Fermented Liquid Feed Reduces Susceptibility of Broilers for *Salmonella enteritidis*

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**ABSTRACT** The presence of *Salmonella* in chickens is a problem because poultry meat is recognized as a source of human salmonellosis. Fermented feed has characteristics like a high number of lactobacilli and high concentration of lactic acid, which could make chickens less susceptible for infection with *Salmonella*. Fermented feed might therefore prevent the colonization of chickens with *Salmonella*. Two studies were performed to quantify the effect of fermented liquid feed on the susceptibility of broilers for *Salmonella*. The fermented feed was prepared by fermenting a dry broiler feed supplemented with 1.4 parts of water. *Lactobacillus plantarum* was used for fermentation. The fermented liquid feed (FLF) contained 10⁹ to 10¹⁰ cfu lactobacilli per gram, and the pH was 4. Individually housed control chickens and FLF-fed chickens were inoculated with 10² to 10⁷ cfu *Salmonella enteritidis* (SE). Colonization was estimated by cloacal swabs and quantitative caecal culture.

The proportion of SE-shedding chickens was decreased in FLF-fed chickens. FLF-fed chickens required a longer time after inoculation or a higher inoculation dose to get the same proportion of infected chickens in comparison with dry feed-fed chickens. The level of cecal colonization with *Salmonella* in the ceca was not different at the end of the experimental period. The results indicate that FLF can hamper the introduction of *Salmonella* in broiler flocks because the chickens are less susceptible for infection. Fermented liquid feed might therefore be a new hurdle in the strategy to control *Salmonella* in chicken flocks.

*(Key words: broiler, fermented liquid feed, *Salmonella)*

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**INTRODUCTION**

Poultry is recognized as a major reservoir for human infections with *Salmonella*. From a public health point of view, it is necessary to control this biological hazard. Hygienic measures (Henken et al., 1992), the use of cecal cultures, such as Broilact that make chickens less susceptible for colonization (Impey et al., 1982; Nuotio et al., 1992), or vaccination can help to control *Salmonella* in poultry flocks. However, not any of these measures guarantees absolute control of *Salmonella*. Therefore, new or additional control measures are needed.

In pig husbandry, it was observed that the prevalence of *Salmonella* is decreased at farms where fermented by-product are applied (Van Schie, 1987; Wolf et al., 1999). Fermented feed influences the bacterial ecology of the gastrointestinal tract and reduces the levels of *Enterobacteriaceae* in the different parts of the gastrointestinal tract in pigs (Winsen et al., 2001).

Fermented feed has several characteristics that may explain the antagonistic effect of this feed against *Salmonella*. First, it is rich in lactic acid bacteria. Although not unequivocally quantified, it is claimed that lactobacilli have a beneficial effect on colonization resistance and immunity in men and animals (Adler and DaMassa, 1980; Soerjadi et al., 1981; Watkins and Miller, 1983; Weinack et al., 1985; Perdigon et al., 1990). Second, digestibility of fermented or soaked feed may be improved, which may alter the composition of the intestinal microflora. And finally, this feed has a high concentration of lactic and other organic acids. Organic acids are detrimental for the survival of *Salmonella* and can hinder its multiplication (Russell and Diez Gonzalez, 1998).

The present experiments were performed to determine whether fermented liquid feed could also play a role in the control of *Salmonella* in chickens. Therefore,
the effect of this feed on the susceptibility of individual chickens was assessed. The success of inoculation of a range of inoculation doses in individually housed and of chickens housed in pairs was determined. Parameters estimated were fecal shedding and cecal colonization level of *Salmonella*.

**MATERIALS AND METHODS**

**Experiment A**

Experiment A was performed to estimate the *Salmonella* dose needed to colonize the main proportion of inoculated chickens and to assess whether transmission to contact chickens occurred for both dry feed and fermented liquid feed (FLF) fed groups. One hundred forty 1-d-old broiler chickens were randomly divided into two groups. At d 8, 32 chickens (males and females) of each feed group were orally inoculated with *Salmonella enteritidis* (SE) and housed individually. Eight chickens were inoculated per inoculation dose and per feed. An overnight SE culture was used to prepare the needed inoculation suspensions. The number of SE in colony-forming units per milliliter in the different individual inocula was $2.8 \times 10^2$, $3.2 \times 10^2$, $5.7 \times 10^3$, and $2.8 \times 10^5$ cfu/mL. These doses are further called $10^2$ to $10^5$.

In order to check shedding of SE of the chickens after inoculation, cloacal swabs were taken from the inoculated chicken at d 1, 2, and 3 postinoculation (PI).

Another chicken (called contact chicken) was placed in each pen with the previous inoculated animal at d 3 PI. Cloacal swabs of these contact chickens were taken at d 4, 5, and 6 PI.

At d 7 PI the chickens of the $10^2$ inoculation dose were euthanized since no *Salmonella* shedding was detected until then. Cecal content was cultured for the presence of SE.

A cloacal swab was taken at d 8 PI of all other chickens. At d 13 PI, all animals were euthanized, and their ceca were isolated aseptically. One gram of cecal content was weighted in 9 ml buffered peptone water (BPW), and serial dilutions were made and plated out for quantitative enumeration.

**Experiment B**

The doses used in experiment A appeared to be sufficiently high to infect all dry feed chickens, but the FLF chickens needed higher doses. To estimate the dose needed to achieve a comparable proportion of FLF-fed chickens that shed SE, experiment B was conducted with only FLF-fed chickens. Fifty 1-d-old chickens were reared in one group in one compartment. After 1 wk, 48 chickens were divided into 24 groups of two chickens each, and these groups were housed in pens. Both chickens were inoculated with SE, eight groups per inoculation dose. The inoculation suspensions contained $4.8 \times 10^3$, $2.2 \times 10^5$ and $4.3 \times 10^7$ cfu SE/mL (further called $10^3$ to $10^7$ cfu). Cloacal swabs were taken during 6 d PI. At d 6 PI the chickens were euthanized, and the cecal contents were cultured for the presence of SE.

**Chickens**

One-day-old Ross broilers were obtained from a parent flock with a *Salmonella*-free history. Fluff and paper pads from the hatching cabin and paper pads from the transport boxes were examined on the presence of *Salmonella*. Before the chickens were experimentally infected and before noninoculated chickens (contact chickens) were placed in the pen with an inoculated animal, fresh feces samples were gathered from the litter, and these were examined on the absence of *Salmonella*. It was concluded that the chickens were reared free of *Salmonella*, as all samples were negative for *Salmonella*. The animal experiments complied with all relevant national regulations and institutional policies.

**Feed**

A broiler feed, based on a commercial composition (see Table 1) was supplied as dry feed to the control groups. The antibiotic and growth promoter-free feed and pelleted feed were γ-sterilized with 0.9 Mrad before delivery.

To prepare the fermented feed, the same broiler feed was used. The feed-to-water ratio in the liquid feed was 1:1.4 (wt/wt). Five hundred grams of this liquid feed was inoculated with 1 mL of an overnight MRS broth culture of *Lactobacillus plantarum* (Urlings et al., 1993). This mixture was incubated in sterile glass jars at 30°C for 24 h. Three of these fresh starter batches were supplemented to 12.4-kg batches of liquid feed, and after mixing, the feed was incubated for 2 d at 30°C. A pH of 4

![Table 1. Composition of the broiler feed](image)

<table>
<thead>
<tr>
<th>Feed ingredients</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>362</td>
</tr>
<tr>
<td>Wheat</td>
<td>206</td>
</tr>
<tr>
<td>Heated soybeans</td>
<td>100</td>
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<tr>
<td>Soybean pulp</td>
<td>205</td>
</tr>
<tr>
<td>Sunflower seed pulp</td>
<td>20</td>
</tr>
<tr>
<td>Fish meal</td>
<td>15</td>
</tr>
<tr>
<td>Animal fat</td>
<td>35</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>20</td>
</tr>
<tr>
<td>Premix (corn)</td>
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</tr>
<tr>
<td>Chalk</td>
<td>13</td>
</tr>
<tr>
<td>Monocalcium phosphate</td>
<td>7.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>3</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>3.3</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>1.8</td>
</tr>
<tr>
<td>Calculated feed values</td>
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</tr>
<tr>
<td>Dry matter, g/kg</td>
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</tr>
<tr>
<td>Protein, g/kg</td>
<td>205</td>
</tr>
<tr>
<td>Fat, g/kg</td>
<td>96.2</td>
</tr>
<tr>
<td>Net energy, MJ/kg</td>
<td>1,095</td>
</tr>
</tbody>
</table>

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was reached after this fermentation. The pH was monitored as control of quality. From a previous study (Heres, unpublished data) it was known that well-fermented feed contained $10^9$ to $10^{10}$ cfu L. plantarum/g. The fermented feed was stored at 4°C until use within 7 d.

Feed was administered in troughs with a wired cover and was refreshed daily. Drinking water was acidified with fumaric acid, acetic acid, and propionic acid (7.5, 14.3, and 21.2 mmol/L, respectively) and was administered in 1-L round drinkers.

**Housing**

The 1-d-old chickens were reared in separate compartments per feed treatment. The chickens were in 2.5-m$^2$ ground cages on litter and had free access to feed and water. On the first day the chickens were fed twice.

Two other compartments, the same in size with 32 pens in each, were used to house the inoculated chickens. The pens were located next to each other. A pen had a closed floor with 1 cm of sawdust and sized 0.5 m × 0.5 m. The sides and back of a pen were covered with plastic sheets at the outside. No contact between animals of different pens was possible. The roof and the front side of the cages were wired and open. Chickens, feed, and water equipment were handled with gloved and 70% alcohol-disinfected hands to prevent spread of microorganisms between different pens. In experiment A, chickens of the two highest inoculation doses were placed in one compartment. The lowest inoculation groups were housed in the other one. In experiment B one compartment was used.

**Bacteriology**

A nalidixic acid resistant strain of SE (PT4) was used for inoculation. Chickens were inoculated by providing them 0.25 mL inoculum with a curved and blunted needle at the pharyngeal side of the tongue, and the chickens swallowed the inoculum. No waste of inoculum was seen.

Samples collected prior to the experiment were incubated in BPW at 37°C for 24 h. The enriched cultures were plated on modified semisolid Rappaport Vassiliadis broth (MSRV) and incubated for 24 h at 42°C. Suspected cultures were subcultured on brilliant green agar (BGA, modified) for 24 h at 37°C. The viable counts in the inoculum suspensions were determined by diluting and plating on BGA with 100 µg/ml nalidixic-acid (24 h at 37°C).

Cloaca swabs of inoculated and contact chickens were directly spread on BGA with 100 µg/mL nalidixic-acid (24 h at 37°C) and also enriched in BPW (24 h at 37°C). Samples with no growth were again plated after enrichment. Positive diagnosis depended on the presence of one or more typical colonies. Confirmation of the cultured colonies was done by serum agglutination.

After necropsy, serial dilutions from cecal content in BPW were plated on the BGA with nalidixic acid and counted after overnight incubation at 37°C.

**Statistical Analyses**

There are three typical features of the data. First, there is dependency between binary observations on the same animal. Second, there is a structure of a waiting time problem: i.e., waiting until an animal starts shedding *Salmonella*. And finally, some data are censored. Any proper model for these data should reflect these typical features. Therefore, statistical analyses to calculate the significance of observed differences were performed as follows. Suppose that an animal becomes positive at time $T$; $T$ will either be in between two time points where observations are collected or it will be beyond the last time point where an observation is collected. In the last case, it is a censored observation. We assume that $T$ follows a proportional hazard model with a Weibull baseline hazard function (Cox and Oakes, 1984).

$$h(t) = h_0(t) \exp (-\eta) \eta = x' \beta, \quad h_0(t) = r t^{-1}$$

Here $h(t)$ is the hazard function, $h_0(t)$ is the baseline hazard function with parameter $r$, and $\eta$ is a linear combination of the treatment and concentration effects $\beta$ with design factor $x$ for a specific chicken.

For each interval $(t_1,t_2)$ between successive time points $t_1$ and $t_2$ where observations are collected, we can calculate the conditional probability $p$ that an animal becomes positive in this interval given that it was negative up to time $t_1$:

$$p = (S(t_1) - S(t_2))/S(t_1),$$

where $S(t)$ is the survivor function, i.e., the probability that $T$ exceeds time $t$. With these probabilities, $p$, a likelihood function for the binary data at the observation times, is calculated.

An algorithm was developed, based on iterated regression, to obtain maximum likelihood (ML) estimates (Cox and Hinkley, 1974). The program was written in the statistical programming language GenStat (Genstat Committee, 2000). Also an analysis was performed based on Markov Chain Monte Carlo (Gelman et al.,

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264, 98 to 100%, pro analyse, Merck Nederland B.V., Amsterdam, The Netherlands.

63, 100% pro analyse, Merck Nederland B.V., Amsterdam, The Netherlands.

605, 100% pro analyse, Merck Nederland B.V., Amsterdam, The Netherlands.


8878, Sigma Chemie, Bornmen, Belgium.
coefficient $b$ was a linear function of dose (in $10^{\log \text{cfu}}$) with the approximate ML analysis. Models were fitted where with the Baysian analysis, which will be regarded as more easier with BUGS than with ML, we proceeded estimates. Because the analysis for a variety of models of generated parameters values were used as point estimates. Because the analysis for a variety of models is much easier with BUGS than with ML, we proceeded with the Baysian analysis, which will be regarded as an approximate ML analysis. Models were fitted where $\eta$ was a linear function of dose (in $10^{\log \text{cfu}}$) with the intercept $a$ depending on the treatment but a common coefficient $b$ for the dose (in $10^{\log \text{cfu}}$). To test for lack of fit, models with quadratic functions of dose (in $10^{\log \text{cfu}}$) were fitted as well. Median waiting times $m$ were calculated as

$$m = (\log(2) \exp(-\eta))^{1/r}.$$  

Plots of $m$ against dose (in $10^{\log \text{cfu}}$) enable estimation of the concentration needed such that on average half of the animals will shed $Salmonella$ at a chosen time or the time we have to wait before half of the animals will shed $Salmonella$ for a chosen concentration. In the second experiment, a probability $se$ was included that an animal which sheds $Salmonella$ is positive according to the test. The extra parameter $se$ is referred to as the sensitivity of the test. In experiment A, $se$ was set to value 1. In experiment B, $se$ was estimated with a homogenous prior distribution on the interval $<0; 1>$. In view of the moderate size of our data sets, it seemed inappropriate to employ more complex models.

Fisher’s exact test was performed to test significance of differences between proportions per inoculation dose and time of sampling. Analysis of variance was performed to compare cecal colonization levels between feeding groups and inoculation dose for inoculated and contact chickens. These statistical tests were performed with S-plus.$^{10}$

<table>
<thead>
<tr>
<th>Days PI</th>
<th>FlF</th>
<th>DF</th>
<th>FlF</th>
<th>DF</th>
<th>FlF</th>
<th>DF</th>
<th>FlF</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>3/8</td>
<td>0/8</td>
<td>5/8</td>
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<td>2</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>4/8</td>
<td>1/8</td>
<td>5/8</td>
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<td>4/8</td>
<td>1/8</td>
<td>5/8</td>
<td>0/8</td>
<td>8/8</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>2/8</td>
<td>6/8</td>
<td>2/8</td>
<td>5/8</td>
<td>5/8</td>
<td>8/8</td>
</tr>
</tbody>
</table>

Final in ceca

cfu SE

<table>
<thead>
<tr>
<th>Days PI</th>
<th>FlF</th>
<th>DF</th>
<th>FlF</th>
<th>DF</th>
<th>FlF</th>
<th>DF</th>
<th>FlF</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>Day 7 0/8</td>
<td>Day 7 2/8</td>
<td>Day 13 6/8</td>
<td>Day 13 8/8</td>
<td>Day 13 8/8</td>
<td>Day 13 8/8</td>
</tr>
<tr>
<td>2</td>
<td>0/8</td>
<td>0/8</td>
<td>6.7 ± 1.8</td>
<td>6.5 ± 1.0</td>
<td>6.0 ± 1.6</td>
<td>6.9 ± 1.5</td>
<td>7.3 ± 0.6</td>
<td>6.6 ± 0.9</td>
</tr>
</tbody>
</table>

$^{1}$SE positives per number of tested chickens.
$^{2}$Colonization level ($\pm$ standard error) in the ceca; no significant differences (ANOVA).
$^{3}$ND = not done.
$^{*}$Significant differences ($p < 0.05$; Fisher’s exact test) between proportions between feed groups per day per dose.

Shedding of $SE$ by inoculated chickens in experiment A is summarized in Table 2 and for experiment B in Table 3. Results of cloacal swabs of the contact chickens in experiment A are shown in Table 4.

Experiment A shows that chickens inoculated with $10^2$ cfu SE, both control and FLF fed, do not shed $Salmonella$ during the first 3 d PI. The inoculated chickens fed with FLF stayed negative till the end of the experiment (i.e., d 7 PI). In the dry feed chickens ($10^2$), only one susceptible contact chicken of the control group became positive at d 3 postcontact. The ceca of this chicken and its inoculated pen mate were positive, and the ceca of one other inoculated control was positive at d 7 PI.

Less FLF-fed chickens then control chickens shed SE during the first 8 d in the $10^3$, $10^4$, and $10^5$ dose groups. At the end of the experiment, no significant difference in number of SE positive ceca was found. In the $10^3$ and $10^5$ dose groups 2, respectively, one chicken was not colonized in the FLF group. In the $10^4$ control chickens, two chickens were not colonized with SE.

Most of the contact chickens that were in a cage with a SE-shedding chicken became contact infected. There are some exceptions on this. First, in the $10^2$ control group, a contact animal became positive 3 d after the first contact, whereas its inoculated pen mate was not detected shedding SE as measured by cloacal swabbing during the first 3 d PI. However, at the end of the experiment, this inoculated chicken showed SE colonization in the ceca. Second, at d 5 after the first contact in the $10^3$, $10^4$, and $10^5$ groups, respectively, one, one, and two of contact chickens that had a positive pen mate did not become $Salmonella$ positive. These were all FLF-fed chickens. Third, in the $10^4$ control chickens and in the $10^5$ FLF chickens, there was one contact chicken that was positive in its cecum, 4.4 and 2.3 in log cfu SE/g respectively, whereas their inoculated pen mate showed no colonization.

In those chickens that showed infection, there were no differences in colonization level of SE in the ceca between control and liquid feed groups, between inocu-
TABLE 3. SE-positive swabs of SE-inoculated fermented liquid feed (FLF) fed broilers chickens during 6 days after inoculation (PI)

<table>
<thead>
<tr>
<th>Days PI</th>
<th>FLF $10^5$ cfu SE</th>
<th>FLF $10^6$ cfu SE</th>
<th>FLF $10^7$ cfu SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chicken Pens$^1$</td>
<td>Chicken Pens$^1$</td>
<td>Chicken Pens$^1$</td>
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<tr>
<td>1</td>
<td>1/16</td>
<td>1/8</td>
<td>4/16</td>
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<td>2</td>
<td>0/16</td>
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<td>1/16</td>
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<tr>
<td>4</td>
<td>2/16</td>
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<td>5</td>
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<td>4/16</td>
</tr>
<tr>
<td>6</td>
<td>4/16</td>
<td>3/8</td>
<td>5/16</td>
</tr>
<tr>
<td>Day 6 ceca</td>
<td>7/16</td>
<td>5/8</td>
<td>10/16</td>
</tr>
</tbody>
</table>

$^1$Pens (experimental units) with one or two positive chickens.

luted and contact infected chickens, or between inoculation doses at the end of experiment A (Tables 2 and 4).

The inoculation doses used in experiment A appeared to be sufficiently high to infect all control chickens. The FLF-fed chickens apparently needed higher doses to become colonized. To estimate the dose needed to get a comparable proportion of FLF-fed chickens that shed SE, experiment B was conducted with only FLF-fed chickens. The $10^5$ inoculation chickens in experiment B served as control for the $10^5$ FLF chickens of experiment A. The number of shedding animals in the $10^5$ group in experiment B was not significantly lower than in experiment A. There was a dose-dependent increase of SE-shedding chickens, inoculated with $10^6$ and $10^7$ (Table 4). The maximum level of 11/16 chickens in the $10^7$ group is lower than the 8/8 in dry feed control animals at $10^5$ in experiment A.

More precise analyses of the bacteriological examination of cloaca swabs revealed that in experiment A, eight out of 21 swabs (38%) from the FLF group were SE positive only after enrichment, whereas from all positive swabs in the control group only seven of 90 (8%) needed enrichment to detect Salmonella. In experiment B (only FLF chickens), 49 of 77 positive swabs (63%) needed enrichment. In the control group, enrichment was only needed for the first swab in a series of SE positive swabs. In the FLF groups, however, more consecutive swabs needed enrichment. As can been seen in Table 3, the number of positive SE ceca at the end of experiment B was in all dose groups higher than the number of positive cloacal swabs at the same day. In the $10^5$ and $10^6$ groups, it was even twice as high. The sensitivity for cloacal swabs to detect colonization was 65% (20 of 31).

No quantitative estimations were made in experiment B, so it is inconclusive whether chickens that showed no Salmonella in the cloacal swabs but had Salmonella positive ceca were colonized at a low level.

The statistical analysis showed that, with only three exceptions, once a chicken is observed to shed Salmonella in experiment A, it appeared to be positive on all subsequent sampling moments. Therefore, a modification was made for these three exceptions, as it makes no sense to estimate an apparently large test sensitivity on the basis of three observations only. Initially, an analysis was performed with main effects for treatment and concentration as factors. Significant differences between treatments and concentrations were found. This shows that on average the time to shedding is larger in the FLF group than in the dry feed chickens, and higher inoculation doses correspond to shorter time to shedding. Subsequently, doses as a factor was replaced by linear and quadratic terms for dose (in log cfu), with common coefficients and a different intercept for the treatments. Results for the treatment effect and baseline hazard parameter $r$ were very similar to the analysis with concentrations as factor. The quadratic function

TABLE 4. SE-positive swabs of dry feed (DF) and fermented liquid feed (FLF)-fed broiler chickens at several days postcontact (PC) with individually housed chickens, inoculated with different doses of SE

<table>
<thead>
<tr>
<th>Inoculation dose (cfu)</th>
<th>10^2</th>
<th>10^3</th>
<th>10^4</th>
<th>10^5</th>
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</thead>
<tbody>
<tr>
<td>Days PC</td>
<td>FLF</td>
<td>DF</td>
<td>FLF DG</td>
<td>FLF</td>
</tr>
<tr>
<td>1</td>
<td>0/8$^1$</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8*</td>
</tr>
<tr>
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<td>0/8$^1$</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8*</td>
</tr>
<tr>
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<td>0/8$^1$</td>
<td>1/8</td>
<td>0/8</td>
<td>1/8</td>
</tr>
<tr>
<td>5</td>
<td>ND$^2$</td>
<td>ND</td>
<td>1/8*</td>
<td>6/8</td>
</tr>
<tr>
<td>ND $^3$</td>
<td>ND</td>
<td>6.7 ± 2.2</td>
<td>6.3 ± 1.1</td>
<td>6.0 ± 1.3</td>
</tr>
</tbody>
</table>

$^1$SE positives/number of tested chickens.

$^2$ND = not done.

$^3$Colonization level (± standard error) in the ceca; no significant differences (ANOVA).

* Significant differences ($P < 0.05$; Fisher’s exact test) between proportions between feed groups per day per dose.
was not significantly different from zero. The final analysis was based on a model with linear effect of dose in the linear predictor of the hazard function with a common coefficient for the treatment and control groups but different intercepts. Pearson’s chi-square statistic indicates that there is some lack of fit for experiment A. This is largely due to two observations that differ considerably from their values expected on the basis of the model. For experiment B, the fit of the model with concentration as a factor was numerically unstable except for the baseline hazard parameter r and the sensitivity se. Oddly enough, the model with a linear and quadratic term behaved much better, although this is an equivalent model since there are only three concentrations in experiment B. Again, the quadratic term was not significant, and for the final analysis, the same model as in experiment A was used with a linear effect of dose. Parameter estimates for both experiments are shown in Table 5. Figure 1 shows the predicted curves for experiment A and B, which nicely illustrates the difference in susceptibility between dry feed and FLF-fed broiler chickens.

**DISCUSSION**

The differences in the proportion of infected chickens observed in the present experiments show the improved resistance against a single oral infection with SE in broilers that are fed with FLF. To infect all pens in the FLF group, 10^7 cfu was needed, whereas 10^4 cfu was sufficient to infect all (n = 8) pens with control chickens. The number of infected chickens in the FLF chickens infected with 10^7 cfu SE (11 out of 16, d 6) chickens is comparable with the 10^5 cfu SE-inoculated dry feed-fed chickens (six out of eight). This implies a 1,000 to 10,000 times difference in SE susceptibility between FLF-fed and dry feed-fed chickens. Interpolation in Figure 1 gives a somewhat lower estimate of a 1.7 log cfu higher dose needed in FLF-fed chickens to achieve the same waiting time for 50% infected chickens. The statistical analyses showed that treatment (fermented feed) and inoculation dose have a significant effect on shedding of *Salmonella* and thus on susceptibility for infection.

For many years, several intervention strategies have been studied for their effect on *Salmonella* in chickens. These strategies were basically aimed to reduce the susceptibility for infection of chickens or to prevent introduction by hygienic husbandry procedures. It is hard to compare other experiments with the present experiments, because there are differences with respect to group size and to the quantification of effect. A quite substantial protection against *Salmonella* was also observed for treatment with intestinal flora (Goren et al., 1984; Goren et al., 1988; Ziprin and Deloach, 1993). Most other intervention methods show less substantial protection. In vaccine studies, a species-specific and only relative protection can be achieved against SE (Cooper et al., 1994; Methner et al., 1997). Lactobacillus cultures did not protect individual chickens (Adler and Damassa, 1980). Also, feed composition (Allen et al., 1997) and addition of organic acid (Izat et al., 1990) did not result in less infected chickens or a lower colonization level of infected chickens.

The elucidation of the mechanism behind the protective effect of FLF was not the objective of the present study. However, there are some hypothetical mechanisms that can explain the improved resistance against colonization of *Salmonella* in the FLF-fed chickens. The first one is the acidifying effect of the fermented feed in the anterior part of the gastrointestinal tract. Because
the pH of the liquid feed is lower than the pH generally observed in the crop, pH 4.5 (Cox et al., 1972), the liquid feed lowers the crop pH (Heres et al., 2003). Moreover, the liquid feed contains a high amount of lactic acid. In combination with the low pH in the crop and the gizzard, the high lactic acid concentration has a very high ability to kill most salmonellae (Rubin et al., 1982; Alakomi et al., 2000). The nondissociated organic acid, of which the concentration rises when the pH decreases, will pass the cell wall of the bacteria. In the internal environment of the bacterial cell, the molecules will dissociate and the internal pH will drop. Enzymatic processes will stop, and the proton motive force will collapse, which will result in cellular death (Russell and Diefenbach, 1997). Probably less Salmonella could pass the crop and gizzard, which would reduce the probability of salmonellae reaching the ceca.

The second reason why fermented feed may work is that it has an effect on the level of Salmonella in the distal parts of the intestine, especially the cecum. The high number of enrichment needed indicates a lower level of colonization early during the experiment, although the final levels of colony-forming units of SE in the ceca were not significantly different between FLF and dry feed groups. The possibly lower level of colonization can be caused by a lower number of Salmonella reaching the cecum. In that case, it is the acidifying effect of the liquid feed in the anterior parts of the intestine that prevents Salmonella bacteria passing the anterior part of the intestinal tract. Another explanation for the lower level of colonization in the distal part of the intestinal tract during the first days of colonization could be the competition between Enterobacteriaceae and Lactobacilli. With the fermented feed, high amounts of L. plantarum are fed. Possibly these lactobacilli compete for niches to colonize; they have a local bactericidal effect on other bacteria, or they compete for nutrients (Adler and Dammassa, 1980; Soerjadi et al., 1981; Watkins and Miller, 1983; Gusil et al., 1999; Pascual et al., 1999). The composition of the intestinal ecosystem could finally also be changed by difference of substrate that enters the cecum. It is imaginable that by soaking and fermentation the digestibility of the feed changes, which may influence the composition of the ecosystem.

With respect to the statistical analyses, there is no explanation for the fact that the sensitivity in experiment B was much lower than in experiment A. It was quite apparent from the data, even before the statistical analysis, that the two experiments were quite different. In experiment B, intermittent shedding was more frequently observed. In experiment B positive swabs were repeatedly followed by Salmonella negative swabs in contrast to experiment A. Note that estimates from the statistical analyses in experiment B are less accurate than in experiment A, as is apparent from the wider confidence intervals in experiment B (Table 5). So, some care should be taken with the results of this experiment. Nevertheless, it is encouraging to note that the modeled estimate of 0.56 for the sensitivity is close to the estimate 0.65 based on the comparison between cloacal swab and cecal culture. Moreover, the estimated values for the intercept and slope for the treatment groups in experiment A and B are quite similar in relation to the width of the confidence intervals.

The trend, also reflected in Figure 1, is that the number of infected animals and pens rose in time. There are at least two possible explanations for the increase of positive animals in time. First, it takes some time before the levels of colony-forming units of Salmonella in the cecum are sufficiently high to detect Salmonella by cloacal swabbing. The higher number of cloacal swabs of the FLF group that needed enrichment before Salmonella was detected suggests that this phenomenon is stronger in FLF-fed chickens than in dry feed-fed chickens. However, if this explanation is correct, there should be a difference in colonization level in the ceca between the two feed groups, which was not observed. Second, new secondary infections between from pen to pen may have occurred, for example through the air. The roofs of the cages were not covered, and so litter might have been thrown in the air by wing movement and may subsequently have been dropped in one of the other cages. Also transmission by smaller dust particles floating through the air may have occurred in this way. This is in accordance with reports that explained the infections that occurred in chicken houses with a rapid transmission by air (Lever and Williams, 1996; Gast et al., 1998). In our experiment, this airborne transmission from cages with a high level of Salmonella shedding to pens with noncolonized chickens cannot be excluded. In other experiments, we observed that 50% of pen roofs and all filters of air outlets were Salmonella positive (results not shown). Also, the two SE-shedding FLF-fed chickens that had a SE-negative penmate can be an explanation for this.

As a pilot for future transmission experiments, susceptible contact animals were placed in contact with inoculated chickens in experiment A. It was shown that despite the effective treatment with FLF, transmission of infection still occurred. Transmission itself and the rate by which it occurs depend on the susceptibility of individual animals and the infectious pressure in the environment. Thus, fermented feed could possibly hamper transmission in a flock, because FLF reduces the chickens’ susceptibility. In a situation that a smaller proportion of chickens becomes colonized after infection, the infective pressure will not rise as fast as in circumstances in which more chickens become infected. Whether transmission to contact animals is hampered after introduction of Salmonella needs to be studied in more detail. The four not infected FLF-fed contact chickens suggest that this could be the case.

In the present experiments, the water in both feeding groups was acidified. Addition of acids to drinking water will have affected the quality of the drinking water. The reason for addition of these acids was to prevent bacterial growth (including Salmonella) in the drinkers. Otherwise, the drinker could cause a continuous chal-
lengen of the chickens. By some farmers, the addition of acids to water is a general practice.

It is known that, after feed withdrawal, organic acids affect the contamination level of crops of chickens (Byrd et al., 2001). On the other hand, organic acids in water do not change the pH in the intestines of chickens, although they increase the levels of organic acids in the crop and gizzard (Thompson and Hinton, 1997). Although it is emphasized that 2.75 mg acid/ml was added, in comparison with the high levels of lactic and acetic acid in the liquid feed (24.4 and 4.8 mg/g, respectively), it is difficult to imagine that 10% extra acids via drinking water made the FLF effective.

The conclusions from these experiments are that there is a significant difference between the FLF and dry feed groups in the proportion of SE-shedding chickens. No differences in Salmonella colonization level in the ceca were seen at the end of the experiment. The unchanged level of colonization of SE in FLF chickens is in contrast with a more frequently needed enrichment step to detect SE in the FLF group and the intermittent shedding of FLF-fed chickens. The results indicate, at least, that FLF offers increased protection against introduction of Salmonella in flocks because the chickens are less susceptible for infection. While Salmonella contamination of broilers and layer hens is a threat for human health, and liquid feeding reduces the susceptibility for infection significantly, FLF is a promising key or complementary strategy to control the Salmonella status of chickens.

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REFERENCES


