualised by electrophoresis and purified with SureClean (Bioline).

The D-Code system (Bio-Rad) was used to separate the amplified DNA from the different bacteria present in the caecal samples. Separation of PCR products and a clone mixture used as a reference ladder (200 ng) was performed on an 8% polyacrylamide gel containing an increasing linear gradient of denaturants (40–60%, Severn Biotech Ltd). Electrophoresis was performed in 1× TAE buffer for 20 min at 200 V, followed by approximately 16 h at 50 V at a constant temperature of 60°C. Gels were stained with SYBR Green I (1:60000 in 1× TAE buffer, Invitrogen) for 40 min and destained in distilled water for a further 40 min. Gels were scanned on a Pharos FX Molecular Imager (Bio-Rad). Individual DGGE lanes were converted into densitometric profiles (TL120 v2006 Phoretix 1D Advanced Software, NonLinear Dynamics), and profiles were analysed as described previously (Tourlomousis *et al.*, 2010). Non-metric multidimensional scaling (nMDS) analysis on the band presence/absence matrix was performed using the free software package PAST (Palaeontology Statistics, <http://folk.uio.no/ohammer/past/>) as described (Oguntoyinbo *et al.*, 2011).

### **Statistical analysis**

Bacterial concentrations,  $x$  (cfu/g), were transformed into  $\log_{10}(1+x)$  prior to statistical data analysis. The correlation between *L. johnsonii* concentrations after oral administration in the several studied segments of the GI tract was evaluated by estimating the Pearson coefficient of linear correlation,  $\rho$ . Similarly the correlation coefficient between the concentrations of *L.*

*johnsonii* and *C. jejuni* in the caecum after the challenge was also estimated. Correlations were considered to be significant if the *P*-value associated to the coefficient was smaller than 0.05.

A two-way ANOVA model was used to detect significant differences in the mean values of bacterial concentrations. One factor was “sampling time” which took discrete values - 14, 15 and 20 days - while the other factor was “treatment” with groups A, B, C and D as described in Table 1. When the effect of the factor “treatment” was found to be significant (*P*-value < 0.05), *F* tests were used to assess the significance of the differences between the multiple means in this model as previously described (Brown and Rothery, 1994).

Correspondence analysis was run on the DGGE profile of each bird sample. Correspondence analysis is a multivariate statistical technique applicable to categorical data and is conceptually equivalent to principal component analysis (PCA) for continuous data. It is applied to find out new dimensions, which are linear combination of the original categories and estimated by maximising the distances between sample profiles. Then, the distance between the sample coordinates in the new dimensions produces a reasonable approximation of the distance between the original sample profiles. DGGE profiles consisted of values equal to 1, if the band was detected in that sample, and 0 otherwise. The two first correspondence analysis dimensions, which are equivalent to the two first principal components of PCA, were selected to evaluate the ability of the DGGE profiles to discriminate between samples of birds subjected to different treatments and/or collected at different sampling times. To do this, a one way ANOVA and a Tukey test for comparison of multiple means were used to find out whether these new variables exhibited significantly different values between the samples. All statistical procedures were carried out using the SAS 9.3 software (Statistical Analysis System, 2009. SAS Institute Inc., Cary, NC).

## RESULTS

### Detection of *L. johnsonii* in the GI tract of chickens

Birds were given two doses of *L. johnsonii* or PBS, on the d of hatch, and one week later. Birds were challenged with *C. jejuni* or PBS one week after that (Table 1). The birds were tested for the presence of *L. johnsonii* at d 14 (just before being challenged with *C. jejuni*), at d 15 (1 d after challenge) and at d 20 (6 d after challenge). On d 14 a single bird was tested from each cage, and all 4 birds inoculated with *L. johnsonii* (treatment groups C and D, *Lj-C* and *Lj-Cj*) tested positive in one or more sites along the GI tract, with the highest recovery being from the caecal contents (Table 2, Figure 1), while the probiotic was not detected in the gut of the 4 mock inoculated birds (treatment groups A and B, C-C and C-Cj). At d 14 the tested bird from group D cage 8 showed a higher level of colonisation and tested positive in all tissues analysed.

**Table 2 and Figure 1 near here**

On d 15, 2 birds from cages 1, 2, 4, 5 and 7 and 3 birds from cages 3, 6 and 8 were sacrificed from each cage. *L. johnsonii* counts were slightly lower than on d 14. Group D (*Lj-Cj*) still showed a clear difference between the cages, with the three birds from cage 8 showing a higher colonisation of the whole intestinal tract (Table 2, Figure 2 (a)), while as at d 14 the colonisation of birds in cage 7 was more similar to those in cages 5 and 6, which were broadly similar to each other in terms of total colonisation. Finally, on d 20 the remaining birds (2 from cages 1, 3, 4, 6 and 8 and 3 from cages 2, 5 and 7) were analysed, showing that only 5 birds out of 10 tested positive for *L. johnsonii* and the counts were on average lower in both groups. All 5 birds tested positive only for one tissue, generally the caecum. At this stage cage 8 no longer showed a higher level of colonisation. The bacterial counts in the caeca of all birds from treatment groups C and D showed that the concentration

of *L. johnsonii* was not significantly affected by the presence of *C. jejuni* ( $P = 0.25$ , Figure 2 (a)).

**Figure 2 near here**

In general, a decreasing trend in the counts of the probiotic was seen in all the tissues with time (Table 2, Figure 1). The tissue showing the highest colonisation was the caecum, with both the highest number of positive birds and the highest counts, followed by the crop, then colon, ileum and finally duodenum. High numbers of *L. johnsonii* in the crop, duodenum or colon were significantly correlated with high numbers in the next part of the GI tract, while high numbers in the crop were significantly correlated with high values in all other tissues tested (duodenum, ileum, caecum and colon) (Table 3).

**Table 3 near here**

### **Effect of pre-dosing chickens with *L. johnsonii* on *C. jejuni* colonisation**

The caecum was the main tissue in which *C. jejuni* was detected after the challenge. Colonisation of the spleen by *C. jejuni* was also detected but at very low levels and only one bird from cage 3 was found to be positive ( $1.82 \log_{10}$  cfu/g) at 1 d post challenge. At 6 d post challenge, one bird from cage 3 and another one from cage 7 were positive with spleen counts of 3.04 and 4.19  $\log_{10}$  cfu/g respectively.

The mean counts of *C. jejuni* from caecal contents at 1 d post challenge were between 4-7  $\log_{10}$  cfu/g in both treatment groups B (C-Cj) and D (Lj-Cj) (Table 4, Figure 2 (b)); looking at individual birds, although the counts of *C. jejuni* in birds treated with *L. johnsonii* varied in a similar range as in those not given the probiotic, we observed a significant negative correlation ( $\rho = -0.92$ ,  $P = 0.026$ ) between the concentration of *C. jejuni* and *L. johnsonii* in the caeca of birds inoculated with both bacteria (Figure 3 (a)).

At 6 d post challenge (d 20) there was more variation depending on the group. When considering all birds of treatment groups B and D, the concentration of *C. jejuni* in the

caecum was significantly reduced when administered together with *L. johnsonii* ( $P < 0.0001$ ) - the mean counts of *C. jejuni* after the administration of the probiotic were  $6.3 \log_{10}$  cfu/g compared to  $8.2 \log_{10}$  cfu/g in the group without the probiotic. However, when cages 7 and 8 were analysed separately, it was clear that this reduction was primarily associated with the birds in cage 8 (Table 4), where *C. jejuni* counts were more than  $4 \log_{10}$  cfu/g lower than in cage 7, although there was no statistically significant correlation between *L. johnsonii* and *C. jejuni* counts in individual birds at this time point (Figure 3 (b)). *C. jejuni* was not detected in samples from groups A and C.

**Table 4 and Figure 3 near here**

### **Effect of the administration of *L. johnsonii* on the chicken intestinal microbial composition**

A correspondence analysis was carried out from the DGGE profiles of each bird in order to investigate the effect of the administered bacteria on the chicken intestinal microbiota. The two first correspondence analysis dimensions, which are equivalent to the two first principal components of PCA, indicated differences in the bacterial population composition of the intestine after administration of bacteria (Figure 4). Tukey grouping showed that the first dimension was significantly different in chickens infected with *L. johnsonii* only, while the second dimension was significantly different between birds infected with either *L. johnsonii* plus *C. jejuni* or *L. johnsonii* only and the others ( $P < 0.05$ ). There was some overlap between these 2 groups, which included the birds from treatment group D sampled at d 14 prior to *C. jejuni* challenge. Profiles from chickens infected with *C. jejuni* only and profiles from control chickens were not significantly different. There was no clear separation between samples from different cages within the same group (Figure 4 (a)), and the time of sampling also did not appear to affect the profile (Figure 4 (b)).

**Figure 4 near here**

## DISCUSSION

Given the continuing increase in the incidence of human campylobacteriosis acquired from eating contaminated chicken, new methods of control to reduce colonisation and/or contamination are highly desirable (Ganan *et al.*, 2012). The provision of probiotics in drinking water or feed from hatching to effect competitive exclusion may help to reduce the burden of *Campylobacter* in the food chain. This study aimed to investigate whether *L. johnsonii* FI9785, which has already shown promise to control *C. perfringens*, could at the same time reduce the colonisation of *C. jejuni*. We used an established 1 d old chick model to assess the effects of an antibiotic tagged derivative of *L. johnsonii* FI9785 upon colonisation and persistence of *C. jejuni*.

After 2 treatments of the probiotic, all birds tested showed colonisation in at least one tissue on the day of challenge with *C. jejuni*, and this was maintained at 1 d post challenge, although at d 20 (6 d post challenge) *L. johnsonii* was below the detection limit in most tissues, with the population being mostly limited to the caecum. As the caecum is the major site of *C. jejuni* colonisation (Beery *et al.*, 1988; Hermans *et al.*, 2011a), the loss of notable *L. johnsonii* populations in the other tissues may not be detrimental to the exclusion of *Campylobacter*. However, the reduction in *L. johnsonii* counts by d 20 suggests that the delivery of higher or more frequent dosages might be required to maintain a high level of persistence. Previous trials with single doses of *L. johnsonii* in 1 day and 20 day chick models demonstrated persistence of the probiotic over 36 days, but also noted declining numbers (La Ragione *et al.*, 2004), giving further weight to the importance of adjusting the dosing schedule to maintain high colonisation until commercial slaughter age. It is possible that there was loss over time of the plasmid encoding chloramphenicol resistance used to aid selection

of the probiotic, leading to an underestimation of *L. johnsonii* numbers. However, this construct is derived from a native plasmid and has shown strong persistence *in vitro* (Horn *et al.*, 2005).

Surprisingly there was a noticeable difference in the colonisation between cages in treatment group D - birds in cage 8 had both higher counts of *L. johnsonii* and a higher number of positive tissues at the earlier sampling times, although counts at d 20 were more similar to those in the other *L. johnsonii*-treated cages. A previous examination of the chicken intestinal microbiota diversity demonstrated population succession at several stages during a 49 d study, with significant differences between caecal libraries taken at 3, 7, 14-28 and 49 days but with an interval of relative stability in the 14-28 day period (Lu *et al.*, 2003). In terms of this study, the fact that the intestinal flora may have been evolving between the first two *L. johnsonii* inoculations, at hatch and d 7, and the challenge date at d 14 after hatch, may explain some of the observed variation in colonisation of the probiotic in the different tissues. Differences in colonisation of the entire GI tract may have been affected by differences in the background microbiota in the cages. The 'cage effect' has been referenced in studies on the microbiota of mice – Shanahan and colleagues found that the cage had a stronger effect on the microbiota composition than the genotype of the mice (Shanahan *et al.*, 2014), while cage also had a significant contribution (up to 30%) to variation in the microbiota of common laboratory strains (Hildebrand *et al.*, 2013), with an indication of synchronisation of the microbiotas within a cage. However, in the present experiments DGGE analysis suggested that profiles clustered on the basis of treatment rather than on the basis of cage. All three birds from cage 8 tested at 1 d post challenge still had good colonisation of the crop; as high numbers in the crop were significantly correlated to high counts in other tissues, it is possible that a high population in the crop may have acted as a reservoir for the rest of the GI tract and contributed to more effective persistence.

The differences in *L. johnsonii* colonisation appeared to have an effect on the persistence of *C. jejuni*; birds from cage 8, whose earlier-sampled birds had established a high level of *L. johnsonii* colonisation, showed 4-5- $\log_{10}$  less *C. jejuni* counts than controls at d 6 post challenge. However, this reduction was not seen in birds given the same treatment in cage 7, whose birds sampled at d 15 did not show *L. johnsonii* colonisation of the whole GI tract. This indicates that although *L. johnsonii* has the potential to control *C. jejuni*, the success of probiotic colonisation is crucial to the efficacy of exclusion. We hypothesise that high probiotic counts at the time of inoculation prevented good colonisation by the *C. jejuni* in the initial inoculum and led to lower counts in later samples. The fact that the birds with lower numbers of *C. jejuni* 6 d post challenge also had low *L. johnsonii* counts at this time suggests that high probiotic numbers might be most effective at the time of challenge. However, given the observed drop in probiotic counts there is the possibility that *C. jejuni* numbers might have increased if the birds had been left until commercial slaughter age. It does have to be considered that the hypothesis is based on a small number of birds and there may have been some other effect on the gut or the microbiota that affected the survival of the pathogen in this cage, and also that probiotic colonisation of all birds in the same cage might not be the same.

The mechanism of the *C. jejuni* reduction is yet to be elucidated. A number of effects of probiotic bacteria on *C. jejuni* have been documented, including inhibition of growth, adhesion and invasion or motility and direct antimicrobial activity (Santini *et al.*, 2010; Campana *et al.*, 2012; Nishiyama *et al.*, 2014; Mohan, 2015), while a culture supernatant of *L. johnsonii* La1 inhibited motility of *Helicobacter pylori*, which is closely related to *C. jejuni* (Isobe *et al.*, 2012). The fact that high numbers of *C. jejuni* in cage 7 coincided with high counts of *L. johnsonii* at d 20 suggest that the probiotic may not have a strong direct inhibitory effect. Birds treated with *L. johnsonii* had different DGGE profiles compared to

both control birds and to those treated with both *L. johnsonii* and *C. jejuni*, although there was some overlap, so it is possible that effects of the probiotic on either the host microbiota or the host tissue may have contributed to the observed decrease in *C. jejuni*. Several studies have shown an effect of probiotics on the chicken intestine and/or microbiome: a lactic acid bacteria-based probiotic increased villus length and goblet number in broilers, while one based on *Bacillus subtilis* affected mucin gene expression (Aliakbarpour *et al.*, 2012), and probiotic *Enterococcus faecium* treatment of chickens challenged with *E. coli* affected bacterial groups, intestinal morphology and immune response (Cao *et al.*, 2013). Analysis of the DGGE profiles of the caecal microbiota showed no clear change from days 14 to 20, while probiotic counts decreased. This suggests that the effect of *L. johnsonii* on the gut microbiota may be a result of changes early in probiotic treatment. There was also no separation between the DGGE profiles of samples from cages 7 and 8, which had differences in the levels of *L. johnsonii* and control of *C. jejuni*. This would indicate that although the changes in the microbiota may contribute to the ability of the probiotic to decrease the pathogen, it is the high numbers of *L. johnsonii* at the time of infection and attempted establishment of the pathogen that appears to be key. However, it cannot be ruled out that another variable affected the birds in cage 8 which led to the differences in colonisation of both *L. johnsonii* and *C. jejuni*.

It was interesting that the DGGE analysis showed no separation between controls and chicken inoculated with *C. jejuni* alone; a recent study using 16S rRNA gene sequencing showed that while *C. jejuni* colonisation did not affect the caecal microbiome alpha diversity, there was a moderate effect on the beta diversity (Thibodeau *et al.*, 2015). In addition to the differences in methodology, both the genotype of the birds and the composition of the microflora may contribute to differences between studies.

There have been a number of earlier studies on competitive exclusion and probiotic treatment to reduce *Campylobacter* in poultry (Hermans *et al.*, 2011b), many of them involving *Lactobacillus* spp. *Lactobacillus gasseri* SBT2055 suppressed *C. jejuni* colonisation by c. 250-fold (Nishiyama *et al.*, 2014); an elegant study on this strain identified a cell surface-associated aggregation-promoting factor APF1 as being important both for colonisation of chickens and for reducing colonisation by *C. jejuni in vivo* (Nishiyama *et al.*, 2015), and it is notable that the genome of *L. johnsonii* FI9785 encodes a protein with 95% amino acid identity to APF1 (FI9785\_RS07055). A probiotic treatment of 7 *Lactobacillus* spp of poultry origin reduced mortality and increased productivity in field trials (Timmerman *et al.*, 2006), while a multispecies probiotic including *Lactobacillus salivarius*, *Lactobacillus reuteri*, *Enterococcus faecium*, *Pediococcus acidilactici* and *Bifidobacterium animalis* was also effective in reducing *C. jejuni* colonisation *in vivo*, giving a mean 6-log<sub>10</sub> reduction compared to controls (Ghareeb *et al.*, 2012). These bacteria were of chicken origin, but interestingly 4 species of *Lactobacillus* and *Lactococcus* isolated from humans were also shown to be effective *in vivo* to prevent *C. jejuni* colonisation (Cean *et al.*, 2015). *Campylobacter* counts were also reduced in trials with commercial products by 1.4-logs (multispecies probiotic Broilact) (Schneitz and Hakkinen, 2016) and 3-logs (probiotic Calsporin) (Guyard-Nicodeme *et al.*, 2016). These studies demonstrate the potential of probiotics to combat high levels of *C. jejuni* in the chicken gut.

A study on *Campylobacter* contamination of broiler carcasses showed a positive correlation between the number of bacteria in the caeca and the number on carcasses (Reich *et al.*, 2008). Consequently, any decrease in the *Campylobacter* colonisation should lead to reduced contamination of the food chain. Mathematical modelling predicted that a 2-log<sub>10</sub> reduction in the number of *Campylobacter* on chicken carcasses could lead to a 30-fold decrease in campylobacteriosis resulting from eating chickens, and showed a linear

relationship between flock prevalence and campylobacteriosis (Rosenquist *et al.*, 2003). Another risk assessment model found that variability in the numbers of *Campylobacter* in broiler faeces and the number on the exterior of carcasses had a large impact on estimated risks, and that a high concentration of *Campylobacter* in the faeces had a dominant impact on human infection (Nauta *et al.*, 2007). This pilot study suggests that reductions can be achieved with successful colonisation of *L. johnsonii* FI9785, but the results show that an adjustment of dosing to ensure high levels of persistence is required and larger numbers of birds need to be analysed in future studies to confirm a significant effect. The use of wire cages does reduce the potential for coprophagy, and thus re-inoculation of the birds with the probiotic strain, compared to rearing on litter in floor pens, and further experiments in a context that simulates field practice would be the logical next step. If a good level of probiotic colonisation can be reliably established at the farm level, this strain could have a significant impact in decreasing campylobacteriosis in humans.

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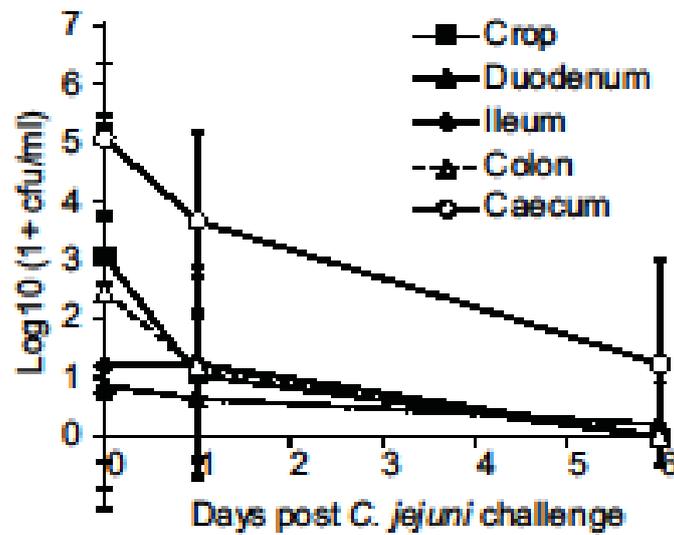
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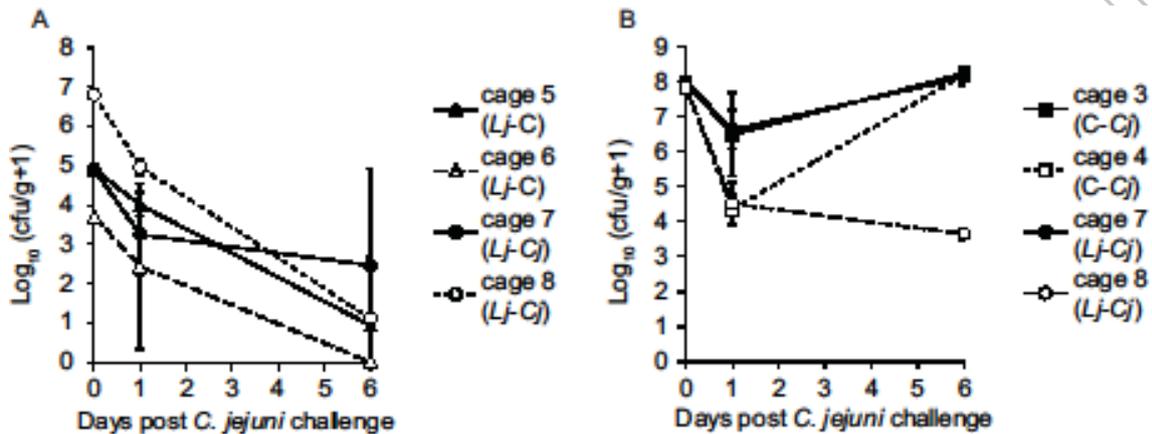
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## FIGURE LEGENDS

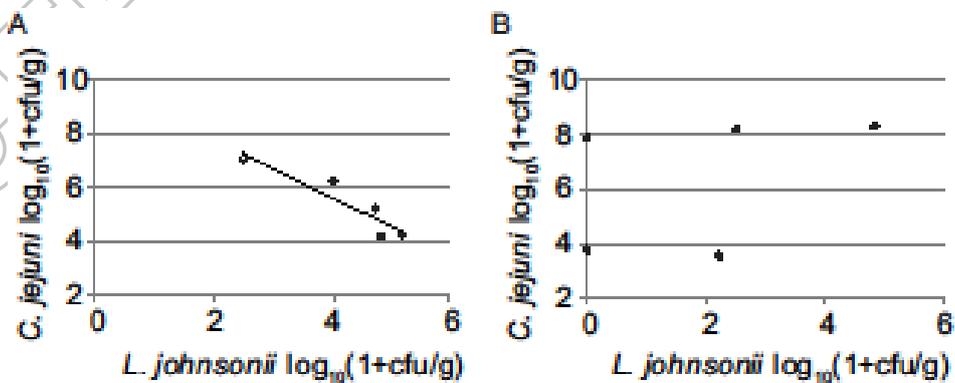
**Figure 1.** *L. johnsonii* counts from all tissues and all birds analysed in this study. Samples were taken at d 14 just before challenge with *C. jejuni* (0 d) and 1 d and 6 d after challenge; symbols represent the mean  $\pm$  standard deviation; samples where *L. johnsonii* was below the limit of detection were taken as 0 cfu/g.



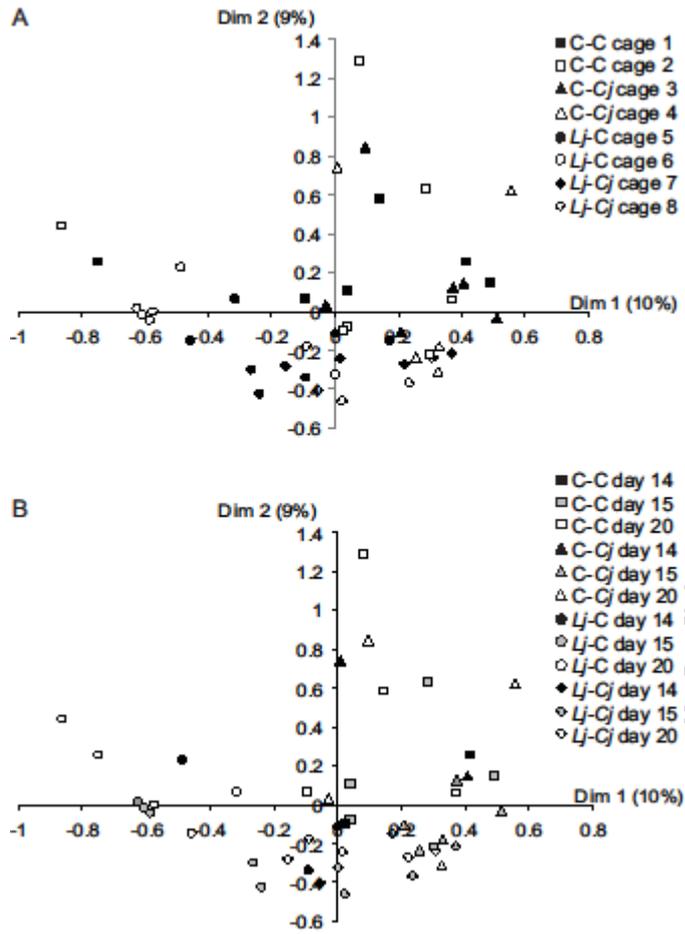
**Figure 2.** Bacterial counts from the caeca. (a) *L. johnsonii* in groups C (*Lj-C*, cages 5 and 6) and D (*Lj-Cj*, cages 7 and 8), (b) *C. jejuni* in groups B (*C-Cj*, cages 3 and 4) and D (*Lj-Cj*, cages 7 and 8). Symbols represent the mean  $\pm$  standard deviation, samples where *L. johnsonii* was below the limit of detection were taken as 0 cfu/g.



**Figure 3.** Scatter plots of bacterial concentrations from the caeca of individual birds from group D (*Lj-Cj*) at (a) d 15 (1 d post challenge,  $\rho = -0.92$ ;  $P = 0.026$ ) and (b) d 20 (6 d post challenge,  $\rho = 0.40$ ;  $P = 0.50$ ). Open, birds from cage 7, filled, birds from cage 8.



**Figure 4.** Correspondence analysis of the DGGE profiles from the caecal microbiota from groups A (C-C), B (C-Cj), C (Lj-C) and D (Lj-Cj) highlighting cage (a) and time (b). Dim, dimension.



**Table 1.** *Treatment regime*

Treatment group	Cages	No. birds tested	Day 0 (hatch)	Day 7	Day 14	Abbreviation
A	1 <sup>a</sup> , 2	5 <sup>a</sup> , 6	PBS	PBS	broth	C-C
B	3, 4 <sup>a</sup>	6, 5 <sup>a</sup>	PBS	PBS	<i>C. jejuni</i>	C-Cj
C	5, 6	6, 6	<i>L. johnsonii</i>	<i>L. johnsonii</i>	broth	Lj-C
D	7, 8	6, 6	<i>L. johnsonii</i>	<i>L. johnsonii</i>	<i>C. jejuni</i>	Lj-Cj

<sup>a</sup>One bird in cage 1 and one in cage 4 found dead at d 12, likely owing to natural causes as only saline had been administered by the time of death.

**Table 2.**  $\text{Log}_{10}(1+\text{cfu/g})$  of *L. johnsonii* in all tissues analysed by cage

Treatment	Cag	$\text{Log}_{10}(1+\text{cfu/g})$ <i>L. johnsonii</i> in individual birds				
Group <sup>1</sup>	e	Crop	Duodenu m	Ileum	Colon	Caecum
<i>Day 14, pre challenge</i>						
C	5	b	b	b	b	4.9
C	6	2.7	b	b	b	3.7
D	7	3.8	b	b	4.4	4.9
D	8	5.8	3.5	4.9	5.4	6.8
<i>Day 15, 1 d post challenge</i>						
C	5	b, b	b, b	b, b	3.1, b	3.8, 4.2
C	6	b, b, b	b, b, b	3.1, b, b	b, b, b	3.8, 3.5, b
D	7	b, b	b, b	b, b	3.1, b	2.5, 4.0
D	8	3.6, 2.7, 4.1	3.2, 3.3, b	3.5, 2.2, 3.7	2.9, 2.7, b	5.2, 4.8, 4.9
<i>Day 20, 6 d post challenge</i>						
C	5	b, b, b	b, b, b	b, b, b	b, b, b	2.8, b, b
C	6	b, b	2.2, b	b, b	b, b	b, b
D	7	b, b, b	b, b, b	b, b, b	b, b, b	4.9, 2.5, b
D	8	b, b	b, b	b, b	b, b	2.2, b

<sup>1</sup>C: Lj-C, D: Lj-Cj.

b: below limit of detection in  $10^{-1}$  dilution,

**Table 3.** Linear correlation coefficient ( $\rho$ ) between the concentrations of *L. johnsonii* in the different tissues assayed over all time points

Tissue	Crop	Duodenum	Ileum	Colon	Caecum
Crop	1	0.698	0.680	0.732	0.653
Duodenum		1	0.814	0.514	0.317
Ileum			1	0.502	0.557
Colon				1	0.656
Caecum					1

$\rho$ : linear correlation coefficient between *L. johnsonii* concentration -  $\log_{10}(1+\text{cfu/g})$  - detected in the collected tissues; significant correlations ( $P < 0.05$ ,  $p$  value associated to the null hypothesis that  $\rho = 0$ ) are highlighted.

**Table 4.**  $\text{Log}_{10}(1+\text{cfu/g})$  of *C. jejuni* in the caecum

Treatment group	Cages	No. birds tested	$\text{Log}_{10}(1+\text{cfu/g})$ <i>C. jejuni</i> in individual birds		
			Day 14 <sup>a</sup>	Day 15	Day 20
B	3	6	8.0	5.53, 6.13, 7.82	8.38, 8.11
B	4	5	7.8	4.67, 4.04	8.27, 8.15
D	7	6	8.0	7.02, 6.24	8.31, 8.20, 7.88
D	8	6	7.8	4.20, 5.21, 4.14	3.76, 3.55

<sup>a</sup> Counts in inoculum