Butyric acid-based feed additives help protect broiler chickens from *Salmonella* Enteritidis infection

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ABSTRACT Sodium butyrate is a sodium salt of a volatile short-chain fatty acid (butyric acid) used to prevent *Salmonella* Enteritidis infection in birds. Three groups of fifty 1-d-old broilers each were fed the following diets: T0 = standard broiler diet (control); T1 = standard broiler diet supplemented with 0.92 g/kg of an additive with free sodium butyrate (Gustor XXI B92); and T2 = standard broiler diet supplemented with 0.92 g/kg of an additive with sodium butyrate partially protected with vegetable fats (Gustor XXI BP70). Twenty percent of the birds were orally infected with *Salmonella* Enteritidis at d 5 posthatching and fecal *Salmonella* shedding was assessed at d 6, 9, 13, 20, 27, 34, and 41 of the trial. At d 42, all birds were slaughtered and 20 of them dissected: crop, cecum, liver, and spleen were sampled for bacteriological analyses. Both butyrate-based additives showed a significant reduction ($P < 0.05$) of *Salmonella* Enteritidis infection in birds from d 27 onward. However, the partially protected butyrate additive was more effective at the late phase of infection. Partially protected butyrate treatment successfully decreased infection not only in the crop and cecum but also in the liver. There were no differences in the spleen. These results suggest that sodium butyrate partially protected with vegetable fats offers a unique balance of free and protected active substances effective all along the gastrointestinal tract because it is slowly released during digestion.

Key words: feed additive, *Salmonella* Enteriditis, butyrate-based additive

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INTRODUCTION

The poultry industry receives pressure from the European Union to control *Salmonella* infections. *Salmonella* is one of the most important causes of foodborne infections in humans, mainly due to the consumption of poultry meat or contaminated eggs (Rabsch et al., 2001). Scientific literature indicates an increase of over 85% in the number of infections caused by *Salmonella* Enteritidis during the last few years from products of poultry origin (Altekruse et al., 2006). Since 2003, all members of the European Union have to put into practice monitoring programs to control these pathogens (European Parliament and European Council, 2003). Industries working on poultry have approached the problem in many ways (White et al., 1997; Doyle and Erickson, 2006). On one hand, vaccination can reduce egg contamination in laying hens but is useless in broilers (Van Immerseel et al., 2005a); on the other hand, intensive hygienic measures for controlling rodent and insect infestations during fattening, together with combinations of antibiotics, probiotics, acidifiers, or short-chain fatty acids, may be useful to prevent bacterial contamination in broilers (Barrow, 1997).

Antibiotics have been thoroughly used for decades with a low cost of implementation and easy application mixed in the food and drinking water. Unfortunately, years of massive application have lead to *Salmonella*-resistant strains (Parry, 2003). Furthermore, some papers indicate that the use of antibiotics to control infections by pathogenic *Salmonella* may induce cross-resistance to other bacterial species (Talan and Moran, 2000). Competitive exclusion by probiotic feed additives has been used extensively throughout the world as possibly one of the most effective methods of prevention (Klose et al., 2006); unfortunately, probiotics do not represent a therapeutic treatment because they do not eliminate infection in infected animals (Mead, 2000). Acidifiers have been used extensively in recent years and apparently have the ability to reduce *Salmonella* shedding in feces by changing the bowel pH. But, as with the antibiotics, some bacteria strains can develop resistance against acidifiers (Heres et al., 2004).

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More recently, a new group of feed additives was introduced in poultry farms based on the bacteriostatic in vitro effects of volatile short-chain fatty acids (**VSCFA**; C $\leq$ 4) on gram-negative bacteria (Hume et al., 1993; Berchieri and Barrow, 1996; Thompson and Hinton, 1997; Hirshfield et al., 2003). Volatile short-chain fatty acids consist on biodegradable weak organic acids that are able to eliminate pathogenic microorganisms without affecting the intestinal microflora (Ricke, 2003a). Despite being composed of organic acids, these compounds are not regarded as acidifiers because the volumes used to prevent bacteria proliferation are very low and because their mechanism of action does not consist in reducing the bowel pH (Hinton and Linton, 1988). The mechanism of VSCFA toxicity is attributed to their ability to diffuse across bacteria in the undissociated form (Warnecke and Gill, 2005). Bacterial cytoplasm pH is kept near neutral and it is often higher than the extracellular media, well above the acid dissociaton constant of VSCFA. Consequently, once VSCFA diffuses into the bacterial cytoplasm, it dissociates to a proton, which disrupts the internal pH, and the corresponding anion. The increase in internal acidity affects the integrity of purine bases, resulting in DNA synthesis and cell proliferation arrest. Dissociated VSCFA anions affect cell growth in a variety of manners. Increased anion concentration has been shown to lead to an increased transport of potassium ions into the cell, which increases cell turgidity. In addition to this unspecific anion effect, there are also effects specific to each organic acid that are not well characterized (Roe et al., 1998). Several authors have reported an increase in expression of proteins in response to extracellular VSCFA, such as the stress-inducing regulons (OppA transporter, RpoS regulon), several amino acid uptake proteins, DNA-binding proteins, and extreme-acid periplasmic chaperones (Kirkpatrick et al., 2001).

Volatile short-chain fatty acids are released at bowel pH, inhibiting the proliferation of pathogen microorganisms. In addition, butyric acid can downregulate expression of genes involved in *Salmonella* invasion at low doses (Van Immerseel et al., 2006).

This work describes the preventive effect against *Salmonella* Enteritidis infection of sodium butyrate in broiler chickens at gastrointestinal (**GIT**) and systemic levels. In addition, it compares the sodium butyrate presentation form: Gustor XXI B92, a nonprotected sodium butyrate, and Gustor XXI BP70, a special mixture of partially protected sodium butyrate (70% of active ingredient: 40% free and 30% protected) with vegetable fats.

MATERIALS AND METHODS

Salmonella Strain

Salmonella enterica serovar *enterica*, type *Salmonella* Enteritidis (Edwards and Kauffmann, 1952) was supplied by the Spanish Collection of Type Cultures (Valencia, Spain). The bacteria were grown in Luria-Bertoni medium for 6 h and titered before inoculation. The number of colony-forming units was determined by counting serial 10-fold dilutions of the bacterial suspension, on brilliant green agar plates (**BGA**). Bacterial suspensions were diluted in sterile PBS afterward, to reach the bacterial titer required to perform the oral inoculation of chickens.

Chickens

Healthy Ross chicken broilers of both sexes were housed on litter floors. Birds were fed and treated at the Experimental Farm of the University of Leon (Spain). They had ad libitum access to feed and drinking water. Environmental conditions of housing were constant during the trial: temperature of 20 $\pm$ 3°C, RH of 60%, and a 12-h photoperiod. Birds received a daily veterinary inspection.

Feed Additives

Gustor XXI B92 and Gustor XXI BP70 are butyrate-based feed additives that were mixed with a conventional broiler chicken feed at a rate of 0.92 g/kg (based on final butyrate concentration). All feeds were supplied by Norel & Nature SA (Madrid, Spain).

In Vivo Trials

The trial studied how commercial feeds supplemented with the feed additives Gustor XXI B92 or Gustor XXI BP70 could protect against *Salmonella* Enteritidis infections. These feeds were tested in challenged broilers under normal farm conditions. Birds were randomly divided into 3 groups of 50 chickens. From the second day posthatch, birds were fed with the experimental diet treatments: T0 = control feed; T1 = control feed supplemented with Gustor XXI B92; or T2 = control feed supplemented with Gustor XXI BP70. Before bacterial inoculation, 1-d-old birds from each experimental group were analyzed for *Salmonella* Enteritidis contamination and certified as being negative before and after enrichment procedure. On d 5 posthatch, a random 20% sample of the birds of every group was inoculated with 10^5 cfu of *Salmonella* Enteritidis per bird, to study the horizontal transmission of the pathogen throughout the rest of the flock.

Cloacal swabs were taken from all (scheduled and unscheduled) birds for bacteriological analysis the day before the infection (d 4 posthatch) and at d 6, 9, 13, 20, 27, 34, and 41 posthatch. On d 42 posthatch, a random sample of 20 out of 50 birds was killed by injecting an embutramide solution (T61, Intervet, Salamanca, Spain); samples of crop, cecum, liver, and spleen were taken to determine the bacteriological burden of the GIT organs liver and spleen.

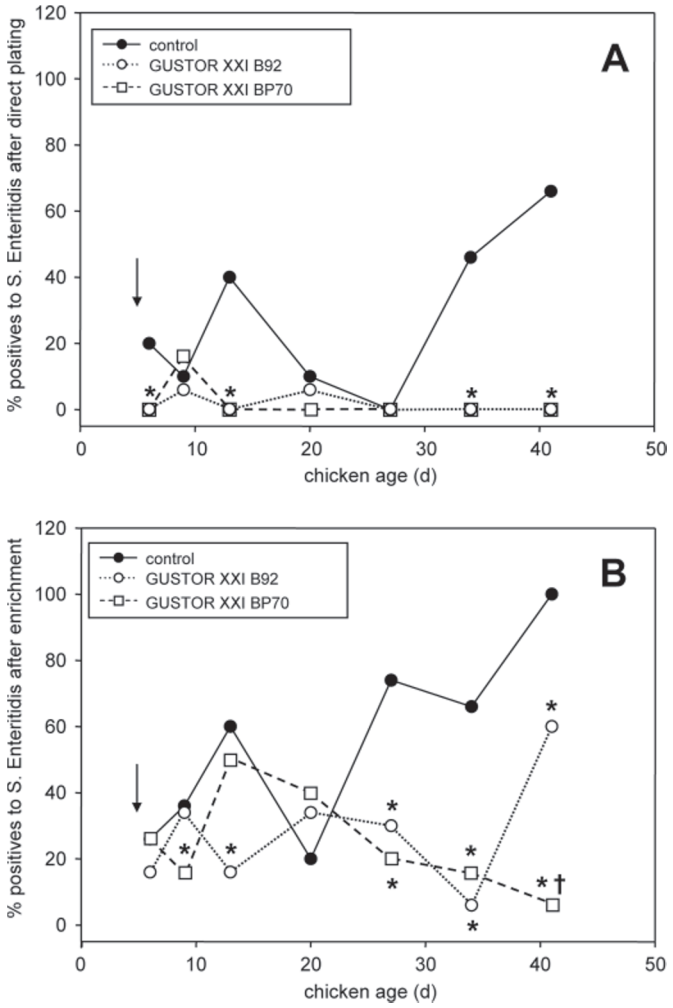

Figure 1. Fecal shedding of *Salmonella* Enteriditis-infected broilers fed with the partially protected Gustor XXI BP70 and the unprotected Gustor XXI B92 butyrate-based additives (Norel & Nature SA, Madrid, Spain). The percentage of positive-infected samples is shown after direct brilliant green agar plating (panel A) and after enrichment in Muller-Kauffmann tetrathionate broth (panel B). Ten Ross chickens from each group were inoculated with 10^5 cfu of *Salmonella enterica* serovar Enteriditis at d 5 after hatch (arrow). The infected birds were then housed with 40 noninfected birds to propagate the infection among the healthy birds. The T1 birds were fed with a diet supplemented with the unprotected butyrate-based additive Gustor XXI B92 (○); T2 chicks were fed with a diet supplemented with the partially protected butyrate-based additive Gustor XXI BP70 (□). The percentage of infected birds was compared with the nonsupplemented (●) control group (T0). *Significant differences between T1 and T2 vs. control ($P < 0.05$; n = 50 birds). †Significant differences between T1 vs. T2 ($P < 0.05$; n = 50 birds).

Bacteriological Analysis

Sterilized peptone-watered moistened swabs (DeltaLab, Barcelona, Spain) were used to take cloacal samples. Cloacal samples were directly inoculated onto BGA plates and incubated overnight at 37°C. When negative, samples were subjected to a preenrichment procedure in buffered peptone water, at 37°C in a shaking incubator to promote aeration. One milliliter of these samples was enriched by adding 9 mL of Muller-Kauffmann tetrathionate broth (Oxoid, Basingstoke, Hampshire, UK) for *Salmonella* and was incubated at 37°C during 48 h in a shaking incubator. After incubation, 100 μL of each sample was plated on BGA.

To determine counts in organs, the cecum, spleen, liver, and crop samples were weighed and homogenized in peptone water buffer under sterile conditions. These solutions were incubated for 18 h at 37°C. After incubation, 1 mL of every suspension was enriched by the addition of 9 mL of Muller-Kauffmann tetrathionate broth (Oxoid) and was incubated at 37°C for 48 h. The samples of each organ were serial diluted (10-fold) in PBS; from every dilution, a 100-μL sample was extracted and isolated on BGA plates. The number of positive samples was associated to those plates where the highest dilution was performed.

Statistical Analysis

Nonparametric Krustal-Wallis test was used to determine intertreatment differences. Once these differences were detected, the Mann-Whitney approach was used to calculate the significance between treatments.

RESULTS

Fecal Shedding of Salmonella-Infected Chicks

Figure 1 shows the fecal shedding of *Salmonella* Enteriditis-infected birds after 6, 9, 13, 20, 27, 34, and 41 d of life in T0 control, T1 Gustor XXI B92-supplemented, and T2 Gustor XXI BP70-supplemented groups. The percentage of *Salmonella*-positive swabs after direct BGA plating is illustrated at the top section (Figure 1A) and after enrichment with Muller-Kauffmann tetrathionate at the bottom one (Figure 1B).

There was a biphasic trend of *Salmonella* shedding in the T0 control group (Figure 1A): up to d 27 posthatch, the percentage of broiler chickens with *Salmonella*-positive cloacal swabs was very low, except for d 6 and 13. From d 27 on, there was an increase in positives, reaching 65% of the birds at the day before slaughtering (d 41). No shedding of *Salmonella* Enteriditis at all was observed in the cloacal swabs collected from chickens fed with T1 Gustor XXI B92 and T2 Gustor XXI BP70. Differences between T1 and T2 groups with the control group T0 were significant ($P < 0.05$) at d 6, 13, 34, and 41 of age. However, no differences in *Salmonella* shedding were observed between the experimentally treated groups.

Salmonella Count After Muller-Kauffmann Tetrathionate Enrichment

Fecal shedding analysis of *Salmonella* in cloacal swabs after Muller-Kauffmann tetrathionate enrichment showed an increase in the percentage of positive

Table 1. Gastrointestinal organ (crops and ceca) colonization of *Salmonella* (intestinal phase) in broiler chickens orally inoculated with 10^5 cfu of *Salmonella* Enteriditis d 5 posthatch[1]

	Ceca			Crops		
Salmonella (cfu/g)	Control	Gustor XXI B92	Gustor XXI BP70	Control	Gustor XXI B92	Gustor XXI BP70
Negative	2	16	18	2	18	16
Positive	18	4	2	18	2	4
10^6 to 10^7	2	1	—	6	—	2
10^7 to 10^8	8	2	2	12	2	2
$>10^8$	8	1	—	—	—	—
Differences		*	*		*	*

[1]Birds were fed with a standard broiler feed supplemented or not with unprotected or partially protected butyric acid additives Gustor XXI B92 or Gustor XXI BP70 (Norel & Nature SA, Madrid, Spain), respectively, during the fattening course. At the slaughter age (d 42 posthatch), a sample of 20 out of 50 birds was killed and dissected for bacteriological analysis.

*Statistical differences vs. control group ($P < 0.05$).

plates in control (T0) and sodium butyrate (T1 and T2)-treated birds (Figure 1B). A large percentage of birds of the T0 group shed *Salmonella* during the fattening period. After d 20, a gradual increase of positive birds was detected corresponding to 80 and 65% (d 27 and 34, respectively) and 100% the day before slaughter age. The group fed with unprotected sodium butyrate showed a significant lower percentage of birds positive to *Salmonella* after d 20 posthatch when compared with the control diet. These differences were not significant during the first period (up to d 20), with the exception of d 13. It is important to note the upturn in the percentage of *Salmonella*-shedding birds (up to 60%) observed the day before the slaughter age (d 41 posthatch) in the unprotected butyrate group. Differences between the control and Gustor XXI B92-treated group were significant at d 13, 27, 34, and 41 of age ($P < 0.05$).

In the birds that received the partially protected sodium butyrate additive, the percentage of birds positive to *Salmonella* after enrichment increased up to 50% (d 13 posthatch), decreasing thereafter to 6% of the birds the day before the slaughter age (Figure 1B). Differences between the control and Gustor XXI BP70-treated group were significant at d 9, 27, 34, and 41 of age ($P < 0.05$).

Colonization of GIT Liver and Spleen

At d 42 posthatch, a sample of 20 out of 50 birds of each group was killed to obtain crop, ceca, liver, and spleen samples to study the *Salmonella* Enteritidis colonization. Samples of these organs were homogenized and immediately enriched with Muller-Kauffmann tetrathionate broth and checked for *Salmonella* (Tables 1 and 2).

Most of the birds (90%) that received T0 had ceca and crop colonized with *Salmonella*, whereas a lower percentage (20%) of chickens presented *Salmonella* in spleen and liver. After the preenrichment procedure, an increase was observed in colony-forming unit counts per gram of fresh GIT organs. The ceca and crop estimated bacteriological burden was $>10^7$ and 10^6 to 10^8 cfu/g, respectively. Bacterial colonization of the internal organs – spleen and liver – was comparatively much lower, with bacterial burdens within the range of 10^4 to 10^6 cfu/g of fresh tissue (Table 1).

The bacterial colonization of the GIT organs in the birds fed with T1 was much lower than in the T0 group. Eighty percent of ceca and 90% of crops were *Salmonella*-negative (vs. 10% each in the T0 control group, $P < 0.001$). There were no significant differences between T0 control and T1 in liver and spleen levels (Table 2).

Table 2. Invasion of liver and spleen by *Salmonella* (systemic phase) in broiler chickens orally inoculated with 10^5 cfu of *Salmonella* Enteriditis d 5 posthatch[1]

	Liver			Spleen		
Salmonella (cfu/g)	Control	Gustor XXI B92	Gustor XXI BP70	Control	Gustor XXI B92	Gustor XXI BP 70
Negative	16	16	20	16	18	18
Positive	4	4	0	4	2	2
10^3 to 10^4	—	1	—	—	—	—
10^4 to 10^5	—	2	—	2	—	2
$>10^5$	4	1	—	2	2	—
Differences			*			

[1]Birds were fed with a standard broiler feed supplemented or not with unprotected or partially protected butyric acid additives Gustor XXI B92 or Gustor XXI BP70 (Norel & Nature SA, Madrid, Spain), respectively, during the fattening course. At the slaughter age (d 42 posthatch), a sample of 20 out to 50 birds was killed and dissected for bacteriological analysis.

*Statistical differences vs. control group ($P < 0.05$).

A significant reduction in bacterial burden was also found in the organs dissected from T2 birds. Ninety percent of ceca and 80% of crops were negative after preenrichment protocol (vs. 10% each of the control group, $P < 0.001$). The incidence of *Salmonella* was 0% in livers of birds from the T2 group, which was significantly less than the T0 and T1 groups. However, there were no differences in spleens when compared with the control group.

Despite both treatments producing a drastic reduction in colonization of GIT organs with no significant differences between them, Gustor XXI BP70 was more effective in clearing *Salmonella* from the liver. Bacteriological analysis of liver samples taken from birds fed with partially protected butyric acid, Gustor XXI BP70, showed significantly lower organ contamination ($P < 0.05$) than those taken from chicks fed with the unprotected Gustor XXI B92 additive.

DISCUSSION

This study shows how sodium butyrate feed additives, in both partially protected and unprotected forms, are able to prevent *Salmonella* colonization of GIT organs (crops and ceca), whereas only the partially protected source of the butyrate salt reduces internal organ colonization (liver). Partially protected sodium butyrate tended to have better results than the nonprotected presentation of the additive in fecal *Salmonella* shedding. Gustor XXI B92 protected at earlier phases of infection and Gustor XXI BP70 had a similar profile at earlier phases but a better profile at the late phase, reducing the percentage of birds having positive cloacal swabs ($P < 0.05$) the day before slaughtering. The results show too that the partially protected source of butyrate with vegetable fats was better than the unprotected source for reducing fecal *Salmonella* shedding during late phases of the experimental infection.

After oral inoculation, *Salmonella* has to move down the crop to colonize the GIT tract where *Salmonella* can grow aerobically. The colonization stage takes place in this highly fermentative environment (Ricke, 2003b). Because Gustor XXI BP92 contains unprotected free sodium butyrate, it should be more effective against bacteria at the acidic pH of the upper portion of the digestive tract (Van Immerseel et al., 2003, 2005b). This is demonstrated with the crop results after enrichment, in which there was a 90% reduction of crop colonization. Thereafter, bacteria adhere to the intestinal cells initiating the diffusion through the epithelial cells (invasive phase; Durant et al., 1999). The vegetable fats protecting sodium butyrate in the partially protected butyrate additive provide better resistance to the acidic pH and allow part of the butyrate to be released further down the intestine (Van Immerseel et al., 2004). Remarkably, the percentage of birds shedding *Salmonella* during the early phase of the infection (d 13 posthatching) was significantly lower ($P < 0.05$) in T1, supplemented with the nonprotected Gustor XXI B92, than in T2, with Gustor XXI BP70 partially protected additive. These trends inverted during the late phase (41 d posthatching), in which a scarce 6% of birds from the T2 Gustor XXI BP70 group shed *Salmonella* in feces vs. 60% of birds in T1 Gustor XXI BP92 ($P < 0.05$). Despite the high prevention of fecal shedding of *Salmonella* obtained with free sodium butyrate, a significant percentage of birds are still carrying the bacteria in significant amounts at the slaughter age. This fact suggests that a partially protected butyric acid-based additive like Gustor XXI BP70 would be a very useful tool to control *Salmonella* Enteriditis contamination, although it does not completely clear chickens from *Salmonella*.

Once the intracellular localization is achieved, *Salmonella* starts an extensive proliferation. Healthy birds recruit macrophages and lymphocytes B and T 24 h after infection. Macrophages engulf bacteria within the intestinal wall, which is the beginning of the systemic phase (Ricke, 2003b). *Salmonella* is an intracellular parasite that survives and multiplies inside the parasitophorous vacuoles of macrophages of internal organs such as the liver and spleen (systemic phase). This internal localization allows *Salmonella* to evade the host immune response (Beal and Smith, 2007). Overall, there was a significant lower contamination of the GIT organs: ceca and crops of the birds treated with both partially protected and unprotected butyrate additives when compared with the control group. However, this effect was not seen in liver and spleen in the case of birds fed T1 Gustor XXI B92. Interestingly, Gustor XXI BP70 prevented liver colonization reducing the bacteriological burden with regards to control and Gustor XXI B92-treated groups.

Dissociated internal butyrate can affect *Salmonella* virulence in a variety of manners. In vitro cell invasion of epithelial cells can be suppressed when butyrate and propionate, but not acetate, are added to the culture media. Lawhon et al. (2002) explained this effect on the basis of changes in *Salmonella* pathogenicity island (**SPI-1**) expression. It has been found that SPI-1 contains *Salmonella* virulence genes arranged in operons required to invade epithelial host cells during early stages of infection. These genes are transcriptionally regulated by the HilA protein, encoded by a gene of the SPI-1 pathogenic island (Durant et al., 2000). These authors noted that butyrate led to a decrease in HilA and some of the genes under its control. These results are supported by DNA microarrays of both *Salmonella* Typhimurium and *Salmonella* Enteritidis cultures grown at low doses of butyric acid that downregulated SPI-1, although it did not alter metabolic gene expression (Gantois et al., 2006).

In conclusion, the additive formulated with partially protected sodium butyrate (Gustor XXI BP70) was shown to be the most effective to decrease the fecal shedding of *Salmonella* in *Salmonella* Enteriditis-infected broilers. The vegetable fat protection allows sodium butyrate to have an effect all along the GIT tract because it is slowly released during digestion. It

has, therefore, a positive effect on bird health by preventing *Salmonella* colonization at the intestinal and systemic phases.

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