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ORIGINAL ARTICLE

Prevention of intestinal *Campylobacter jejuni* colonization in broilers by combinations of in-feed organic acids

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Abstract

Aim: We have tested the effect of various combinations of formic acid and sorbate on *Campylobacter jejuni* colonization in broiler chickens to reduce the colonization of this zoonotic pathogen in broiler chicken flocks.
Methods and Results: Chickens were offered feed supplemented with different concentrations and combinations of formic acid and/or potassium sorbate. We found little or no effect on the *Camp. jejuni* colonization levels in chickens that were given feed supplemented with formic acid alone. A combination of 1·5% formic acid and 0·1% sorbate reduced the colonization of *Camp. jejuni* significantly, while a concentration of 2·0% formic acid in combination with 0·1% sorbate prevented *Camp. jejuni* colonization in chickens. This inhibition was replicated in two independent trials with a combination of three different *Camp. jejuni* strains.
Conclusions: Our results show a novel and promising intervention strategy to reduce the incidence of *Camp. jejuni* in poultry products and to obtain safer food.
Significance and Impact of the Study: To ensure food safety, a reduction of the carcass contamination with *Camp. jejuni* through reduced colonization of this pathogen in broiler chicken flocks is important. A range of organic acids as additives in feed and drinking water have already been evaluated for this purpose. However, no studies have yet shown a complete inhibition of *Camp. jejuni* colonization in broiler chickens.

Introduction

Campylobacter jejuni is one of the leading causes of diarrhoeal disease and food-borne gastroenteritis in humans in the developed world (Park 2002). This bacterium is zoonotic, and poultry is an important source of transmission to humans (Solomon and Hoover 1999). *Campylobacter jejuni* is able to colonize the gastrointestinal (GI) tract of chickens, with the principal site of colonization being the lower GI tract, especially the caecum (Beery *et al.* 1988; Stern *et al.* 1988; Shane 1992). Once *Camp. jejuni* enters the broiler flocks, this bacterium spreads rapidly via horizontal transmission and is currently impossible to control (Humphrey *et al.* 2007). The *Camp. jejuni*-positive broiler flocks can cause carcass contamination during slaughter (Rosenquist *et al.* 2006; Allen *et al.* 2007). Despite major intervention efforts targeting the lower GI tract (Hariharan *et al.* 2004), no successful approaches have been developed for reducing the colonization of *Camp. jejuni* in broiler chickens.

Organic acids have been tested as additives in drinking water and feed for the reduction of *Campylobacter* and *Salmonella* in chickens (Byrd *et al.* 2001; Chaveerach *et al.* 2004; Hilmarsson *et al.* 2006; Van Immerseel *et al.* 2006; Solis de los Santos *et al.* 2008a; Van Deun *et al.* 2008). Studies have shown that caprylic acid in feed has the

potential of reducing the *Camp. jejuni* load in chicken caecum (Solis de los Santos *et al.* 2008a,b), thus reducing carcass contamination during slaughter. Still, no studies have yet demonstrated the complete inhibition of *Camp. jejuni* colonization by the use of feed additives.

Until now, the synergistic effects of antimicrobial treatments have not been thoroughly investigated. Sorbic acid and some of its salts (especially potassium sorbate) are among the most widely used antimicrobial agents for food preservation (Chaveerach *et al.* 2002). In addition, formic acid has been shown to have a strong bactericidal effect on *Camp. jejuni* compared to other weak organic acids (Chaveerach *et al.* 2002). Acidifying litter with formic acid has demonstrated a reduction in other pathogens, such as *Clostridium perfringens*, in broilers (Garrido *et al.* 2004). To the best of our knowledge, the combination of formic acid and sorbate has not been tested for antimicrobial effects in chickens. The aim of this work was to investigate the combined effect of formic acid and sorbate on the colonization of *Camp. jejuni* in broiler chickens. Both colonization dynamics of *Camp. jejuni* in the GI tract of the chicken and of the total microflora in the lower GI tract were studied. We present results showing that a combination of sorbate and formic acid completely prevented the colonization of *Camp. jejuni* in both crop and caecum. The mechanistic and practical relevance of these findings is discussed.

Materials and methods

Experimental infections

All *in vivo* experiments were started with 1-day-old conventional broiler chickens (Ross 308) of mixed sex. In each treatment group in all experiments, only one type of feed was used during the whole study period. The feed used in all experiments was equivalent to commercially pelleted grower feeds for broilers, with wheat, soya bean meal and oats as the main ingredients. The ionophorous anticoccidial agent narasin (declared contents 70 ppm), but no antibacterial growth promoter, was added to feed used in all the experiments with the exception of Experiment 2. The feed used in Experiment 2 contained neither anticoccidials nor antibacterial growth promoters. For all the experiments, environmental samples from the experimental room, and swabs from chickens collected on day 0 and the day before inoculation, were collected and examined for the presence of *Camp. jejuni*. All samples were found to be negative for *Camp. jejuni*. Chickens inoculated with *Camp. jejuni* appeared healthy and showed no signs of disease. All *in vivo* experiments were approved by the Norwegian governmental committee for experimental animals (http://www.mattilsynet.no/fdu/).

Bacterial challenge procedure

The following *Camp. jejuni* strains were used in our experiments: C484 isolated from a poultry leg (Rudi *et al.* 2005), C523 and C534 isolated from poultry faeces (Rudi *et al.* 2005), G109 isolated from caecal dropping (Skånseng *et al.* 2007) and G125 isolated from dog faeces (Skånseng *et al.* 2007).

Selection of challenge strains was performed as described in the Appendix S1. To make the inoculum, a single colony was inoculated into 10-ml buffered peptone water (BPW) and incubated at 37 ± 1°C for 24 h. The culture was serially diluted in BPW, and the appropriate dilutions were used for the inoculation of the chickens used in the experiments. In Experiments 1–3, it was decided to use a concentration of *c.* 4-$\log_{10}$ CFU ml^{-1} in the inoculum (manuscript in preparation), as the total in a mixture or of a single strain. The chickens were individually inoculated by crop instillation with *c.* 1·5 ml of the bacterial suspension (depending on the concentration of the inoculum and the infection dose), using a 2-ml syringe with an attached flexible tube. The negative controls were inoculated with sterile BPW.

Effect of in-feed formic acid and sorbate on *Campylobacter jejuni* colonization

All birds were started in cages with floors (0·6 × 0·5 m) covered with dry, unused wood shavings. Chickens were inoculated with the challenge strains at 13–15 days of age ('Bacterial challenge procedure'). Each treatment group contained eight chickens per experiment, and the different treatments groups tested are summarized in Table 1.

Table 1 Different combinations of formic acid and sorbate tested as feed additives against *Campylobacter jejuni* colonization in chickens in Experiments 1a–c. In-feed organic acids were used as from day 1 and throughout the entire experimental period

Treatment	Combination groups*
1	Negative control†
2	Positive control‡
3	1·0% formic acid
4	2·0% formic acid
5	1·0% formic acid + 0·1% sorbate
6	1·5% formic acid + 0·1% sorbate
7	2·0% formic acid + 0·1% sorbate
8	0·1% sorbate

*Chickens in all groups, except the negative control group, were inoculated with *Camp. jejuni* at 13–15 days of age.
†Basic feed (no additives), not inoculated with *Camp. jejuni*.
‡Basic feed (no additives), inoculated with *Camp. jejuni*.

Experiment 1a (Treatments 1, 2, 3 and 7)
One challenge strain (C484) was used in this experiment. The experiment ended on Day 15 postinoculation (p.i.), and caecal contents were collected from all chickens. Crop material (contents as well as mucosal tissue) was collected on Day 15 p.i. from five chickens in each experimental group.

Experiment 1b (Treatments 1, 2, 5 and 8)
A mixture of the three challenge strains (C484, G109 and G125) was used. Caecal contents were collected on Day 13 p.i.

Experiment 1c (Treatments 1, 2, 4, 6 and 7)
The same mixture of challenge strains as in Experiment 1b was used. Caecal contents were collected on Day 14 p.i.

Colonization mechanisms of *Campylobacter jejuni*

Experiments 2 and 3 were carried out to study the colonization dynamics of *Camp. jejuni* in chicken GI tract. Samples and data from Experiment 1 were also used for this purpose.

Survival of Campylobacter jejuni in crop
Five broiler chickens (Experiment 2) were inoculated on Day 14 with *Camp. jejuni* strain C484 (see 'Bacterial challenge procedure'). Samples from crop and caecum were examined for *Camp. jejuni* on Day 11 p.i. Samples from the mucosal membranes and from the luminal contents of both organs were examined separately.

Following collection of luminal crop contents for analyses based on real-time PCR and cultivation, the entire crop was removed from the carcass and divided into two equally sized parts. The mucosal membranes were flushed with sterile physiological saline for the removal of luminal material before the surface was scraped off with a sterile scalpel blade. The mucosal scrapings and the wall from each crop half were pooled as one sample before *Camp. jejuni* analysis. One-half was examined by real-time PCR, and the other half of the crop was examined by cultivation for *Camp. jejuni*.

The caecal mucosa was flushed with a sterile physiological saline solution and rubbed gently with a sterile surgical glove finger to remove luminal contents. Mucosal scrapings from the narrow part of one caecum and the wide part of the other caecum were pooled as one sample, and a corresponding pooled sample was collected from the opposite parts of the caecae. One of these caecal samples was examined by cultivation from each bird, whereas the other sample was examined using real-time PCR.

Colonization dynamics of Campylobacter jejuni in chicken GI
Chickens were challenged with a mixture of three *Camp. jejuni* isolates (see 'Bacterial challenge procedure'), (Experiment 3). Three chickens were sacrificed on Days 1, 3, 6, 8 and 13 p.i. Samples were taken from crop mucosa, proventricular mucosa, small intestinal mucosa, caecal mucosa and cloacal mucosa from each chicken.

Growth and feed uptake

The birds were weighed individually immediately after they were put down at the end of the experiments 1a and 1c. Total body weight per cage was recorded immediately after the birds were put down at the end of the experiment 1b, and mean individual body weight per treatment group was calculated. Remaining feeds were weighed after termination of experiment 1a, and feed uptake per bird was calculated based on the differences between amounts of purchased and remaining feeds. Feed uptake was not recorded in experiments 1b and 1c.

Campylobacter jejuni quantification and identification

Sample types and examination
Cloacal swabs were used for the pre-inoculation control of birds (qualitative cultivation). Swabs were also used for the pre-inoculation control of the experimental premises (qualitative cultivation). Luminal contents of the caecae were used for the quantitative PCR-based examinations (real-time PCR). Mucosal membrane was used for detection of *Camp. jejuni* in other segments of the chicken GI tract.

Cultivation procedures
For the detection of *Camp. jejuni*, each swab sample was immersed in 1·5-ml BPW in a test tube. The test tubes were shaken briefly on a whirl mixer, and a loopful of broth was plated on modified charcoal cephoperazone desoxycholate agar and further processed as described in NMKL no. 119, 2007. The plates were briefly incubated in anaerobic jars under microaerophilic conditions at 41·5 ± 1·0°C for 44 ± 4 h. A total of five typical colonies from each presumptive positive sample were subcultured on blood agar plates and incubated at 37 ± 1°C for 44 ± 4 h. Colonies with a typical colony morphology and typical appearance by light microscopy were positive in the catalase and oxidase tests. Presumptive *Campylobacter* spp. was further identified to species level by using a multiplex-ID PCR (Johannessen *et al.* 2007).

To enumerate *Campylobacter* from cloacal swabs, the swabs were moistened in BPW and weighed both before and after taking the faecal sample. The number of

Campylobacter present in 1 g of faeces was subsequently calculated. Samples of luminal contents from the caecum were initially diluted 1 : 10 with BPW and further serially diluted in BPW. Samples of caecal mucosa were immersed in 1·5-ml BPW in a test tube and shaken with a whirl mixer before initial dilution 1 : 10 with BPW and further serial dilution in BPW. The detection limit was 2 $\log_{10}$ CFU g^{-1}.

DNA isolation

Swabs with caecal lumen contents were mixed separately with 1 ml of Solution 1 (25 mmol l^{-1} Tris–HCl pH 8·0, 10 mmol l^{-1} EDTA pH 8·0). DNA isolation and purification was further performed using an automated procedure with silica particles (Bioclone Inc., San Diego, CA, USA) as described earlier by Skånseng *et al.* (2006).

For crop samples from Experiment 1a, 200 μl of the crop fluid was diluted 1 : 4 in 4 mol l^{-1} guanidinium thiocyanate (GTC) and further treated in the same way as the caecum samples. For the detection of *Camp. jejuni* in the mucosal membrane (Experiment 1), a part of the membrane was transferred to a FastPrep® tube (Qbiogene Inc., Carlsbad, CA, USA) containing 250 mg of glass beads (106 microns and finer; Sigma-Aldrich, Steinheim, Germany) and 500-μl 4 mol l^{-1} GTC. The samples were homogenized for 40 s in a FastPrep instrument (Qbiogene) and further treated in the same way as the lumen samples.

Quantitative real-time PCR

A quantification of *Camp. jejuni* was performed relative to the total flora as previously described by Skånseng *et al.* (2006). Universal 16S rDNA primers and a probe (Nadkarni *et al.* 2002) were used for the quantification of the total flora. A *Camp. jejuni*-specific real-time PCR was performed using the primer- and probe set described by Nogva *et al.* (2000). The real-time PCR was performed as previously described by Skånseng *et al.* (2007).

In Experiment 3, *Camp. jejuni* in the mucosal samples were quantified relative to the chicken 18S rRNA (Hillier *et al.* 2004). From the published sequence of chicken 18S rRNA, we designed a TaqMan primer and probe set using Primer Express® software v3.0 (Applied Biosystems, Foster City, CA, USA). The designed primers were K18S Forward (5′-GGG TCG GGA GTG GGT AAT TT-3′) and K18S Reverse (5′-AGC CTG AGA AAC GGC TAC CA-3′). The K18S Probe (5′-CGC GCC TGC TGC CTT CCT TG-3′) was modified with TAMRA at 3′-end and FAM at 5′-end. A standard curve, with a 10-fold dilution of DNA isolated from chicken material, was made, and the efficiency of the primer/probe set was found to be 0·84 (R^2 = 0·99).

Statistical analysis

A two-sample *T*-test with a 95% confidence interval was performed on the data from the relative quantification of *Camp. jejuni* from Experiments 1a–c, using Minitab® 15.1.0.0 software (Minitab Inc., State College, PA, USA). The two-sample *T*-test is a hypothesis test for the means of two populations to determine whether they are significantly different. Treatment 1 (negative control, Table 1) was assigned as the negative group and tested for a significant difference from the other treatments. We tested the hypothesis that the difference between the means was less than the hypothesized difference (H_1: $\mu_1-\mu_2 < 0$) for the effect of treatments. We also tested the effect of experiments by the hypothesis that the differences between the levels of *Camp. jejuni* relative to the total flora within treatments were different from zero (H_0: $\mu_1-\mu_2 \neq 0$). We assumed that a *P*-value lower than 0·05 confirms that there was a significant difference among treatments and experiments.

An analysis of variance (anova) was performed on the results from the relative quantification of *Camp. jejuni* in Experiment 2, using Minitab® 15.1.0.0 software (Minitab Inc.). anova tested that the means of the colonization levels among the different colonization sites were equal. The null hypothesis stated that the colonization level means were equal, while the alternative hypothesis stated that at least one was different. We assumed that a *P*-value <0·05 confirmed a significant difference in colonization levels among colonization sites.

Results

Effect of feed additives on *Campylobacter jejuni* colonization

Three experiments (Experiment 1a–c, Table 1) were performed using sorbate and formic acid. In all three experiments, negative and positive control treatments tested negative and positive for *Camp. jejuni*, respectively, which was confirmed by both cultivation and real-time quantification.

No effect was observed on the *Camp. jejuni* colonization in the caecum by use of 1·0% formic acid alone, 0·1% sorbate alone or a combination of 1·0% formic acid and 0·1% sorbate supplemented in the feed (Table 1). However, when the concentration of formic acid in feed was increased to 1·5% (Treatment 6) and 2·0% (Treatment 7), these treatments yielded complete inhibition of *Camp. jejuni* colonization in combination with 0·1% sorbate, except for one outlier in Treatment 6 (one sample with 2·3 $\log_{10}$ CFU g^{-1} *Camp. jejuni* at Day 14 p.i. was detected by cultivation). The complete inhibition of

Camp. jejuni colonization by the use of Treatment 7 was confirmed in two independent experiments (Experiments 1a and 1c). Real-time quantification (Fig. 1) showed that that addition of 2·0% formic acid to feed (Treatment 4) gave a variable colonization level of *Camp. jejuni* in chicken caecum, which was confirmed by cultivation. Real-time data varied between no detected *Camp. jejuni* (<−4 log) and −1·04 log relative to the total flora, cultivation data varied between no detected *Camp. jejuni* and 8·8 $\log_{10}$ CFU g^{-1}. The PCR-based quantification levels of positive samples were *c.* −2 $\log_{10}$ relative to the total flora. Mean colonization levels detected by cultivation were *c.* 8·5-$\log_{10}$ CFU g^{-1} for Treatment 3 (Day 15 p.i.) and roughly 6-$\log_{10}$ CFU g^{-1} for Treatments 5 and 8 (Day 8 p.i.).

The effect of treatment was tested by the use of a two-sample *T*-test. All treatments were tested for significant differences from Treatment 1 (negative control). Samples with no detected *Camp. jejuni* were given a threshold value depending on the amount of total flora detected in the sample. Treatment 1 (negative control) was known to contain levels of *Camp. jejuni* below the detection level as confirmed by cultivation, and this group contained only samples with values <−4 log relative to total flora. Further, none of the other treatments contained cultivation negative samples with values >−4 log relative to total flora. A threshold value of *Camp. jejuni*-negative samples at −4 log was therefore established. Treatments 6 and 7 showed a colonization level (no detected *Camp. jejuni*) similar to Treatment 1 (negative control), indicating that these treatments prevented *Camp. jejuni* colonization in chickens. Treatments 2, 3, 5 and 8 had mean *Camp. jejuni* levels that did not differ significantly from the mean of Treatment 2 (positive control).

Finally, we tested the effects of experiments on *Camp. jejuni* colonization levels (Fig. 1). The two-sample *T*-test on samples from Treatment 2 (positive control) showed that there was an effect from the experiments, but this effect was less than the effect of the treatments.

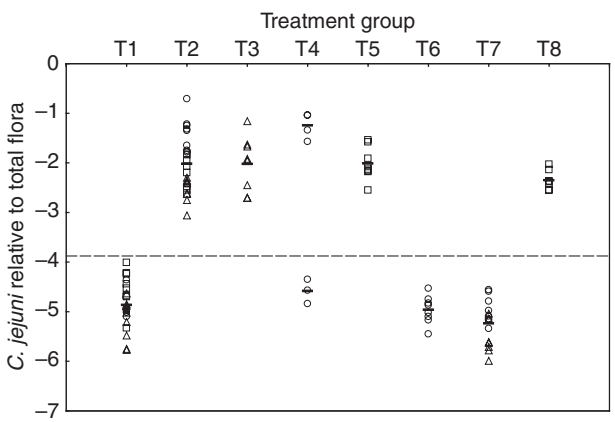

Figure 1 Colonization of *Campylobacter jejuni* in chicken caecum Days 13–15 p.i. with *Camp. jejuni* (Experiment 1a–c). The amount of *Camp. jejuni* relative to total flora measured with real-time PCR. Samples from Experiment 1a (△), samples from Experiment 1b (□), and samples from Experiment 1c (○). The mean value for each treatment is marked with (—). For Treatment 4, the samples are divided into two different groups, one group for the detected *Camp. jejuni* and another group for the samples with detection limit (no detected *Camp. jejuni*). The dashed line indicates the detection limit of *Camp. jejuni*.

Effect of feed additives on growth and feed uptake

There was a substantial (11%) variation in mean body weight between the negative control groups in experiments 1a and 1b, even though body weights were recorded at the same day of age (Table 2). Supplementation of the feed with 1% formic acid (with or without 0·1% sorbate) was tested twice. The data indicate that 1% formic acid did not influence growth rate in a consistent manner (Table 2). Supplementation of the feed with 1·5% formic acid and 0·1% sorbate was tested once. The data indicate *c.* 13% reduced body weight at day 27 in this experiment, when compared with the positive control group. Supplementation of the feed with 2·0% formic acid and 0·1% sorbate was tested twice. In both cases, a substantially (16 and 25%) reduced body weight was

Table 2 Mean body weight per bird and mean accumulated feed uptake per bird at the end of experiments 1a, 1b and 1c

Treatment groups*	Mean body weight†			Accumulated feed uptake†		
Experiment	1a	1b	1c	1a	1b	1c
1. Negative control‡	1790	1606	1743	2·638§	NR	NR
2. Positive control¶	1711	1681	1650	2·638§	NR	NR
3. 1·0% formic acid	1700	NT	NT	2·788	NT	NT
4. 2·0% formic acid	NT	NT	1389	NT	NT	NR
5. 1·0% formic acid + 0·1% sorbate	NT	1671	NT	NT	NR	NT
6. 1·5% formic acid + 0·1% sorbate	NT	NT	1442	NT	NT	NR
7. 2·0% formic acid + 0·1% sorbate	1437	NT	1238	2·725	NT	NR
8. 0·1% sorbate	NT	1769	NT	NT	NR	NT

NT, not tested; NR, not recorded.

*Chickens in all groups, except the negative control group, were inoculated with *Campylobacter jejuni* at 13–15 days of age.

†Mean body weight given as grams per bird, accumulated feed uptake given as kg feed per bird. Data from experiments 1a and 1b collected at 28 days of age, data from experiment 1c collected at 27 days of age.

‡Basic feed (no additives), not inoculated with *Camp. jejuni*.

¶Basic feed (no additives), inoculated with *Camp. jejuni*.

§Data from treatment groups 1 and 2 were pooled.

found. A similar impairment of growth was found in a single test of the effect of 2% formic acid alone. Supplementation of 0·1% sorbate was tested once and was recorded with 5% higher body weight than the positive control group.

Feed uptake was recorded only in experiment 1a. One test of 1·0% formic acid indicated a 5·7% increase in feed intake compared with the control groups, and one test indicated a 3·3% increase in feed intake associated with 2% formic acid and 0·1% sorbate.

Colonization mechanisms of *Campylobacter jejuni* in the GI tract

Real-time PCR and cultivation examinations were used on mucosa and lumen contents to determine the survival and potential colonization of *Camp. jejuni* in the crop (Experiment 2). Based on real-time PCR, we found that the level of mucosal *Camp. jejuni* relative to total flora was higher than the corresponding counts for the luminal contents of all five chickens examined (Table 3). The crop mucosa contained significantly higher ($P < 0{\cdot}006$, using ANOVA) relative levels of *Camp. jejuni* than the lumen contents. The relative level of *Camp. jejuni* in the mucosa of caecum was slightly higher than in the lumen contents, although not significantly higher. The crop mucosa contained *c.* 2-$\log_{10}$ values more *Camp. jejuni* relative to total flora than the lumen contents, while the difference in the caecum was *c.* 1-$\log_{10}$ in value between the mucosa and the lumen contents. This indicates that mucosal microflora has a relatively higher level of *Camp. jejuni* than the lumen contents.

The colonization dynamics of *Camp. jejuni* in five segments of GI tract of the chicken was studied in Experiment 3. The colonization of *Camp. jejuni* was first established in the caecae and cloaca (Table 4). Cultivation showed that all segments were positive for *Camp. jejuni* on Day 6 p.i., and the bacterial counts increased during this period. The level of *Camp. jejuni* was higher ($\log_{10}$ 8·0–10·0 CFU g^{-1}) in the lower part (caecum and cloacae) than in the upper part ($\log_{10}$ 4·0–5·0 CFU g^{-1}) of the GI tract (crop, proventriculus and small intestine).

Table 3 Colonization of *Campylobacter jejuni* in mucosa and luminal contents from caecum and crop (Experiment 2) in five chickens, measured by relative quantification to the total flora (real-time PCR) and by absolute numbers (cultivation)

	Quantification in caecum		Quantification in crop	
Sample	Relative*	Absolute†	Relative	Absolute
Mucosa				
1	−3·06	7·7	−2·62	3·5
2	−2·92	7·1	−2·23	4·8
3	−1·37	8·7	−2·62	3·7
4	−3·46	7·8	−1·98	3·2
5	−1·91	8·2	−4·21	5·2
Lumen				
1	−4·07	8·0	−4·76	4·5
2	−4·23	8·0	−4·25	2·6
3	−2·43	8·8	−3·64	4·7
4	−3·34	7·2	−4·63	<2·0
5	−3·34	8·8	−5·34	4·2

*The amount of *Camp. jejuni* relative to the total flora, given in $\log_{10}$, measured by real-time PCR.

†Amount of *Camp. jejuni* in $\log_{10}$ CFU g^{-1} material, determined by cultivation.

Discussion

In this work, we have demonstrated that chickens offered feed supplemented with a particular combination of formic acid and sorbate were protected against *Camp. jejuni* colonization during the infection trials. This effect on

Table 4 Colonization dynamics of *Campylobacter jejuni* in chicken gastrointestinal tract, Experiment 3. Samples were collected at Days 1, 3, 6, 8 and 13 p.i., and *Camp. jejuni* were quantified relative to the total flora (real-time PCR) and with absolute numbers (cultivation)

	Crop		Proventriculus		Small intestine		Caecum		Cloaca	
Day p.i.*	Relative†	Absolute‡	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute
1	–	–	–	–	–	–	–	+	–	+
3	–	+	–	–	–	+	+	+	+	+
6	+	++	+	++	–	++	+++	+++	+++	+++
8	+	NT§	+	NT	+	NT	++	NT	++	NT
13	+	NT	+	NT	–	NT	+++	NT	++	NT

*Days postinoculation.

†*Camp. jejuni* measured with real-time PCR relative to the amount of chicken DNA (see 'DNA isolation'). The categories are empirically determined. –, below detection limit (≤−4·37 $\log_{10}$ relative); +, between −4·36 and −3·50; ++, between −3·49 and −3·00; +++, ≥−2·99.

‡*Camp. jejuni* detected by cultivation. The categories are empirically determined. –, below detection limit; +, between 0·1 and 3·0 ($\log_{10}$ CFU); ++, between 3·1 and 6·0; +++, ≥6·1.

§NT – *Camp. jejuni* is not tested by cultivation at these time points.

Camp. jejuni colonization was not seen when sorbate and formic acid were tested separately as feed additives. The effect of different concentrations of formic acid with sorbate was also tested, and the highest concentration of formic acid in combination with sorbate gave the best prevention against colonization of *Camp. jejuni* in the chicken GI tract. The bactericidal effect of weak organic acid depends on low pH for optimal activity (Brul and Coote 1999; Chaveerach *et al.* 2002), which may explain that the best effect against *Camp. jejuni* was found when using 2·0% formic acid in the feed. However, our results demonstrated that formic acid alone was insufficient, suggesting that the bactericidal effect of sorbate was of major importance, whereas the main contribution of formic acid was to lower the pH. The activity of sorbates against bacteria has not been assumed to be as comprehensive as their action against fungi, although sorbates have been shown to inhibit the growth of a wide variety of bacteria. The use of sorbates ranges from food preservation and cosmetics, to the reduction of pathogen colonization in patients with pneumonia (Sofos and Busta 1981; Sofos *et al.* 1986; Tulaimat *et al.* 2005). Still, the mechanism with which sorbate acts as a bactericidal agent, preventing the establishment of a *Camp. jejuni* colonization in the chicken GI tract, has not been fully explained.

We studied the survival of *Camp. jejuni* in the crop and the colonization dynamics in the chicken GI tract. In the crop, the amount of *Camp. jejuni* relative to total flora was significantly higher in the mucosa than in the lumen contents, meaning that the mucosa consisted of a relatively high amount of *Camp. jejuni*. For that reason, it was assumed that the crop was a potential reservoir for *Camp. jejuni* colonization. However, the colonization dynamics study showed that the colonization of *Camp. jejuni* was first established in the lower GI tract before further colonization in the upper GI tract. Although the principle site for the colonization of *Camp. jejuni* is in the lower GI tract, our experiments suggest that the effect of the feed additives on *Camp. jejuni* was in the upper GI tract, as there will be a full colonization in the lower GI tract if *Camp. jejuni* overcomes the barrier functions in the upper GI tract. Our results suggest that modification of the barrier in the upper GI tract was effective against the colonization of *Camp. jejuni* in the lower GI tract, independently of where colonization was first established.

We observed that chickens given feed with added sorbate and formic acid had reduced total bacterial numbers in the crop (Appendix S1). It has previously been seen that the addition of formic acid to feed increases the concentration of formic acid in the crop, thus reducing the concentration of lactic acid (Thompson and Hinton 1997). This reduction in the amount of lactic acid may be attributed to a reduction in lactic acid bacteria in the crop. Lactic acid bacteria in crop are important because of its influence on total microflora in the lower GI tract, as well as in the prevention of the colonization of pathogens (Nurmi and Rantala 1973; Juven *et al.* 1991). Yet, it has been observed that a combination of organic acids (formic, acetic and propionic acids) yields a higher bactericidal effect against *Camp. jejuni* although the reason for these synergistic effects is unknown (Chaveerach *et al.* 2002).

Analyses of total flora in caecum from all treatment groups (Appendix S1) did not show any changes in the composition of total flora arising from the addition of acid in the feed. The total flora seemed to be more similar within experiments than between treatments groups, indicating that the total flora in the caecum is not influenced by these feed additives and the effect of the acids is in the upper GI tract. This is in accordance with the findings of Thompson and Hinton (1997), who could not detect a reduction in pH in the lower GI tract after the addition of acid in feed. An observation of the colonization level of *Camp. jejuni* in the positive control (Treatment 2) was that the distribution of the colonization level was dependent on the experiment the sample originated from. This indicates that total flora could have an effect on the colonization ability of *Camp. jejuni* in caecum, which has been previously mentioned by Skånseng *et al.* (2007). These results support the importance of collecting data on the total microflora during *Camp. jejuni* studies. The use of multiple strain challenges is also likely to be of importance, because different *Camp. jejuni* strains may interact differently with other members of the microflora.

We recorded final body weights in all three experiments investigating the effect of these acids on *Camp. jejuni* colonization. Our data suggest that whereas 1% formic acid with or without 0·1 sorbate does not influence growth rate, supplementation of the feed with 2% formic acid (with or without 0·1% sorbate) is likely to impair growth substantially. Feed uptake was recorded in one experiment. Our data indicate that neither 1% formic acid nor 2% formic acid with 0·1% sorbate was associated with reduced feed consumption, suggesting that the birds did not disapprove the taste of the feeds.

Further work is required to find optimal concentrations and method of use of organic acids in feed, ensuring a preventive effect against *Camp. jejuni* and sustainable production results of the chickens. Such optimization is important to enable the implementation of this preventive approach in conventional broiler production.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Total flora analysis.

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