Microencapsulated Short-Chain Fatty Acids in Feed Modify Colonization and Invasion Early After Infection with *Salmonella* Enteritidis in Young Chickens

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ABSTRACT Short-chain fatty acids (SCFA) are widely used as feed additives in poultry for the control of pathogenic bacteria, such as *Salmonella enteritidis*. Recently, a new range of products was developed in which SCFA are encapsulated in mineral carriers, resulting in a slow release during the transport of these carriers through the intestinal tract. To test the efficacy of this type of products against early colonization after *Salmonella* infection in poultry, a challenge experiment with *S. enteritidis* was performed. Five groups of 20 chickens were given feed with no supplement or feed supplemented with acetic acid (0.24%), formic acid (0.22%), or propionic acid (0.27%) as film-coated microbeads or butyric acid (0.15%) as spray-cooled microcapsules. The 5 groups were challenged with 5.10^3 cfu *S. enteritidis* at d 5 and 6 posthatch, and samples of ceca, liver, and spleen were taken at d 8 and analyzed for the number of colony-forming units of *Salmonella* per gram of tissue. Feed supplementation with acetic acid, and to a lesser extent formic acid, resulted in an increase of colonization of ceca and internal organs. Birds receiving propionic acid-coated microbeads as feed supplement were colonized with *Salmonella* to the same extent as controls. Butyric acid-impregnated microbeads in the feed, however, resulted in a significant decrease of colonization by *S. enteritidis* in the ceca but not in liver and spleen.

(Key words: chicken, microbead, *Salmonella*, short-chain fatty acid)

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INTRODUCTION

*Salmonella* enteritidis is still one of the leading causes of foodborne infections in the world, mainly due to the consumption of food prepared from poultry meat and eggs (Rabsch et al., 2001). Measures for reducing the risk of introduction of *Salmonella* on the farm and for controlling *Salmonella* in chicken flocks are widely applied in Western countries (Van Immerseel et al., 2002a).

Short-chain or volatile fatty acids (SCFA) have been used as feed additives in poultry for many years. The SCFA are bacteriostatic or bactericidal in vitro for gram-negative bacteria, provided that there are sufficient undissociated acid molecules present and that they are in contact with the bacteria for a sufficiently long time (Thompson and Hinton, 1997). It is commonly accepted that the SCFA diffuse into the bacterial cell in undissociated form, which is favored by low pH. Inside the bacterial cell, the acid dissociates, resulting in reduction of intracellular pH and anion accumulation (Russell and Diez-Gonzalez, 1998; Van Der Wielen et al., 2000). It is proposed that the SCFA in the feed will have antibacterial effects in the crop but will have no effects further down in the gastrointestinal tract (Thompson and Hinton, 1997). The SCFA however can be impregnated in or coated on micropearls, from which they are released slowly during transport in the gastrointestinal tract. In this way SCFA could also reach the small intestine and the ceca, the latter being the predominant site for *Salmonella* colonization (Anonymous, 1997). The principle of microencapsulation and continuous slow release of the encapsulated products was recently developed and has potential as a way to target probiotic bacteria as well as chemical compounds to the intestinal environment (Cheu et al., 2001; Favaro-Trindade and Grosso, 2002). It has been shown that feed supplementation with mixtures of propionic and formic acid, coated on a carrier, resulted in decreased intestinal colonization, mortality, and morbidity after *Salmonella* infection (Hinton and Linton, 1988; Iba and Berchieri, 1995; Berchieri and Barrow, 1996). Data on the effects of acetic and butyric acid on colonization of *Salmonella* in chickens in vivo are lacking.

Abbreviation Key: BGA = brilliant green agar; SCFA = short-chain fatty acids.
After *Salmonella* infection, bacteria spread rapidly to internal organs, and peak numbers of bacteria in internal organs and ceca are detected at about 3 d postinfection (Van Immerseel et al., 2002b). Decreased colonization at this early point after infection will lead to a decrease in contamination of the environment and as a consequence to a decrease in infection pressure. Moreover, young chickens are immunologically immature and are very susceptible to infection. Therefore we assessed the efficacy of microencapsulated and coated SCFA in reducing the colonization of ceca and internal organs early after infection of young chickens by *Salmonella enteritidis*. For this purpose, microbeads carrying formate, acetate, butyrate, or propionate were supplemented to the feed.

**MATERIALS AND METHODS**

**Salmonella Strain**

*Salmonella enteritidis* phage type 4, strain 76Sa88, a well-characterized strain isolated from a poultry farm, (Desmidt et al., 1996, 1998; Van Immerseel et al., 2002b) was used in our experiments. The strain was grown for 6 h in Luria-Bertoni medium (LB), after which the number of colony-forming units per milliliter was determined by plating 10-fold dilutions of the bacterial suspension on brilliant green agar (BGA). Then the bacteria were diluted to the inoculation titer in PBS.

**Chickens**

White Leghorn specific pathogen free (SPF) chickens of both sexes were hatched and housed in isolation. The birds were grown on litter floors. Before the start of the experiment, 20 chickens were euthanized, 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 autoclaved drinking water and irradiated feed (25 kGy of γ-irradiation) supplemented with the feed additives described below, ad libitum. The experiment was performed under supervision of the ethical committee of the faculty of veterinary medicine, Ghent University.

**Feed Additive**

Different short-chain fatty acids were used as feed additives. Formic, acetic, and propionic acid were coated as a film on microbeads, whereas microbeads containing butyric acid were made by spray cooling. Concentrations of different components of the microbeads are shown in Table 1. For all products, 0.5% micropearls was included in the feed so that final infeed concentrations of SCFA were 0.217% for formic acid, 0.242% for acetic acid, 0.275% for propionic acid, and 0.156% for butyric acid.

**In Vivo Trial**

Chickens were randomly divided into 5 groups of 20 chickens. From the day of hatch, 4 groups received feed supplemented with the above described feed additives, whereas 1 group received unsupplemented feed. The birds were orally inoculated with 2.10³ cfu *S. enteritidis* 76Sa88 at d 5 and 6. At d 7, cloacal swabs were taken from 5 birds per group to detect *Salmonella* bacteria. At d 8, chickens were euthanized by intravenous T61 injection. Samples of cecum, liver, and spleen 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 overnight at 37°C, thereafter samples were enriched by addition of 1 mL of this suspension to 9 mL brilliant green tetraphionate broth. After incubation overnight, a drop of this suspension was plated on BGA.

Samples of ceca, liver, and spleen were homogenized, and 10-fold dilutions were made in buffered peptone water 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 were performed as described above. Samples that were negative after titration but positive after *Salmonella* enrichment were presumed to contain 0.5 × 10¹ (ceca), 10³ (liver), or 2 × 10³ cfu/g (spleen). Samples that were negative after enrichment were presumed to have 0 cfu/g. The mean colony-forming units per gram of tissue was calculated for each group. The SPSS 9.0 software was used for statistical analysis. The nonparametric Kruskal-Wallis test was used to check for intertreatment effects. Because for each organ, intertreatment effects were detected, the nonparametric Mann-Whitney test was used to determine significant differences among the treatment groups (P < 0.05).

**RESULTS**

**Bacteriological Analyses**

One day after the second infection four-fifths of the birds from the group that received acetic acid as feed supplement had positive cloacal swabs compared with none of the birds in other groups.

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2Oxoid, Basingstoke, England.
3Iffa-Credo, Brussels, Belgium.
4SODA Feed Ingredients Ltd., Dublin, Ireland.
5Oxoid, Basingstoke, England.
Table 2 shows the number of birds classified according to cecal bacterial counts of Salmonella (log-scaled intervals). In the control group, ceca of 6 out of 20 chickens were only positive after enrichment, whereas 9 out of 20 chickens had more than $10^6$ cfu/g Salmonella in their ceca. In the group that had received formic acid as feed supplement, 10 out of 20 birds had more than $10^6$ cfu/g Salmonella in the ceca, and only 1 was positive after enrichment. Thus, more chickens had intermediate Salmonella titers compared with the controls. In the group receiving acetic acid as feed supplement, 16 out of 20 chickens had a Salmonella count of more than $10^9$ cfu/g, from which 13 had more than $10^7$ cfu/g of cecum. In the group supplemented with propionic acid, 7 birds had more than $10^6$ cfu of Salmonella per gram of cecum, whereas 8 out of 20 were positive only after enrichment. Finally, in the group receiving butyric acid impregnated microbeads, no birds had a cecal Salmonella count of more than $10^6$ cfu/g, whereas 11 out of 20 were only positive after enrichment.

Table 3 shows the number of birds classified according to bacterial counts of Salmonella in liver and spleen. Whereas for the control and butyric acid groups only a few birds had bacterial counts of more than $10^2$ cfu/g of spleen, 5 birds in the propionic acid group had Salmonella between $10^2$ and $10^3$ cfu/g spleen. In the formic acid group, 11 out of 20 birds had a Salmonella count of more than $10^2$ cfu/g, from which 6 had more than $10^3$ cfu/g, and 1 had more than $10^4$ cfu/g. In the acetic acid group, 17 out of 20 birds had more than $10^2$ cfu/g spleen, 6 had more than $10^3$ cfu/g, 5 had more than $10^4$ cfu/g, and 1 had more than $10^5$ cfu/g spleen. For liver, comparable results were obtained (Table 3).

The mean log colony-forming units per gram of ceca, liver, and spleen for the different groups are shown in Figure 1. The mean log colony-forming units S. enteritidis per gram of cecum was significantly higher in the acetic acid-treated group than in all other groups. The butyric acid-treated group had a significantly lower mean log colony-forming units per gram of cecum than all other groups, except the propionic acid-treated group. For liver and spleen, the acetic and formic acid groups had significantly higher mean log colony-forming units per gram than all other groups.

**DISCUSSION**

Short-chain fatty acids (SCFA) have been used for years in poultry to control Salmonella (Van Immerseel et al., 2002a). Experimental use of single propionic and formic acid as feed or water additives yielded variable results in protection experiments against Salmonella, depending on the experimental design (Patten and Waldroup, 1988; Izat et al., 1990a,b; McHan et al., 1992). Information on the effects of butyric or acetic acid supplementation in the feed on Salmonella infections to date was lacking.

It is believed that the direct (nonencapsulated) incorporation of SCFA in feed or drinking water only results in activity in the crop and not further down the gastroin-
TABLE 3. Colonization of the liver (L) and spleen (S) at d 8 after hatch (inoculation with 10^3 cfu Salmonella enteritidis 76Sa88 on d 5 and 6) in chickens fed a diet supplemented with formic, acetic, propionic or butyric acid or no feed additives

<table>
<thead>
<tr>
<th>Colonization</th>
<th>Control</th>
<th>FOR</th>
<th>ACE</th>
<th>PROP</th>
<th>BUT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L^a</td>
<td>S^a</td>
<td>L^bc</td>
<td>S^b</td>
<td>L^cd</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Positive after enrichment</td>
<td>19</td>
<td>11</td>
<td>8</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>10^2 &lt; x &lt; 10^3 cfu/g</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>10^3 &lt; x &lt; 10^4 cfu/g</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>10^4 &lt; x &lt; 10^5 cfu/g</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>More than 10^5 cfu/g</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

^a,b,c,dWithin the same organ, groups not having equal superscripts are significantly different (P < 0.05).

1FOR = formic acid-impregnated microbeads; ACE = acetic acid-impregnated microbeads; PROP = propionic acid-impregnated beads; BUT = butyric acid-impregnated microbeads.

2Number of chickens in a group of 20 that has a given amount of Salmonella bacteria in the L or S.

testinal tract (Thompson and Hinton, 1997). Recently, a delivery system for feed ingredients was developed to improve protection, bioavailability, and sustained slow selective release of feed ingredients, such as polyunsaturated fatty acids (Anonymous, 1997). This is done by microencapsulation and coating of these feed ingredients in micropearls. Microencapsulation of SCFA would result in a slow release of the SCFA after uptake by the chicken so that the SCFA also reach the lower intestinal tract and the ceca.

The choice of the SCFA to be encapsulated in the micropearls seems to be important for control of Salmonella. In the present study it was found that different SCFA encapsulated in micropearls in the feed resulted in different levels of colonization of ceca and internal organs shortly after S. enteritidis inoculation in young chickens. The effects of the different SCFA on colonization with Salmonella can be explained by the alteration of Salmonella virulence gene expression and invasion of epithelial cells after contact with the respective SCFA. In a previous study, we observed that exposure of S. enteritidis 76SA88 to acetate or formate for 4 h resulted in an increase in invasion of the intestinal epithelial cell line DIV-1. Contact of the bacteria with propionic and butyric acids resulted in a decrease in invasion (Van Immerseel et al., 2003). The expression of 2 transcriptional activators of genes of Salmonella pathogenicity island I, which are involved in invasion of intestinal epithelial cells, are induced by SCFA in S. typhimurium. At pH 6, expression of these genes increased with time after exposure of the bacteria to acetate, whereas this was not the case after exposure to propionate or butyrate (Durant et al., 2000). More recently, it has been shown that expression of hilA and invF, regulators of gene

FIGURE 1. The mean log colony-forming units per gram in ceca (diagonal stripe), liver (white), and spleen (gray) at d 8 after hatch of chickens that were orally inoculated with 10^3 cfu Salmonella enteritidis 76Sa88 at d 5 and 6. Chickens received diets supplemented with formic acid (FOR), acetic acid (ACE), propionic acid (PROP), or butyric acid (BUT) or feed additives (CTRL). For each organ, values not having a common letter are significantly different (P < 0.05).
expression in pathogenicity island I of *Salmonella*, are upregulated after contact of *S. typhimurium* with acetate at pH 6.7. Also, expression of sipC, an effector protein of the type 3 secretion system, was upregulated (Lawhon et al., 2002). The underlying mechanism was shown to be the activation of the BarA/SirA system by acetylphosphate through phosphorylation. The BarA/SirA system in turn is involved in activation of hilA and invF (Lawhon et al., 2002). Propionic and butyric acids led to decreased hilA, invF, and sipC expressions due to an unknown mechanism (Lawhon et al., 2002).

The ceca are the predominant site for *Salmonella* colonization (Desmidt et al., 1997, 1998). The pH of the cecal lumen in chickens is about 6 (Thompson and Hinton, 1997; Van Der Wielen et al., 2000). In the present study a reduction in colonization of the ceca was observed with propionic and butyric acids, whereas an increase was observed with formic and acetic acids early after challenge infection. Although an in vitro study is not an exact replication of the in vivo system, changes in expression of genes of pathogenicity island I due to the contact with SCFA probably play a role in colonization of ceca and internal organs. It is unclear whether prolonged contact with SCFA may perpetuate this effect observed early after infection. At later stages, the adaptive immune response may play a role.

Microbeads containing butyric acid were developed by spray cooling, whereas for technical reasons the other SCFA products were developed by film coating. The formulation of beads differed among the 4 different preparations. The amount of hydrogenated vegetable oil in the butyric acid-impregnated microbeads (452 g/kg) was, for example, higher than in all other SCFA products (100 to 150 g/kg) used in this study. The relevance of these differences for anti-*Salmonella* activity is not clear, but the formic and propionic acid preparations clearly had a different effect, although having the same amount of vegetable oil. Moreover, the percentages of the free acids differed among the different products. Even then, it was clear that the free acids exerted a different effect on the bacteria.

In conclusion, the choice of which SCFA is encapsulated in micropearls for feed supplementation of chickens seemed to affect the anti-*Salmonella* activity. Butyric acid-impregnated microbeads resulted in decreased cecal colonization shortly after inoculation of young chickens with *S. enteritidis*. Formic and propionic acids, which are most commonly used in coated commercial preparations, however, seemed to have some antagonistic effects on colonization and invasion. Butyric acid encapsulated in micropearls seemed to be a good candidate for use as a feed additive to reduce *Salmonella* colonization of ceca after early infection in young chickens.

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