Supplementation of Coated Butyric Acid in the Feed Reduces Colonization and Shedding of *Salmonella* in Poultry

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ABSTRACT Short-chain fatty acids have been widely used as feed additives to control *Salmonella* in poultry. Data on the use of butyric acid in poultry are lacking. In this study, powder form and coated butyric acid were compared in their ability to reduce *Salmonella* colonization of ceca and internal organs shortly after infection of young chickens with *Salmonella enteritidis*. In the first trial, 4 groups of 25 specific pathogen free layer chickens were given feed either supplemented with powder form butyric acid, coated butyric acid, a combination of powder form and coated butyric acid (all groups received a total of 0.63 g of butyric acid/kg) or nonsupplemented feed. The specific pathogen free layer chickens were orally infected with 10^6 cfu of *S. enteritidis*. Coated butyric acid significantly decreased cecal colonization 3 d post-infection compared with control chickens, and powder form butyric acid had no effect. To study long-term shedding and colonization of *Salmonella* in broilers given coated butyric acid as feed additive (0.63 g of active product butyric acid/kg), 10 Ross broiler chickens were infected at d 5 with 10^5 cfu of *S. enteritidis* and housed together with 40 noninfected broilers. A control group received nonsupplemented feed. The group of broilers receiving coated butyric acid had a significantly lower number of broilers shedding *Salmonella* bacteria, but cecal colonization at slaughter age was equal for both groups. In conclusion, butyric acid decreases cecal colonization shortly after infection, decreases fecal shedding, and as a consequence, decreases environmental contamination by *S. enteritidis*-infected broilers. However, complete elimination can probably only be achieved with a combined approach using both hygienic measures and different protection measures, as the broilers still carried *S. enteritidis* bacteria in the ceca at slaughter age, although at enrichment level.

(*Key words*: butyric acid, *Salmonella enteritidis*, broiler, shedding)

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INTRODUCTION

As a result of new European regulations, all member states of the European Union have to implement monitoring and control programs for *Salmonella* in poultry (European Parliament and European Council, 2003a, b). In laying hens, vaccination can reduce shedding and egg contamination (Davies and Breslin, 2003a; Van Immerseel et al., 2005). Vaccination is not recommended for broilers because of the short lifetime of these chickens (Van Immerseel et al., 2005). For broilers, a combination of maternal vaccination, intensive hygienic measures, and the use of antibacterial feed additives can aid in preventing and controlling the infection (Methner and Steinbach, 1997; Van Immerseel et al., 2002; Davies and Breslin, 2003b).

Short-chain fatty acids have been used extensively in the field to control *Salmonella* in broilers. These acid preparations have been empirically used for their antibacterial action against *Salmonella*. Basically, there are 2 types of preparations on the market: uncoated and coated acid products. Uncoated products are powders or liquids that are used to supplement feed or drinking water, mainly to kill *Salmonella* in these matrices. After uptake, actions of these acid preparations are limited to the crop because of resorption (Thompson and Hinton, 1997). All acids that are antibacterial to *Salmonella* can potentially be used in these uncoated preparations (Al-Chalaby et al., 1985; Hume et al., 1993; Moore et al., 2004). In the coated or encapsulated products, mineral or lipid carriers are used. The aim of coating or encapsulation is to carry the acids down to the intestinal tract of the chickens. In this way, also the gut epithelial cells could be exposed to the acids, and the composition of the intestinal microbiota can po-

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Abbreviation Key: BGA = brilliant green agar; SPF = specific pathogen free.

tentially be modified by the action of the acids. Moreover, the acids are thought to be in closer contact with *Salmonella* at the site where *Salmonella* mounts a crucial step in the pathogenesis, being invasion of the epithelial cells. In the case of coated products, care should be taken in choosing the ideal acid compounds, as more complex interactions than simply antibacterial activity can play a role. It was shown that coated butyric acid was superior in controlling *Salmonella* colonization, compared with coated formic and especially acetic acid (Van Immerseel et al., 2004a). Butyric acid is known to decrease virulence gene expression and invasion of *Salmonella* in epithelial cells in vitro; acetic acid has opposite effects (Lawhon et al., 2002; Van Immerseel et al., 2004b). To our knowledge, only one study analyzing the effect of butyric acid on *Salmonella* colonization has been reported in chickens, using specific pathogen free (SPF) layer-type chicks (Van Immerseel et al., 2004a). No studies have been reported for broilers.

The aim of this study was to compare the efficacy of uncoated and coated butyric acid preparations in controlling *Salmonella* colonization early after oral inoculation of SPF layer chickens with *Salmonella enteritidis*. Moreover, a trial was conducted to evaluate the effect of coated butyric acid on shedding of *Salmonella* in broiler chickens until slaughter age in *S. enteritidis*-infected broilers.

MATERIALS AND METHODS

Salmonella Strain

Salmonella enteritidis phage type 4, Strain 76Sa88, a well-characterized strain isolated from a poultry farm (Desmidt et al., 1997, 1998), was used in the experiments. The strain was grown for 6 h in Luria-Bertoni medium (LB), whereafter the number of colony-forming units per milliliter was determined by plating 10-fold dilutions of the bacterial suspension on brilliant green agar (BGA; Oxoid, Basingstoke, England). Then, the bacteria were diluted in PBS to reach the inoculation titer.

Chickens

Trial 1. Specific pathogen free Lohmann White chickens (Iffa-Credo, Brussels, Belgium) were hatched and housed in isolation. Before the start of each experiment, 20 chickens were euthanized at hatch, and serum samples were taken for the detection of maternal antibodies against *S. enteritidis* by means of a previously described anti-*S. enteritidis* ELISA (Desmidt et al., 1996). All birds were seronegative. Chickens received ad libitum autoclaved drinking water and irradiated feed (25 kGy of γ-irradiation) supplemented with the feed additives described subsequently.

Trial 2. One-day-old Ross broiler chickens were obtained from a local hatchery. Chickens were derived from a vaccinated parent flock. The presence of maternal antibodies was not tested, but the 1-d-old broilers were randomly divided in 2 groups of 50 birds. Chickens received tap water and a wheat-based diet.

Feed Additives

Feed additives used in both trials contained butyric acid as a basic component. The first product was butyrate in powder form [i.e., a white or off-white powder containing 98% sodium salt of n-butyric acid (Admix C, INVE Nutri-Ad, Kasterlee, Belgium)]. A second feed additive contained the sodium salt of n-butyric acid (30%) in microencapsulated (coated) form (Admix 30% coated, INVE Nutri-Ad).

Experimental Setup

Trial 1. Specific pathogen free Lohmann White chickens were randomly divided in 4 groups of 25 chickens each. At day of hatch, cloacal swabs were taken for detection of *Salmonella*. From the day of hatch, 3 groups received feed supplemented with different additives. The first group received the powder form of butyric acid at a concentration of 0.63 g/kg. A second group received the coated product at a concentration of 2.5 g/kg. A third group received 0.315 g of the powder/kg and 1.25 g of the coated product/kg. Thus, all 3 groups received the same amount of the active product butyric acid (or sodium salt). One group received nonsupplemented feed. All SPF layer chickens were orally inoculated with 10^6 cfu of *S. enteritidis* 76Sa88 at d 5 using a plastic tube. At d 6, cloacal swabs of all animals were taken to detect *Salmonella* bacteria. At d 8, all chickens were euthanized, and samples of cecum, liver, and spleen were taken for bacteriological analysis.

Trial 2. One-day-old Ross broiler chickens were randomly divided in 2 groups of 50 chickens each. Cloacal swabs of all birds were taken for detection of *Salmonella*. From the day of hatch, one of the groups received a wheat-based starter diet until d 10, after which the diet was changed to a broiler diet. The other group received the feed supplemented with 2.5 g of the coated butyric acid product/kg. Ten of 50 broilers of both groups were orally inoculated with 10^5 cfu of *S. enteritidis* 76Sa88 at d 5 posthatch. At d 6, 9, 13, 20, 27, 34, and 41 of life, cloacal swabs of all birds were taken and bacteriologically analyzed. At d 42, all broilers were killed by intravenous T61 injection (Intervet, Mechelen, Belgium), and samples of ceca were taken for bacteriological analysis.

Bacteriological Analysis

Cloacal swabs were directly inoculated on BGA plates, which were incubated overnight at 37°C. When negative after direct inoculation, samples were pre-enriched in buffered peptone water (Oxoid) overnight at 37°C, whereafter samples were enriched by addition of 1 mL of this suspension to 9 mL of brilliant green tetrathionate broth (Oxiod). After incubation overnight, a drop of this suspension was plated on BGA. The percentage of chickens positive for *Salmonella* was calculated, and statistical analysis was performed with the SPSS 10.0 software using binary logistic regression, determining differences be-

Table 1. Caecal *Salmonella* colonization in specific pathogen free layer chickens that were orally inoculated with 10^6 cfu of *Salmonella enteritidis* 76Sa88 on d 5 and examined on d 8 of age[1]

	CTRL (n = 25)	Powder (n = 25)	Coated (n = 25)	COMBI (n = 25)
Negative	0[2]	0	2	1
Positive after enrichment	6	8	12	8
$X < 10^4$ cfu/g	2	1	3	2
$10^4 < X < 10^5$ cfu/g	3	1	0	6
$10^5 < X < 10^6$ cfu/g	4	3	4	5
$>10^6$ cfu/g	10	12	4	3
Group differences	A	A	B	B

[1]The chickens were fed a diet supplemented with butyric acid in powder form, coated form, a combination of half doses of powder and coated form (COMBI), or no feed additives (CTRL). Concentration of the active product butyric acid was 0.63 g/kg of feed in each group. Treatment group differences in the distribution of the number of chickens with a bacterial number as indicated in the table are shown in the last row of the table. Group differences not sharing the same letter are significantly different ($P < 0.05$).

[2]Number of chickens in a group of 25 that were negative or had a given amount of *Salmonella* bacteria in the ceca.

tween the percentages of *Salmonella*-positive animals (after direct plating or enrichment) at the different time intervals.

Samples of ceca, liver, and spleen were homogenized, and 10-fold dilutions were made in BPW starting from 5-, 10-, and 20-fold dilutions for ceca, liver, and spleen, respectively. For each dilution, 6×20 μL were inoculated on BGA. After incubation overnight (37°C), the number of colony-forming units per gram of tissue was determined by counting the bacterial colonies. For samples that were negative after titration, pre-enrichment and enrichment was performed, as described previously. The SPSS 10.0 software was used for statistical analysis. Statistical analysis was performed to determine whether there were differences between treatment groups in the distribution of the number of chickens having a bacterial number in a certain range, as specified in Tables 1 to 3. The nonparametric Kruskal-Wallis test was used to check for

Table 2. *Salmonella* colonization in liver of specific pathogen free layer chickens that were orally inoculated with 10^6 cfu of *Salmonella enteritidis* 76Sa88 on d 5 and examined on d 8 of age[1]

	CTRL (n = 25)	Powder (n = 25)	Coated (n = 25)	COMBI (n = 25)
Negative	5[2]	6	5	5
Positive after enrichment	10	12	14	18
$< 10^2$ cfu/g	3	1	0	0
$10^2 < X < 10^3$ cfu/g	1	3	4	2
$>10^3$ cfu/g	6	3	2	0
Group differences	A	A	A	B

[1]The chickens were fed a diet supplemented with butyric acid in powder form, coated form, a combination of half doses of powder and coated form (COMBI), or no feed additives (CTRL). Concentration of the active product butyric acid was 0.63 g/kg of feed in each group. Treatment group differences in the distribution of the number of chickens with a bacterial number as indicated in the table are shown in the last row of the table. Group differences not sharing the same letter are significantly different ($P < 0.05$).

[2]Number of chickens in a group of 25 that were negative or had a given amount of *Salmonella* bacteria in the liver.

Table 3. *Salmonella* colonization in spleen of specific pathogen free layer chickens that were orally inoculated with 10^6 cfu of *Salmonella enteritidis* 76Sa88 on d 5 and examined on d 8 of age[1]

	CTRL (n = 25)	Powder (n = 25)	Coated (n = 25)	COMBI (n = 25)
Negative	12[2]	10	12	15
Positive after enrichment	2	1	4	7
$X < 10^3$ cfu/g	3	3	1	2
$10^3 < X < 10^4$ cfu/g	6	10	4	1
$>10^4$ cfu/g	2	1	4	0
Group differences	A	A	A	B

[1]The chickens were fed a diet supplemented with butyric acid in powder form, coated form, a combination of half doses of powder and coated form (COMBI), or no feed additives (CTRL). Concentration of the active product butyric acid was 0.63 g/kg of feed in each group. Treatment group differences in the distribution of the number of chickens with a bacterial number as indicated in the table are shown in the last row of the table. Group differences not sharing the same letter are significantly different ($P < 0.05$).

[2]Number of chickens in a group of 25 that were negative or had a given amount of *Salmonella* bacteria in the spleen.

inter-treatment effects. When inter-treatment effects were detected, the nonparametric Mann-Whitney test was used to determine significant differences between the treatment groups (Maxwell and Delaney, 1990).

RESULTS

Trial 1

Cloacal swabs taken before inoculation with *S. enteritidis* were negative. Cloacal swabs taken 1 d post-infection showed that 10 of 25 SPF layer chickens shed *Salmonella* in the control group, 8 of 25 chickens in the group receiving the powder form of butyric acid, 4 or 25 in the group receiving coated butyric acid, and 3 of 25 in the group receiving the combined product as feed additive.

The number of colony-forming units per gram of ceca revealed more distinct differences. As can be seen in Table 1, colonization of ceca of groups that received coated butyrate or the combined powder and coated form was significantly lower than the control group or the group that received the powder form of butyrate ($P < 0.05$).

Colonization of liver was rather low for all groups. Statistical analysis on these data showed significant lower colonization of the liver in the group receiving the combined butyrate feed additive compared with the other groups ($P < 0.05$; Table 2).

Concerning spleen, significantly lower colonization of the group that received the combined butyrate product as feed additive was observed compared with the control group or the group that received the powder form of butyrate ($P < 0.05$; Table 3).

In all the statistical comparisons just mentioned, differences in the distribution of the number of chickens having a bacterial number between certain ranges were compared.

Trial 2

Cloacal swabs of all birds taken before inoculation with *Salmonella* enteritidis were negative. In the control group,

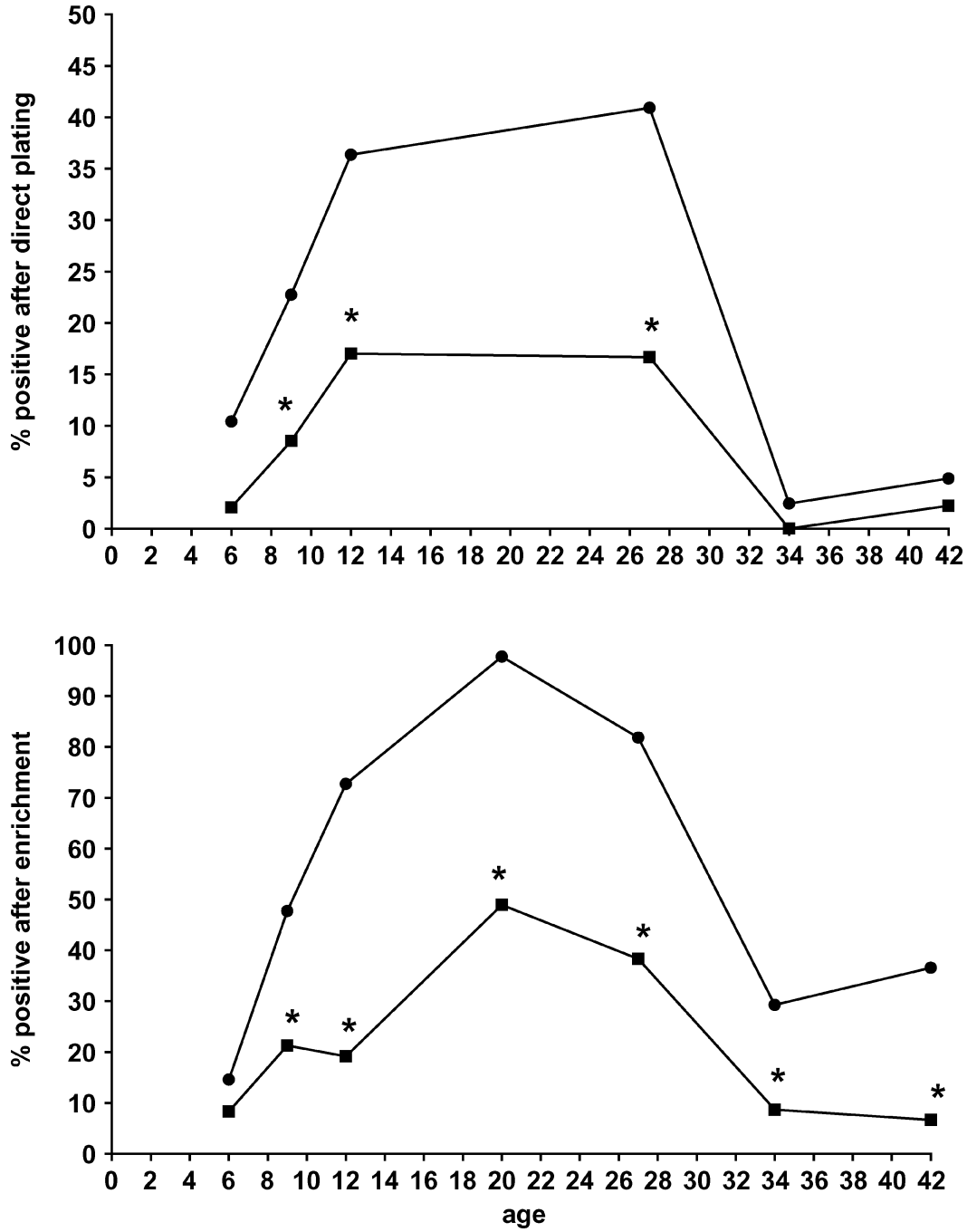

Figure 1. The percentage of *Salmonella*-positive swabs over time (d of age) is shown after direct inoculation on brilliant green agar (upper graph) and after enrichment (lower graph). Infection was carried out using a seeder model. Ten Ross broilers were infected by oral inoculation with 10^5 cfu of *Salmonella enteritidis* 76Sa88 at d 5 post-hatch. The infected broilers were then housed with 40 non-inoculated broilers. Broilers were given feed supplemented with coated butyric acid (0.63 g/kg of butyric acid) (■) and non-supplemented feed (●). *Significant differences between groups ($P < 0.05$; n = 50).

the percentage of broiler chickens having *Salmonella*-positive cloacal swabs after direct plating increased to 35 to 40% at 1 and 3 wk after seeder bird inoculation (d 12 and 27 of age). At slaughter age, however, only 4.8% of birds were positive in direct plating. In the group of broilers that received butyric acid as feed additive, 15% of the broilers had *Salmonella*-positive cloacal swabs after direct plating at 1 and 3 wk after seeder bird inoculation (d 12 and 27 of age), where after the percentage of broilers having *Salmonella*-positive cloacal swabs after direct plating decreased to 2.2% at slaughter age (Figure 1). Differences between both groups were statistically significant at d 9 ($P < 0.1$) and at d 12 and 27 of age ($P < 0.05$).

Analysis of total numbers of positive cloacal swabs (including those positive at enrichment level) showed an increase in the percentage of chickens having *Salmonella*-positive cloacal swabs to 97% at d 20 of age (d 15 post-inoculation of seeders) in the control group. Thereafter,

a decrease was detected to 36% at slaughter age. In the group of broilers that received butyric acid as feed additive, the percentage of chickens having *Salmonella*-positive cloacal swabs increased to 48% at d 20 of age (d 15 post-inoculation of seeders) and then decreased to 6% at slaughter age. Differences between both groups were statistically significant at all time points starting from d 9 of age (d 4 post-inoculation of seeder birds; $P < 0.05$).

Bacteriological analysis of ceca at slaughter age showed 68% of the broilers being positive for *Salmonella* at enrichment level (no direct positives) in both the control group and the group that received butyric acid as a feed additive.

DISCUSSION

In the present study, coated butyric acid was superior to uncoated butyric acid in reducing *Salmonella* colonization of the ceca and internal organs of SPF layer chickens shortly after infection with *S. enteritidis*. A combination of powder and coated butyric acid was most efficient in decreasing liver and spleen colonization of *Salmonella* in SPF layer chickens shortly after infection, but the exact reason is unclear. Uncoated acids are known to be taken up by the chickens by resorption from the upper alimentary tract. Therefore, the action of uncoated acids is limited to the crop, and coated acids can potentially lead to release of the acids further down in the gastrointestinal tract (Thompson and Hinton, 1997). It seems likely that *S. enteritidis* bacteria that have passed the crop alive can colonize the gut without being affected by the antibacterial action of the uncoated feed additive. Coated acids can influence the *S. enteritidis* bacteria at the site of colonization, i.e., in the gut. It is known that butyric acid reduces virulence gene expression and invasiveness in *S. enteritidis*, and a decrease in invasion has been proposed to lead to decreased cecal colonization (Porter and Curtiss, 1997; Lawhon et al., 2002; Van Immerseel et al., 2004b). The influence of butyric acid on composition of the gut microbiota is currently unknown.

Fecal shedding of *Salmonella* in *S. enteritidis*-infected broilers is strongly reduced when coated butyric acid is used as a feed additive in broiler feed. Indeed, both direct plating and enrichment showed large differences in the number of broilers shedding *Salmonella* bacteria. Shedding of *Salmonella* decreased over time in both groups of broilers. Only a low percentage of broilers were shedding *Salmonella* at slaughter age, mainly at enrichment level. Caecal colonization was decreased in our experiments shortly after infection when SPF layer chickens were given feed supplemented with coated butyric acid. Despite this, there were no differences in cecal *Salmonella* colonization at slaughter age between the control group and the group of broiler chickens that received coated butyric acid in their feed when broiler chickens were infected with *S. enteritidis*. Decreased cecal colonization early post-infection will probably result in decreased fecal shedding, and it can be hypothesized that, also in the broiler chickens, the cecal colonization was decreased shortly after infection, as the shedding pattern in the group fed with butyric acid-supplemented feed was lower than for that for control broilers shortly post-infection. The fact that the ceca of most broilers in both groups were positive, although at enrichment level and at slaughter age, illustrates that broilers can still carry *Salmonella* without shedding at a high degree. This low amount of *Salmonella* bacteria inside the host is one of the major risk factors for public health, as detection by cultivation of litter can be negative while the chickens can suddenly start shedding in high numbers because of stress conditions. Moreover, it seems to be very difficult to completely clear chickens from *Salmonella*, as seen in our study and in many others (Berthelot-Herault et al., 2003; Beal et al., 2004; Van Immerseel et al., 2004c).

In conclusion, butyric acid can be used to decrease fecal shedding of *Salmonella* and, as a consequence, environmental contamination of *Salmonella* in *S. enteritidis*-infected broilers. However, complete elimination can probably only be achieved with a combined approach using both hygienic measures and different protection measures.

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