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Effect of carrots and maize silage on colonization of hens by *Ascaridia galli* and *Salmonella enterica* serovar Enteritidis

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Summary

This study investigated the effect of dietary supplementation of a basal diet with carrots or maize silage on single infections with *Ascaridia galli* and *Salmonella enterica* serovar Enteritidis and concurrent infections of these 2 pathogens. Eighteen experimental subgroups, each of 14 Lohman Tradition pullets, were infected with either *A. galli* or *S. enterica* serovar Enteritidis alone or combinations of *A. galli* and subsequently *S. enterica* serovar Enteritidis or vice-versa. Each series of 6 subgroups were allocated to one dietary treatment, either an organically produced basal diet given *ad libitum* alone or supplemented with 50 g of carrots or 50 g of maize silage per pullet per day. Uninfected control subgroups were established for each dietary treatment.

Supplementation of maize silage rather than carrots increased excretion of *S. enterica* serovar Enteritidis. Within the group of hens supplemented with maize silage, those infected with *S. enterica* serovar Enteritidis and then *A. galli* gave the highest excretion of *S. enterica* serovar Enteritidis. Hens supplemented with carrots exhibited higher weight gains than those given the 2 other diets. Hens infected with *S. enterica* serovar Enteritidis and/or *A. galli* showed lower weight gains than their respective controls. Hens fed the basal diet alone had lower *A. galli* egg counts than the supplemented hens. Furthermore, hens infected with *S. enterica* serovar Enteritidis and then *A. galli* a week later had female worms with a higher fecundity than hens infected with *A. galli* alone.

Key words: chicken; *Ascaridia galli*; *Salmonella enterica* serovar Enteritidis; concomitant infection; organic feed supplementation

Introduction

Organic poultry farming has increased in Denmark during the last 15 years due to changes in consumers' preferences. Organically produced table eggs stand for 13 % of the Danish table eggs production (Anon., 2004a). However, organic production systems allow many pathogens, including *Ascaridia galli* and *Salmonella enterica* serovar Enteritidis, to be introduced and to establish (Permin *et al.*, 1999) because of lower biosecurity compared to confined production systems and difficulties in cleaning and disinfecting the premises. For example *A. galli* is found in all organic poultry farms in Denmark (Permin *et al.*, 1998). In April-June 1998, 5.8 % of the Danish layers flocks were found infected with salmonella; the prevalence fell to 0.2 % in April – June 2002 as a result of successive phases of the extensive control program for salmonella in Danish poultry, which started in 1989 (Anon., 2004b). Eggs are the major source of infections with *S. enterica* serovar Enteritidis for humans in Denmark (Mølbak & Neimann, 2002) being responsible for approximately 30 % of all cases of human salmonellosis in 2002 (Anon., 2004a). Furthermore, *S. enterica* serovar Enteritidis was predominant in Denmark during the last decade and was responsible for 56.5 ± 9.2 % of the food-borne infections in humans from 1994 to 2002 (Anon., 2004a). Evidence for the role of *A. galli* in the transmission of *Salmonella enterica* serovar Typhimurium was given by Chadfield *et al.* (2001). Worms were exposed in vitro to *S. enterica* serovar Typhimurium; bacteria adhered to the outer coating of their eggs, which it could penetrate. Those eggs were able to cause salmonellosis in day-old chicks (Chadfield *et al.*, 2001).

Danish law requires that the organic poultry producers feed hens with at least 80 % of organically produced diets and

allows 20 % to be of non-organic origin but after 2005 organic poultry must be fed with 100 % organic produced diets (Anon., 2004a). In addition, hens in organic egg production should have access to some rough feed ingredients e.g. different kind of silages, carrots or sugar beet pulp. It has been shown that supplementation with silages or carrots positively influence the development of the gastrointestinal tract and the composition of the microflora. In particular the number of coliform bacteria decreased, which may be important considering these bacteria as indicator for salmonella (Steenfeldt *et al.*, 2001).

The aim of the present investigation was to study the effect of carrots and maize silage given as supplements to an organically produced balanced compound diet on the colonization of *A. galli* and *S. enterica* serovar Enteritidis in young hens under organic farming conditions.

Materials and Methods

Animals and experimental facilities

The experiment took place at the experimental farm of the Royal Veterinary and Agricultural University, Tåstrup, Denmark. Two hundred and fifty-two Lohman Tradition pullets aged 6 weeks raised on a farm free from *A. galli* and *S. enterica* serovar Enteritidis were utilised in this experiment. Prior to infection, faecal samples and faecal swabs of all pullets were checked for the absence of *A. galli* eggs and *S. enterica* serovar Enteritidis, respectively, using methods as described below.

Experimental design, housing and diets

The pullets were fed with an organic starter diet until 6 weeks of age when they were equally divided into 3 groups of 84 chickens. During the experimental period (7 – 23 weeks of age) all groups were fed an organic grower diet (Sydvestjysk Andel 21476, Bramming, Denmark), which was given *ad libitum*. The calculated contents of crude protein and metabolizable energy in this diet were 14.5 % and 11.4 MJ ME kg^{-1}, respectively (Table 1). No essential amino acids were added to the diet since these ingredients are prohibited in diets for pullets and layers in the organic egg production. One group was supplemented with organically produced carrots and another group received organic maize silage. Carrots and maize silage were given to supplemented groups at the dose of 50 g per pullet per day every morning at the same time in a large tray placed in the middle of the hen house. These supplements were purchased from an organic farm located near Copenhagen. The adaptation period to the diet was set to 5 weeks. It was decided to use the same diet throughout the experiment even though the hens would be 23 weeks old at the end of the experiment. In practice, a change in diet would be normally accomplished at 15 – 16 weeks of age. As the effect of the supplements on the colonization of bacteria and the establishment of *A. galli* in the intestine was the main purpose of the study, a shift in diet composition could influence the results and make their interpretation difficult.

Wooden houses of 6 m^2 were equally divided in 2 parts by a fence of wire providing 0.2 m^2 per hen, which complies with the Danish law with regard to stocking density under organic farming conditions. Each part hosted a subgroup of 14 pullets. The litter was constituted of wood shavings. Temperature and humidity were similar inside the houses where pullets had free access to water and feed. At the age of 11 weeks each group of 84 pullets belonging to the same dietary group were randomly divided into 6 subgroups of 14 pullets resulting in a total of 18 subgroups. *A. galli* and/or *S. enterica* serovar Enteritidis inoculations were performed in each series of 6 subgroups given the same feed (Table 2). The experiment lasted 17 weeks in total.

Infection material

A. galli eggs were recovered from mature female worms' uteri and cultivated in 0.1N H_2SO_4 at 18°C, until they became infective, according to the procedure of Permin *et al.* (1997). Female worms were collected from hens raised in an organic farm. The *S. enterica* serovar Enteritidis phage type 4 strain JEO 4203 was isolated from a naturally infected Danish commercial layer flock and was previously characterised (Aabo *et al.*, 2002). The strain had been stored in Luria Bertani (LB) broth (CM Lab) containing 15 % glycerol at -80°C since the original isolation. Two days before inoculation, the purity was checked by culturing the strain aerobically overnight on blood agar plates containing 5 % citrated bovine blood at 37°C. Five colonies were inoculated into LB broth and shaken in an aerobically incubator overnight at 37°C. Challenge inocula were prepared from the overnight broth culture and serially diluted with saline to concentration of 1 x 10^6 CFU of bacteria per 0.1 ml. The concentration was confirmed by plating tenfold dilution on LB agar (CM Lab). The infection dose was given in 1 ml saline.

Experimental records

The pullets were observed twice a day at the same hour for clinical signs of ascaridiosis (loss of appetite, drooping wings, ruffled feathers, anaemia, diarrhoea or mortality according to Permin and Hansen, 1998) and salmonellosis (morbidity, depression, reluctance to move, huddle dejections, eyes closed, ruffled feathers, dropping wings, diarrhoea with dirty vent according to Wray and Davies, 2001). All pullets were weighed each week on an electronic scale (Geniweigher GM-11K, J.R.O.) with a precision of ± 50 g. Individual faecal samples were taken every week from all *A. galli*-infected pullets from week 4 post-infection (p.i.). Pooled faecal samples were taken before infection and every week until week 3 p.i. from *A. galli*-infected pullets and every week throughout the experiment from the controls.

Each *S. enterica* serovar Enteritidis-infected pullet was swabbed in the cloacae prior to infection to check for the absence of *S. enterica* serovar Enteritidis and each week p.i. to determine the excretion of bacteria. Three times during experiment, i.e. before infection, 1 week p.i., and at the end of the experiment, 4 pullets of each subgroup were sacrificed and intestinal contents were obtained from the gizzard, ileum and caeca for the determination of pH and

Table 1. Composition and chemical analysis of the organic basal diet and supplements

Ingredients	Basal diet	Maize silage	Carrots
Composition			
Organic wheat	27.18		
Organic oats	24.53		
Organic barley	20.00		
Peas	12.51		
Soybean, hulled, toasted	6.05		
Fishmeal	3.00		
Organic sunflower seed cake	2.09		
Organic green lucerne meal	1.54		
Calcium carbonate	1.45		
Monocalcium phosphate	0.89		
Sodium bicarbonate	0.33		
Vitamins and mineral mixture[a]	0.25		
Betain monohydrate	0.08		
Sodium chloride	0.07		
Grindazym GPL 15,000®[b]	0.03		
Chemical analysis (in % dry matter, DM)			
Dry matter	90.09	42.61	11.42
Ash	5.53	3.34	7.49
Protein (N x 6.25)	17.19	7.45	8.76
Fat-HCL	6.24	3.89	1.64
Ca	1.05	0.22	0.36
P, total	0.63	0.22	0.26
Gross energy, MJ/ kg DM	11.40	16.47	14.24
Starch	44.31	34.35	t
Glucose	0.17	0.18	13.74
Fructose	0.09	0.05	12.09
Sucrose	1.63	0.04	32.47
Cellulose (Cel.)	2.90	17.55	7.15
Non-cellulose polysaccharides (NCP)			
Rhamnose	0.10	0.10	0.40
Fucose	0.10	t	t
Arabinose	2.30	2.15	1.35
Xylose	2.80	11.50	0.30
Mannose	0.30	0.20	0.45
Galactose	2.10	0.75	2.10
Glucose	1.40	1.30	0.45
Uronic acids	1.20	1.30	8.40
Non-starch polysaccharides (NSP = Cel. + NCP)	13.20	34.95	20.60
Lignin	3.00	7.55	1.40
Dietary Fiber (NSP + lignin)	16.20	42.5	22.50

t: trace [a] Supplying per kg diet: vitamin A 13,000 IU; vitamin D_3 3,000 IU; vitamin E (α-tocopherol) 30 mg; vitamin B_1 1 mg; vitamin B_2 5 mg; vitamin B_6 3 mg; vitamin B_{12} 0.03 mg; d-pantothenic acid 5 mg; niacin 50 mg; betaine anhydrate 680 mg; folic acid 2 mg; biotin 0.1 mg; $FeSO_4$, 7 H_2O 25 mg; ZnO 60 mg; MnO 100 mg; $CuSO_4$, 5 H_2O 5 mg; KI 0.5 mg; Na_2SeO_3 0.3 mg; [b] Xylanase IUB EC 3.2.1.8 12,000 enh.; β-glucanase IUB EC 3.2.1.4 5,000 enh.

counts of dominant intestinal bacteria. The body and the emptied gizzard of each chicken were weighed on an electronic scale for the determination of the relative gizzard weight (g/100 g body weight). Therefore, 10 pullets remained in each subgroup after 1 week p.i. until the termination of the experiment.

Laboratory analyses

Faecal *A. galli* egg counts were obtained by the use of a modified McMaster method (Henriksen & Aagaard, 1976; Permin & Hansen, 1998) with a lower limit of detection of 20 eggs per g of faeces (EPG). The counts were made on individual basis for *A. galli*-infected pullets every week

Table 2. Infection schemes of *Ascaridia galli* and *Salmonella enterica* serovar Enteritidis for each series of 6 subgroups of pullets receiving the same feed

Sub-group[a]	Sub-group size	Infection	Dosage
A	14	*A. galli*[b]	1000 ± 57 eggs
B	14	*S. enterica* serovar Enteritidis[b]	10^6 CFU/ml
C	14	*A. galli* and *S. enterica* ser. Enteritidis[c]	1000 ± 57 eggs + 10^6 CFU/ml
D	14	*S. enterica* ser. Enteritidis and *A. galli*[c]	10^6 CFU/ml + 1000 ± 57 eggs
E	14	Control Location 1[d]	Sham infected with tap water
F	14	Control Location 2[d]	Sham infected with tap water

[a]The experiment totalised 18 subgroups. Three different diets were provided: an organic produced feed given alone or added with 50 g per pullet per day of carrots or maize silage. Each diet was given to 6 subgroups of pullets; [b]The infection was performed at 11 weeks of age; [c]The first inoculation was performed at 11 weeks of age and the second at 12; [d]For biosecurity reasons all subgroups infected with *S. enterica* serovar Enteritidis were located one mile from those infected with *A. galli* alone

from week 4 p.i. until the end of experiment, on pooled experiment and *A. galli*-infected pullets before week 3 p.i. The cloacal swabs for determination of the excretion of *S. enterica* serovar Enteritidis were immediately put in phosphate buffered saline (PBS) after sampling before they were transported to the laboratory for incubation at 37°C overnight. The samples were checked for *S. enterica* serovar Enteritidis as follows: faecal samples were incubated for pre-enrichment in Phosphate buffered peptone water (Difco, Detroit, USA) at 37°C for 18 to 20 hours. Samples were subsequently grown on Modified Semisolid Rappaport-Vassiliadis media (MSRV; Oxoid CM0910) for a further 18 to 20 hours at 42°C before plating out BLSF agar (Merck, Darmstadt, Germany) and incubated 18 to 20 hours. Adult worms and larvae collections at necropsy were performed according to the procedure used by Permin and Hansen (1998). The length of adult worms was measured with a ruler (Linex, 1015 M, Denmark).

The pH-value in the contents of gizzard, ileum and caeca was measured with a combined glass/reference electrode (GK 2401C, Radiometer, Denmark). Enumerations of total anaerobic bacteria, lactic acid bacteria, enterococci, coliform bacteria and *Clostridium perfringens* were conducted in the contents of gizzard, ileum and caeca of 4 pullets in each subgroup according to the procedure described by Engberg *et al.* (2004). The number of bacteria requiring anaerobic conditions was determined using anaerobic roll tubes containing glucose-cellobiose agar supplemented with chicken fecal extract (Barnes & Impey, 1974) incubated at 38°C for 5 days. Presumptive lactic acid bacteria were enumerated on MRS agar (Merck, 1.10660, Germany) incubated in an anaerobic cabinet at 38°C for 48 hours. Presumptive enterococci were counted on Slanetz and Bartely agar plates (Merck 1.05289) after aerobic incubation at 38°C for 48 hours. Presumptive coliform bacteria and lactose negative enterobacteria were counted on Mac Conkey agar (Merck 1.05465) incubated aerobically at 38°C for 24 hours as red and colorless colonies, respectively. Numbers of presumptive *Clostridium perfringens* were counted on tryptose sulphite cycloserine (TSC)-agar plates according to the method of the Nordic Committee on Food Analysis (1997).

Chemical analyses of basal diet and supplements

The DM content of ingredients and diets was determined by drying at 105°C for 8 hours. Protein (N x 6.25) was determined by the Kjeldahl method (987.02) using a Kjell-Foss 16200 autoanalyser. Energy was determined by a LECO AC 300 automated calorimeter system 789 – 500 (LECO, St Joseph, Michigan, USA). Ash was analysed according to the method 923.03 of The Association of Official Analytical Chemists (AOAC, 1990), and fat (hydrochloric acid-fat) was extracted with diethyl ether after acid-hydrolysis (Stoldt, 1952). Amino acids were analysed as described by Mason *et al.* (1980). Monomers (glucose, fructose and sucrose), and oligosaccharides (raffinose, stachyose and versbascose) were extracted with 50 % (v/v) ethanol at 60°C and quantified by gas-liquid chromatography (GLC) using the method of Bach Knudsen and Li (1991). Starch was analysed by the enzymatic-colorimetric method of Bach Knudsen (1997). Total non-Starch polysaccharides (NSP) and their constituent sugars were determined as alditol acetates by GLC for neutral sugars and by colorimetric method for uronic acids using a modification of the Uppsala procedure (Theander *et al.*, 1994) as described by Bach Knudsen (1997). Cellulose was determined as the difference in glucose content of NSP when the swelling step with 12 M sulphuric acid was included and omitted, respectively, and the content of cellulose, non-cellulosic polysaccharides (NCP) and soluble NSP were calculated as previously described (Bach Knudsen, 1997). Klason lignin was measured gravimetrically as the residue obtained of the treatment with 12 M sulphuric acid (Theander *et al.* 1994). All analyses were performed in duplicate.

Statistical analyses

Data of weight gains, egg counts, worm recoveries and *S. enterica* serovar Enteritidis' excretions were stored in Excel™ (Microsoft Corporation) and analysed with SAS® 8.2 Release (SAS Institute Inc., Cary, NC, USA). Weight gains were compared by a repeated measurements procedure with animal as random using the model:

$Y_{ij} = \mu + \beta\,(diet_{ij}, infection_{ij}, week_{ij}) + \gamma\, wd0 + \Sigma_{ij}$, with i for hen, j for week pi and the weight at the day of infection (wd0) as covariate. Logistic regression analysis was performed for the presence or the absence of *S. enterica* sero-

var Enteritidis' excretions in faecal swabs and for excretion over time: Logit (p) = a (diet, infection mode)* time.
Worm burdens, faecal egg counts, fecundities and worm lengths data were transformed to show a normal distribution. Transformed data was analysed by a repeated measurements procedure using the model: $Y_{ij} = \mu + \beta$ ($diet_{ij}$, $infection_{ij}$, $week_{ij}$) + Σ_{ij}, with i for hen, j for week pi.
Statistical analysis of results obtained from measurements in the intestinal content (pH and bacteria numbers) was performed separately for each sampling time and each intestinal segment using the General Linear Models procedure (GLM) of SAS® according to the following general model: $Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}$, where Y_{ijk} was the observed dependent variable; μ was the overall mean; α_i was the effect of diet; β_j was the effect of mode of infection; $(\alpha\beta)_{ij}$ was the interaction between diet and mode of infection; and ε_{ijk} was the random error. In cases in which the overall effect was significant ($P < 0.05$), means were compared pairwise (pdiff). Results are given as least square means with a pooled standard error (SE). The level of significance for all calculations was $P < 5$ %.

Results

Chemical analyses of basal diet and supplements
The chemical analyses of the organic diet and the two supplements are given in Table 1. The protein content in the diet was analysed to be 15.5 % and slightly higher than expected (14.5 %). Two samples of the maize silage and the carrots were analysed as the chemical composition of especially silages sometimes vary to a high content. Samples were collected for analysis in the beginning and at the end of the experiment. The results showed that the chemical composition of the two samples of maize silage and carrots was very similar. The dry matter (DM) content in the maize silage was 42 % and, as expected, the content of NSP was high, being on average 35 % DM. Approximately 50 % of NSP is cellulose, reflecting the very low content of soluble NSP in maize silage. The lignin content was on average 7.5 % DM giving a total content of dietary fibre of more than 40 % DM. The protein content was around 7.5 % DM and the starch content 34 % DM. The sugar content was very low. The DM in carrots is generally low, being 11 % in the carrots used in the present study. The content of NSP is much lower (20 % DM), and contrary to the silage, the soluble NSP constitute as much as 50 % of the total NSP content. There are only trace levels of starch in carrots, but the sugar content is high being on average 58 % DM (sum of glucose, sucrose and fructose). The protein content in carrots was 8.7 – 8.8 % DM and comparable to the protein level seen in maize silage.

Pre-trial examinations
Faecal samples were negative to all gastrointestinal helminths and protozoans prior to infection (*A. galli*, *Heterakis gallinarum*, *Capillaria* spp., *Eimeria* spp.). Likewise the cloacal swabs were negative to *S. enterica* serovar Enteritidis.

Clinical records
Differences between the 3 dietary treatments with regard to weight gains were significant ($P = 0.02$). Hens supplemented with carrots exhibited higher weight gains than those supplemented with maize silage ($P = 0.006$) and those receiving the organic basal diet alone ($P = 0.03$). Weight gains between the 2 latter groups were similar ($P = 0.6$). Infected hens of both locations showed lower weight gains than their respective controls ($P = 0.0001$) whereas no differences in weight gains were found between hens infected with *A. galli* or *S. enterica* serovar Enteritidis alone and those infected with both pathogens. However, no clinical signs of disease were observed in the hens from any subgroup throughout the experiment. Few hens exhibited post mortem lesions: 3 hens infected with *S. enterica* serovar Enteritidis and subsequently *A. galli* showed necrosis on the surface of the spleen, fibrinous peritonitis and splenomegaly, and purulent peritonitis, respectively. Two hens infected with *A. galli* and subsequently *S. enterica* serovar Enteritidis showed small areas of necrosis on the surface of the spleen. Three non-infected hens demonstrated combinations of various lesions: ovaritis, salpingitis, peritonitis, necrosis on the surface of the spleen, perihepatitis and the presence of fibrin.

Excretion of S. enterica serovar Enteritidis
Differences between the dietary treatments with respect to *S. enterica* serovar Enteritidis excretion were significant in case of the combination *S. enterica* serovar Enteritidis and then *A. galli* a week later ($P = 0.008$); hens supplemented with maize silage demonstrated higher *S. enterica* serovar Enteritidis excretion than those supplemented with carrots ($P = 0.002$). The swabs taken in hens fed the organic diet alone were negative. Differences in *S. enterica* serovar Enteritidis excretion between modes of infection (*S. enterica* serovar Enteritidis + *A. galli*, *A. galli* + *S. enterica* serovar Enteritidis, *S. enterica* serovar Enteritidis alone) were significant when the hens were supplemented with maize silage ($P = 0.002$, Fig. 1 and 2); hens infected with *S. enterica* serovar Enteritidis followed by *A. galli* a week later had a higher *S. enterica* serovar Enteritidis excretion than those infected with *S. enterica* serovar Enteritidis alone ($P = 0.02$) and those infected with *A. galli* and then *S. enterica* serovar Enteritidis a week later ($P = 0.002$). *S. enterica* serovar Enteritidis excretions of hens infected with *S. enterica* serovar Enteritidis alone and those infected with *A. galli* and then *S. enterica* serovar Enteritidis were similar ($P = 0.1$).

Faecal egg and worm counts
A significant effect of the dietary treatment on *A. galli* egg counts was found ($P = 0.02$; Table 3). Hens supplemented with either carrots or maize silage had higher faecal egg counts than those given the basal diet alone ($P = 0.01$ and 0.02, respectively). Faecal egg counts of hens given the supplements were not statistically different ($P = 0.8$).

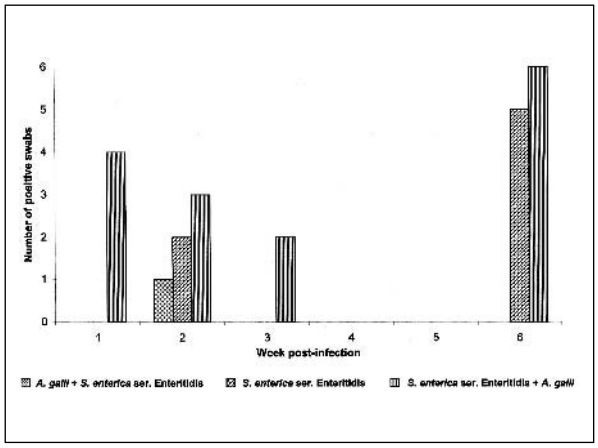

Fig. 1. *Salmonella enterica* serovar Enteritidis excretion in chickens supplemented with maize silage (n = 10 faecal swabs taken every week in each subgroup)

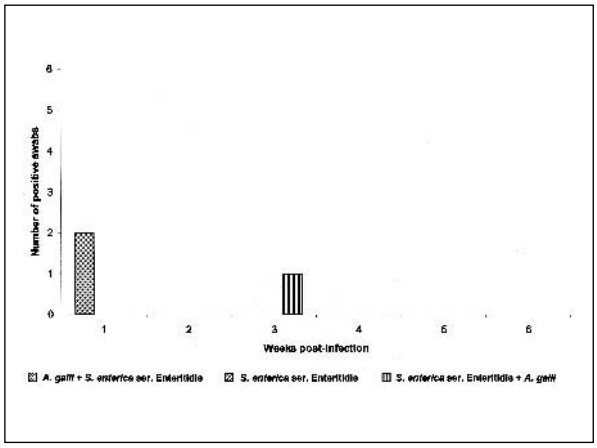

Fig. 2. *Salmonella enterica* serovar Enteritidis excretion in chickens supplemented with carrots (n = 10 faecal swabs taken every week in each subgroup)

Table 3. Faecal egg counts of *Ascaridia galli* infected hens of all groups (mean of 10 hens per group)

Diet	Group	Week post-infection				
		4	6	8	10	12
Basal diet	*Ascaridia galli*	0	2	0	18	248
	A. galli + *Salmonella enterica* serovar Enteritidis	0	0	0	18	166
	S. enterica serovar Enteritidis + *A. galli*	0	22	140	336	692
Basal diet + carrots	*Ascaridia galli*	0	0	8	32	154
	A. galli + *S. enterica* serovar Enteritidis	0	127	264	382	804
	S. enterica serovar Enteritidis + *A. galli*	0	38	126	204	540
Basal diet + maize silage	*A. galli*	0	6	116	192	808
	A. galli + *S. enterica* serovar Enteritidis	0	0	32	144	538
	S. enterica serovar Enteritidis + *A. galli*	8	80	330	376	700

Table 4. The effect of feed on relative gizzard weight (g/100 g body weight) and gizzard pH of experimental hens

	Basal diet	Basal diet + carrots	Basal diet + maize	SEM	*P*-value
	Relative gizzard weight g/100g body weight				
Prior to infection	3.76	3.36	3.66	0.18	0.29
1 week post-infection	3.00 [b]	3.13 [b]	3.34 [a]	0.27	0.04
End of experiment	2.39 [b]	2.44 [b]	2.95 [a]	0.13	< 0.001
	pH of gizzard contents				
Prior to infection	3.03	2.50	2.07	0.29	0.20
1 week post-infection	2.95	2.74	2.58	0.33	0.20
End of experiment	2.63 [b]	3.20 [a]	2.09 [b]	0.39	< 0.001

Different superscripts indicate significant difference ($P < 0.05$)

Moreover, significant differences in *A. galli*'s egg counts were found between modes of infection ($P = 0.0004$). Hens infected with *S. enterica* serovar Enteritidis and then *A. galli* a week later showed higher faecal egg counts than those infected with *A. galli* and then *S. enterica* serovar Enteritidis ($P = 0.02$) and those infected with *A. galli* alone ($P = 0.0001$). *A. galli* egg counts of the 2 latter groups were similar ($P = 0.1$). Differences in female worms' fecundities between modes of infection were significant ($P = 0.0004$). Female worms harboured by hens infected with

Table 5. Effect of feed and mode of infection on the counts of intestinal bacteria (log CFU/g digesta) in the contents of gizzard, ileum and caeca of hens, 12 weeks post-infection (data of *Clostridium perfringens* not shown)

	Diet			Mode of infection					Statistical analysis			
	Basal diet	Basal diet + maize silage	Basal diet + carrots	No infection	*Ascaridia galli*	*Ascaridia galli* + *Salmonella enterica* serovar Enteritidis	*Salmonella enterica* serovar Enteritidis	*Salmonella enterica* serovar Enteritidis + *Ascaridia galli*	SEM	Feed	Infection	Feed x Infection
Coliform bacteria												
Gizzard	3.06 [b]	3.06 [b]	4.28 [a]	3.80	3.70	3.20	3.92	2.71	0.705	0.010	0.193	0.563
Ileum	6.24	5.90	6.30	6.21	6.76	5.72	6.28	5.78	0.505	0.380	0.113	0.001
Caeca	7.03	6.94	7.17	7.07	7.03	6.93	7.07	7.10	0.389	0.631	0.980	0.686
Lactose negative enterobacteria												
Gizzard	2.34	2.43	2.76	2.59	2.59	2.41	2.75	2.21	0.354	0.147	0.399	0.313
Ileum	6.24	5.90	6.30	4.90	4.71	3.96	4.77	4.62	0.659	0.868	0.392	0.049
Caeca	5.54	5.38	5.75	5.62	5.43	5.49	5.62	5.63	0.588	0.570	0.988	0.769
Lactic acid bacteria												
Gizzard	6.95 [a]	6.25 [b]	7.32 [a]	7.12	6.80	7.12	6.80	6.37	0.528	0.009	0.341	0.719
Ileum	8.48	8.11	8.47	8.33	8.37	8.34	8.81	7.93	0.466	0.344	0.294	0.451
Caeca	9.29 [b]	9.24 [b]	9.63 [a]	9.44	9.56	9.18	9.30	9.45	0.176	0.003	0.106	0.030
Enterococci												
Gizzard	4.51 [b]	4.61 [b]	5.70 [a]	5.09	5.19	4.68	5.23	4.52	0.655	0.011	0.564	0.493
Ileum	7.11 [b]	6.89 [b]	7.56 [a]	7.18	7.27	7.05	7.52	6.92	0.345	0.013	0.311	0.005
Caeca	6.91 [b]	6.93 [b]	7.34 [a]	7.25	7.03	6.97	7.20	6.87	0.291	0.037	0.379	0.730
Total anaerobe bacteria												
Gizzard	6.17	6.34	6.62	6.79	6.29	6.03	6.50	6.26	0.617	0.502	0.522	0.197
Ileum	8.21	8.13	8.08	8.19 [b]	7.99 [b]	7.73 [b]	8.94 [a]	7.85 [b]	0.380	0.873	0.009	0.159
Caeca	9.88	9.88	9.74	9.85	9.84	9.63	9.69	10.16	0.266	0.618	0.203	0.819

Different superscripts indicate significant differences ($P < 0.05$)

than those harboured by hens infected with *A. galli* alone ($P = 0.0003$) while other comparisons of female worms' fecundities between modes of infection and between diets gave no significant differences. With respect to the counts of worms, no significant differences were found between the 3 dietary treatments ($P = 0.1$) and between the modes of infection ($P = 0.2$).

Gizzard characteristics and microbial composition of the digestive tract

As shown in Table 4, the gizzard weight in relation to body weight was higher in hens receiving supplemental maize silage as compared to hens fed carrots or hens receiving the basal diet without supplements. Differences in gizzard weights became significant with time ($P = 0.035$, after 1 week p.i.) and were most pronounced at the end of the experiment ($P = 0.003$). The same pattern was evident with respect to the development of the pH in the gizzard contents, which at all sampling times was lowest in the group fed with maize silage, but was significantly lowest at the end of experiment ($P < 0.001$). With respect to the pH in ileal contents a significant effect of the diet treatment was found prior to infection ($P = 0.028$), where hens supplemented with maize silage had a higher ileal pH than hens receiving carrots and non-supplemented hens. One week p.i., significant effects of the dietary treatments on pH in gizzard and intestinal contents and microbial composition were not obtained (results not shown). In contrast, the effect of the mode of infection was more pronounced at this point of time. The pH in gizzard contents was approximately 0.6 pH-units higher in hens infected with *A. galli* and *S. enterica* serovar Enteritidis in both combinations as compared to the other groups (3.16 vs. 2.50).

The numbers of lactose-negative enterobacteria in gizzard and ileal contents were significantly lower ($P < 0.01$), and the numbers of lactic acid producing bacteria (lactic acid bacteria and enterococci) in gizzard contents were over 10 times higher ($P < 0.05$) in both groups with the combined infections as compared to the other groups. At the end of experiment distinct differences were found between hens in relation to the dietary treatment (Table 5). Hens receiving supplemental carrots had over 10 times higher numbers of coliform bacteria ($P = 0.01$) in the gizzard contents as compared to the other two dietary groups. Furthermore, carrot-fed hens had significantly higher numbers of lactic acid producing bacteria in the digestive tract. With respect to lactic acid bacteria this difference was observed in the contents of the gizzard and the caeca, whereas enterococci counts were significantly higher throughout the entire digestive tract. Differences due to the mode of infection (Table 5) were seen with respect to the numbers of total anaerobe bacteria, which were about 10 times higher in ileal contents of hens infected with *S. enterica* serovar Enteritidis alone ($P < 0.01$) as compared the other groups.

Discussion

The present experiment showed a general increase of *S. enterica* serovar Enteritidis excretion during concomitant infection with *A. galli* compared to a single infection with *S. enterica* serovar Enteritidis. However, hens given organic feed with a supplement of maize silage and infected with *S. enterica* serovar Enteritidis followed by *A. galli* a week later excreted more *S. enterica* serovar Enteritidis than those inoculated with *S. enterica* serovar Enteritidis alone and fed likewise. Thus, it is suggested that *A. galli* infection added to an established salmonella infection facilitates colonization of the intestine. Synergistic relations between parasitic helminths and members of the family of Enterobacteriaceae, were previously reported by many authors (Aitken *et al.*, 1978; Wade & Gaafar, 1981; Adedeji *et al.*, 1989; Steenhard *et al.*, 2002). Many explanations have been given to this phenomenon. Bacteria may use the lesions developed during the migratory phase of helminths' larvae for invasion/colonization (Aitken *et al.*, 1978; Tørnehøj *et al.*, 1992; Steenhard *et al.*, 2002). Helminths could also act as vectors of bacteria by carrying them towards the organs they migrate in (Adedeji *et al.*, 1989; Bottjer *et al.* 1978; Chadfield *et al.*, 2001). For instance, Chadfield *et al.* (2001) demonstrated that *A. galli* might acts as a vector of *S. enterica* serovar Typhimurium in chickens. Therefore, it is suggested that the histotrophic phase of the establishment of *A. galli* might facilitate *S. enterica* serovar Enteritidis' infection in the present investigation.

S. enterica serovar Enteritidis, when added to *A. galli* infection a week later, was excreted at a lower rate than in the inverse scheme of inoculation. There was no synergy in this case as the level of *S. enterica* serovar Enteritidis excretion was similar to the level observed with a single bacterial infection. Okulewicz and Zlotorzycka (1985) speculated that *A. galli* has even an inhibitory effect on the intestinal microflora after they had counted fewer microorganisms (10 Gram-negative bacteria, including salmonella, 10 Gram-positive bacteria, *Candida* spp. and other fungi) on the surface of the worms and in the intestinal contents of *A. galli*-infected hens than in the intestinal contents of uninfected control hens. However, with an inhibitory effect of *A. galli* we could expect to have in the present investigation lesser *S. enterica* serovar Enteritidis excretion when *A. galli* infection preceded *S. enterica* serovar Enteritidis inoculation than in the infection with *S. enterica* serovar Enteritidis alone, which was not the case.

The present investigation showed that the excretion of *S. enterica* serovar Enteritidis in concomitant infection with *A. galli* 1 week later was more pronounced in hens given the maize silage as supplement compared to the hens receiving supplemental carrots or the basal diet without supplement. It has been shown that different kinds of dietary fibres exhibit different effects on the intestinal colonisation with several pathogens. In a number of studies with broiler chickens, it has been demonstrated that the dietary addition of various sources of soluble carbohydrates, which are fermentable in the lower gastrointestinal tract, has an inhibitory effect on the establishment of salmonella (Spring *et al.*, 2000; Fernandez *et al.*, 2000, 2002). Experiments with

pigs showed that soluble fibres, such as inulin and sugar beet pulp, which are easily fermented in the lower part of the intestine, decreased the establishment and fecundity of *Oesophagostomum dentatum*. In contrast, more insoluble carbohydrates, such as oat hull meal, which are more resistant to degradation and fermentation, provide more favourable conditions for the establishment of this parasite (Petkevičius *et al.*, 1999, 2001, 2003). The positive effect of soluble fibres fermented in the lower part of the intestine was to some extent explained by the change in microflora composition, increased production of organic acids (short chain fatty acids and lactic acids), which decrease pH in the intestinal environment and create an unsuitable environment for parasites and bacteria (Petkevičius *et al.*, 2004). In the present study, the supplementation of maize silage, which is characterised by a high content of insoluble non-fermentable NSP, could have created a favourable environment for *S. enterica* serovar Enteritidis, and there could be an additional interaction effect caused by the infection with *A. galli*. Although carrots contain considerably higher amounts of soluble NSP as compared to maize silage, lesser excretion of *S. enterica* serovar Enteritidis was found in hens receiving the carrots as supplement. The reason for this is probably the very high content of sugar (>50 % of DM), which is easily digested and fermented in the upper part of the small intestine leaving less fermentable carbohydrates for microbial fermentation in the lower digestive tract. The high sugar content also provides additional energy for the bird, which may explain the higher weight gain of the carrot-supplemented hens as compared to hens supplemented with maize silage and the non-supplemented hens.

Some differences between the dietary treatments were found with respect to the composition of the microflora as well as the gizzard size and pH in gizzard contents. At the end of experiment, higher numbers of lactic producing bacteria (lactobacilli and enterococci) were observed in the gastrointestinal contents of hens given carrots as supplement (Table 5). As mentioned earlier, this finding is very likely due the higher amount of easily fermentable carbohydrates (sugar) in carrots as compared to maize silage. Due to its coarse structure, maize silage stimulates the muscular activity of the gizzard resulting in an increase of the gizzard weights. Simultaneously, the pH in gizzard contents decreases in relation to maize silage supplementation, probably due to a stimulation of gastric HCl secretion. This effect counts in favour of maize silage supplementation, as the acidic environment in the gizzard provides a barrier against pathogenic bacteria such as acid intolerant Gram-negative enterobacteria, like coliform bacteria and salmonella (Engberg *et al.*, 2004; Bjerrum *et al.*, 2005). Therefore, negative effect of using maize silage in terms of increased *S. enterica* serovar Enteritidis excretion observed in the present study was not expected. In an earlier experiment where maize silage was used as a feed supplement to laying hens until the age of 53 weeks significantly higher gizzard weights and a lower pH in gizzard contents were observed compared to a control without access to supplements (Steenfeldt *et al.* 2001). In addition, the number of coliform bacteria in the small intestine tended to be reduced by feeding silage compared to the control indicating a positive effect on the microflora due to the barrier function of the gizzard. However, this effect was only observed in older hens that had received the dietary supplement for 33 weeks, which suggests that the intestinal system including the microflora probably needs time to adapt to the very high fibre supplement over time. The present experiment only involves the early laying period, so possible long-term effects of high fibre material on the development of the gastrointestinal tract and on the microflora has not been studied.

Except for a lower weight gain than the controls, hens infected with *A. galli* showed no clinical signs of ascaridiosis throughout the experiment. Ackert and Wiseman (1944), Dubinský *et al.* (1973), Permin and Ranvig (2001) also reported that growing chickens could compensate from a moderate infection with *A. galli* when fed a diet that fulfilled their nutritional requirements. Furthermore, chickens infected with *A. galli* at the age of 4 weeks were able to resist to a moderate infection when fed a standard diet (Idi *et al.*, 2004). In addition, no clinical signs of salmonellosis were observed. Clarke and Gyles (1993) reported that host resistance to salmonella increases with age and older animals need higher doses to develop the disease. In addition, as reported for *Pasteurella multocida* (Dahl *et al.*, 2002), *A. galli* infection had no effect on the symptoms of the concomitant disease.

Higher *A. galli* faecal eggs' excretion and fecundity were recorded in hens inoculated with *S. enterica* serovar Enteritidis and then *A. galli* a week later compared to hens given *A. galli* alone. Furthermore, the hens inoculated with *S. enterica* serovar Enteritidis and then *A. galli* a week later had also the highest *S. enterica* serovar Enteritidis excretion. Mixed infections of *A. galli* and *S. enterica* serovar Enteritidis in chickens remain a threat for public health as bacterial excretion may be increased. It is therefore recommended to improve the biosecurity of organic poultry flocks in order to lower the prevalence of these pathogens. The use of maize silage to feed organic hens has to be considered with caution in case the status of the flock concerning zoonotic infection is unknown, but further studies related to the effect of maize silage on salmonella infection should be performed involving a longer experimental period to obtain results from older hens. More comprehension of the mechanisms underlying the synergy between *A. galli* and *S. enterica* serovar Enteritidis will also help to improve means of prevention.

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