Microencapsulated sorbic acid and nature-identical compounds reduced *Salmonella* Hadar and *Salmonella* Enteritidis colonization in experimentally infected chickens[1]

E. Grilli,*[2] B. Tugnoli,* A. Formigoni,* P. Massi,† P. Fantinati,‡ G. Tosi,† and A. Piva*

*Dipartimento Scienze Mediche Veterinarie, University of Bologna, 40064 Ozzano Emilia, Provincia di Bologna, Italy; †Iszler, 47122 Forlì, Provincia di Forlì-Cesena, Italy; and ‡Vetagro SpA, 42124 Reggio Emilia, Provincia di Reggio Emilia, Italy

ABSTRACT The reduction of *Salmonella* prevalence in broilers is a priority in European Union agricultural policies because treatment with antibiotics is forbidden by Regulation (EC) 2160/2003. Two trials were conducted to evaluate the efficacy of a microencapsulated blend of sorbic acid and nature-identical compounds (i.e., chemically synthesized botanicals; SAB) on the reduction of the cecal prevalence and contents of *Salmonella enterica* serovars Hadar and Enteritidis in experimentally infected chickens. In the first trial, 125 one-day-old Lohmann specific-pathogen-free chickens were assigned to one of the following treatments: negative control (not challenged and not treated), positive control (challenged and not treated), SAB0.3, SAB1, or SAB5 (challenged and treated with the microencapsulated blend included in the feed at 0.03, 0.1, or 0.5%, respectively). At 30 d of age, birds were infected with 10^6 cfu of *Salmonella* Hadar, and after 5, 10, or 20 d postinfection, 5, 10, and 10 birds per treatment, respectively, were killed and the cecal contents and liver and spleen samples were analyzed for *Salmonella* Hadar. In the second trial, 100 one-day-old Ross 708 chickens were assigned to 1 of 5 treatments: control (not treated), SAB0.3, SAB1, SAB2, or SAB5 (treated with the blend included in the feed at 0.03, 0.1, 0.2, or 0.5%, respectively). At 7 d of age, the birds were challenged with 10^5 cfu of *Salmonella* Enteritidis, and after 7, 14, or 24 d after challenge, 5, 5, and 10 birds per treatment, respectively, were killed and cecal contents were analyzed for *Salmonella* Enteritidis. Results showed that in the early stage of infection *Salmonella* prevalence was high in both studies, whereas at the end of the observation periods, the blends at 0.03, 0.1, and 0.5 in the challenge with *Salmonella* Hadar and at 0.2 and 0.5% in the challenge with *Salmonella* Enteritidis significantly reduced (by 2 $\log_{10}$ cfu) the cecal content of *Salmonella*. This study showed that intestinal delivery of microencapsulated sorbic acid and nature-identical compounds can result in a 100-fold reduction of *Salmonella* at the intestinal level in broilers at slaughter age.

Key words: broiler, microencapsulation, nature-identical compound, *Salmonella*, sorbic acid

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INTRODUCTION

In Europe, *Salmonella enterica* serovars Enteritidis and Hadar are 2 of the 5 most frequently isolated serotypes in human salmonellosis, along with *Salmonella enterica* serovars Infantis, Typhimurium, and Virchow (European Commission, 2003). The reduction of their prevalence in food animals, and in particular in breeding flocks of *Gallus gallus*, laying hens, and broilers is regulated by European Union Regulation 2160/2003 (European Parliament and European Council, 2003) and further implemented by European Union Regulation 1003/2005 (European Parliament and European Council, 2005). The "Community Summary Report on Trends and Sources of Zoonosis and Zoonotic Agents and Foodborne Outbreaks in the European Union" (European Food Safety Authority, 2010) reported *Salmonella* as the most frequent cause of foodborne outbreaks in 2008, with poultry meat being the most implicated food, followed by meat from swine.

The numbers indicate a slight decrease in people affected in *Salmonella* outbreaks when compared with 2007 (131,468 cases in 2008 vs. 151,998 in 2007), and this trend, confirmed over the last 4 yr, could be linked to the application of national monitoring programs within each country, as established by Regula-

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[2]Corresponding author: ester.grilli@unibo.it

tion 2160/2003 (European Parliament and European Council, 2003). Although important results have been obtained over the years regarding laying hen production, because European standards have been achieved in many countries, no significant levels of reduction in *Salmonella* prevalence have yet been obtained for breeders and broilers.

In Italy, *Salmonella* Hadar is the first cause of broiler breeder culling, and no antibiotic treatment is allowed to prevent or treat it, whereas *Salmonella* Enteritidis is the most frequently isolated strain associated with poultry meat. To overcome the problem of *Salmonella* in animal husbandry, no single strategies can be pursued, but multiple approaches targeting each stage of production must be adopted. Because, in Europe, postslaughter treatments such as carcass washing with chlorine derivatives are not allowed, preslaughter intervention strategies are now the priority. In this context, a critical role can be played by feed additives that can help prevent or reduce the intestinal proliferation of foodborne pathogens, thereby reducing the risk of carcass contamination.

Organic acids (**OA**) have long been used as food preservatives because of their antifungal properties, and in the last 10 yr, they have also been used extensively as alternatives to antibiotic growth promoters. The efficacy of OA in the prevention and treatment of foodborne pathogens is still under investigation and is sometimes controversial (Van Immerseel et al., 2004a,b), even though some promising results have been achieved (Van Immerseel et al., 2004a,b, 2005; Jarquin et al., 2007).

Botanicals and their chemically derived counterparts, nature-identical compounds (**NIC**), have proven antimicrobial properties against a variety of microorganisms, including enteric ones (Si et al., 2006). One of the limits associated with the efficacy of OA and NIC against foodborne pathogens is due to their low resistance to stomach pH, which can limit the availability of undissociated forms at the intestinal level, where colonization occurs. The microencapsulation of OA and NIC in a lipid matrix can help to overcome this problem (Piva et al., 2007b).

The purpose of this study was therefore to assess the efficacy of a lipid-microencapsulated blend of sorbic acid and NIC in reducing the cecal prevalence and content of *Salmonella* in chickens experimentally infected with *Salmonella* Hadar or *Salmonella* Enteritidis, as well as possibly reducing *Salmonella* Hadar translocation to the liver and spleen.

MATERIALS AND METHODS

Salmonella Cultures and Growth Conditions

Salmonella Hadar strain 313086/2008, isolated from a laying hen flock, and *Salmonella* Enteritidis strain 166153/2008, isolated from a parental line of Ross 708, were used in the experiments. The strains were grown in brain hearth infusion broth (**BHI**, Oxoid, Basingstoke, UK) through 2 subsequent 24-h incubation periods before being enumerated through plating 10-fold serial dilutions onto brilliant green agar (**BGA**, Oxoid). Bacterial dilutions were prepared in buffered peptone saline solution.

Feed Additive

An experimental sorbic acid-based blend (**SAB**; Vetagro SpA, Reggio Emilia, Italy) containing 25% sorbic acid, 6% thymol, and 6% carvacrol was added to the feed (Piva and Tedeschi, 2004). The blend ingredients were microencapsulated through a spray-chilling method with a lipid-embedding matrix to allow their slow release into the gastrointestinal tracts of the birds (Piva et al., 2007b).

Antimicrobial Assay

The ingredients in the SAB were dissolved in BHI broth with 10% of ethanol. This stock solution was buffered to pH 6.5, filter-sterilized (0.22-μm-diameter pores; Millipore, Billerica, MA), and then diluted in fresh sterile BHI to reach final concentrations of 0.1, 0.05, 0.03, and 0.02%. The *Salmonella* strains (313086/2008 and 166153/2008) were incubated with the solutions at a concentration of 10^6 cfu/mL at 37°C. After an overnight incubation, the optical density of the cultures was measured through their absorbance at 600 nm (UltroSpec 3000, Pharmacia Biotech, Biochrom Ltd., Cambridge, UK). The experiment was made in triplicate for each strain. The minimal inhibitory concentration was defined as the lowest concentration of the substance that did not increase optical density.

Chicken Growth Conditions

All chickens used in both trials were housed in isolation units (0.9 m^2) equipped with bedding straw, drinkers, heating lamps, and a filtered air supply. The L:D program was 23:1 during the first days and was then gradually decreased to 16:8 within 1 wk.

Birds were fed a mash diet that was previously tested for the presence of *Salmonella*. Moreover, before the beginning of each experiment, 20 chickens were killed to check serological-specific anti-*Salmonella* Enteritidis antibodies through a competitive ELISA (IDDEX SE Ab test, IDDEX Laoratories Italia, Milan, Italy).

Both studies were conducted at the Istituto Zooprofilattico di Forlì (Forlì, Italy) in accordance with published guidelines for animal welfare and protection (European Council, 1986).

Experimental Setup

Trial 1: Challenge with Salmonella Hadar. A total of 125 one-day-old female Lohmann specific-pathogen-free chickens were divided in 5 isolation units, with each unit assigned to 1 of the following 5 dietary treatments:

negative control (not challenged), positive control (challenged and not treated with the blend), SAB0.3, SAB1, or SAB5 (challenged and treated with 0.03, 0.1, or 0.5% of SAB, respectively). Birds received SAB from the beginning of the study (d 0) via the feed, and the feed and water supply were allowed ad libitum. At 30 d of age (d 30), birds were challenged via endoesophageal inoculation with 1 mL of saline solution containing 10^6 cfu of *Salmonella* Hadar. The negative control group was challenged with sterile saline water only. After the challenge, birds were monitored for clinical signs of salmonellosis and potential mortality, and at d 32, cloacal swabs were randomly collected within each unit to assess the presence of *Salmonella*. At 5 and 10 d postchallenge (d 35 and 40, respectively), 5 and 10 birds per unit were killed, and cecal contents were collected to perform *Salmonella* counts. The remaining 10 birds per unit were killed after 20 d postchallenge (d 50), and samples from the spleen and liver as well as from cecal contents underwent *Salmonella* analysis.

Trial 2: Challenge with Salmonella Enteritidis. A total of 100 one-day-old female Ross 708 broilers were obtained from a local hatchery and randomly allocated into 5 isolation units. Each isolation unit was assigned to 1 of the following 5 dietary treatments: control (challenged and not treated), SAB0.3, SAB1, SAB2, or SAB5 (birds challenged and treated with 0.03, 0.1, 0.2, or 0.5% of SAB, respectively). The blend was added from the beginning of the study and was mixed with mash feed provided ad libitum.

At 7 d of age, birds were orally challenged with 10^5 cfu of *Salmonella* Enteritidis, and at 2 d after the challenge, cloacal swabs were collected randomly within each group to check for *Salmonella*. At 7, 14, and 24 d postchallenge, 5, 5, and 10 birds per treatment, respectively, were killed and cecal contents were analyzed for the presence and counts of *Salmonella*.

Salmonella Analysis

Samples collected from both experiments were assessed for the presence of *Salmonella* following an amended ISO procedure (ISO, 2002). Briefly, cloacal swabs, 5 g of cecal contents, or 5 g of homogenized liver and spleen pooled together were preenriched in buffered peptone water (Oxoid) overnight at 37°C; thereafter, samples were enriched by transferring them into modified Rappaport Vassiliadis broth (Oxoid) and incubated overnight at 41°C. After incubation, a drop of the new suspension was striped onto xylose-lysine-desoxycholate agar and BGA (Oxoid). Direct counts of *Salmonella* were performed by serially diluting 1-g samples in saline solution and plating the solution onto BGA. After an overnight incubation at 37°C, the number of colonies was counted.

The mean $\log_{10}$ colony-forming units per gram of cecal content was analyzed with a nonparametric Kruskall-Wallis test, followed by Dunn's post hoc test to compare means among treatments (GraphPad Prism 4.0, GraphPad Software Inc., Palo Alto, CA).

RESULTS

Antimicrobial Assay

The lowest concentration of SAB that was tested in vitro (0.02%) completely prevented the increase in optical density of both *Salmonella* Hadar and *Salmonella* Enteritidis and was therefore defined as the minimal inhibitory concentration.

Trial 1: Challenge with Salmonella Hadar

The negative control (not challenged) remained negative throughout the study, and it was excluded from the statistical analyses. Challenged birds never demonstrated signs of illness or suffering despite the massive dose of infection (6 $\log_{10}$ cfu). At 2 d after the infection, cloacal swabs were 100% positive for *Salmonella.* At 5 d after the infection, 5 birds per group were killed and cecal contents were still 100% positive for *Salmonella*. The quantitative assessment revealed that birds fed SAB1 had numerically the lowest content of *Salmonella*, whereas those fed SAB0.3 and SAB5 had the highest. Nevertheless, none of the group differed significantly from the control (Table 1).

At 10 d after infection, 10 birds per group were killed, and 100% of cecal samples were positive for *Salmonel-*

Table 1. *Salmonella* cecal content (cfu/g) at 5 d postchallenge in chickens infected with 10^6 cfu of *Salmonella* Hadar and fed an experimental microencapsulated blend of sorbic acid and nature-identical compounds[1]

Salmonella content	PC[ab] (n = 5)	SAB0.3[a] (n = 5)	SAB1[b] (n = 5)	SAB5[ab] (n = 5)
Negative	0	0	0	0
$x < 10^2$	2	0	5	2
$10^2 < x < 10^3$	3	3	0	1
$10^3 < x < 10^4$	0	2	0	1
$10^4 < x < 10^5$	0	0	0	1

[a,b]Groups without a common superscript are significantly different ($P < 0.05$).

[1]Blend provided by Vetagro SpA (Reggio Emilia, Italy). PC = positive control, chickens challenged and not treated; SAB0.3 = chickens challenged and treated with 0.03% of the blend; SAB1 = chickens challenged and treated with 0.1% of the blend; SAB5 = chickens challenged and treated with 0.5% of the blend.

Table 2. *Salmonella* cecal content (cfu/g) at 10 d postchallenge in chickens infected with 10^6 cfu of *Salmonella* Hadar and fed an experimental microencapsulated blend of sorbic acid and nature-identical compounds[1]

Salmonella content	PC (n = 10)	SAB0.3 (n = 10)	SAB1 (n = 10)	SAB5 (n = 10)
Negative	0	0	0	0
$x < 10^2$	0	1	1	0
$10^2 < x < 10^3$	0	3	3	0
$10^3 < x < 10^4$	9	4	3	9
$10^4 < x < 10^5$	1	2	3	1

[1]Blend provided by Vetagro SpA (Reggio Emilia, Italy). PC = positive control, chickens challenged and not treated; SAB0.3 = chickens challenged and treated with 0.03% of the blend; SAB1 = chickens challenged and treated with 0.1% of the blend; SAB5 = chickens challenged and treated with 0.5% of the blend.

la. No differences in *Salmonella* counts were detected (Table 2).

At the end of the experiment, the remaining 10 subjects per group were killed; cecal contents and spleen and liver samples were checked for both the presence and counts of *Salmonella*. Four out of 10 spleen and liver samples in the control group were positive for *Salmonella*, whereas spleen and liver samples in the SAB0.3, SAB1, and SAB5 groups resulted 7/10, 8/10, and 5/10 positive, respectively; the number of colony-forming units per gram was lower than the detection limit (2 $\log_{10}$ cfu/g) in each sample. Cecal contents from control birds resulted in 10/10 samples positive for *Salmonella*, and those from the SAB0.3, SAB1, and SAB5 groups resulted in, respectively, 9, 8, and 9 samples positive for *Salmonella*. *Salmonella* counts were lower for all of the treated groups compared with the control group ($P < 0.001$; Table 3).

Trial 2: Challenge with Salmonella Enteritidis

At 2 d after the challenge, all the swabs were positive for *Salmonella*. After 7 d, the total number of cecal samples was positive for *Salmonella* and the concentrations were established as 7 $\log_{10}$ cfu/g for the control group and 5, 7, 5.2, and 5.2 $\log_{10}$ cfu/g for the SAB0.3, SAB1, SAB2, and SAB5 groups, respectively. The Kruskal-Wallis test detected a difference among treatments ($P = 0.03$), and the SAB2 group in particular was different from all the other groups except the SAB0.3 group ($P < 0.05$; Figure 1). At 14 d postinfection, all the birds were positive for *Salmonella*, and cecal counts were 4.5, 4.4, 5.0, 4.2, and 4.2 $\log_{10}$ cfu/g for the control, SAB0.3, SAB1, SAB2, and SAB5 groups, respectively. *Salmonella* in the SAB1 group was higher than those in the SAB2 and SAB5 groups ($P < 0.05$; Figure 1). At the end of the experiment, 100% of the birds belonging to the control, SAB0.3, and SAB1 groups were positive for *Salmonella*, whereas only 7/10 birds for both the SAB2 and SAB5 groups were positive. Furthermore, *Salmonella* counts were decreased by 2 $\log_{10}$ cfu in both the SAB2 and SAB5 groups compared with the control group, whereas the SAB1 group showed a numerical reduction of 0.5 $\log_{10}$ cfu without reaching significant *P*-values (5.2, 2.9, and 3.1 for the control, SAB2, and SAB5 groups, respectively; $P < 0.01$; Figure 1).

DISCUSSION

Organic acids have long been used as antimicrobials in food preservation and as feed additives. Many studies have clearly demonstrated the efficacy of OA in improving the feed efficiency and growth of food animals (Partanen and Mroz, 1999; Mroz, 2003), as well as their efficacy in preventing and ameliorating *Salmonella* colonization (Van Immerseel et al., 2006; Dunkley et al., 2009).

The mechanism by which OA should exploit their antibacterial action is explained well by the anion model (Russell and Diez-Gonzalez, 1998; Van Immerseel et al., 2006), even though it is clear that different acids

Table 3. *Salmonella* cecal content (cfu/g) at 20 d postchallenge in chickens infected with 10^6 cfu of *Salmonella* Hadar and fed an experimental microencapsulated blend of sorbic acid and nature-identical compounds[1]

Salmonella content	PC[a] (n = 10)	SAB0.3[b] (n = 10)	SAB1[b] (n = 10)	SAB5[b] (n = 10)
Negative	0	1	2	1
$x < 10^2$	0	9	6	9
$10^2 < x < 10^3$	4	0	0	0
$10^3 < x < 10^4$	6	0	2	0

[a,b]Groups without a common superscript are significantly different ($P < 0.05$).

[1]Blend provided by Vetagro SpA (Reggio Emilia, Italy). PC = positive control, chickens challenged and not treated; SAB0.3 = chickens challenged and treated with 0.03% of the blend; SAB1 = chickens challenged and treated with 0.1% of the blend; SAB5 = chickens challenged and treated with 0.5% of the blend.

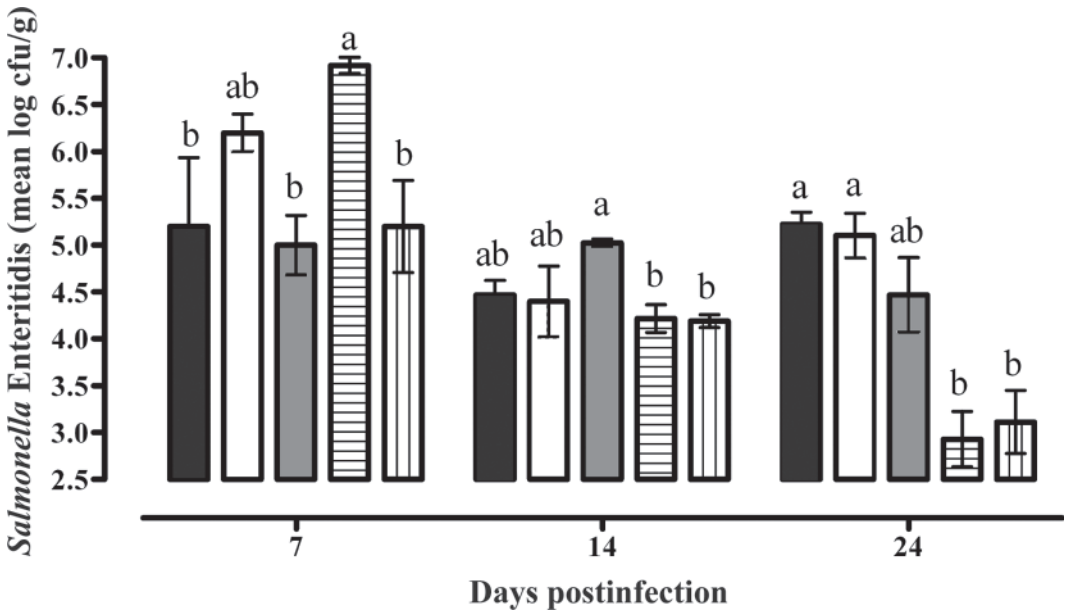

Figure 1. Mean $\log_{10}$ cfu of *Salmonella* Enteritidis per gram of cecal content at each time point after infection. Black bars represent the control (chickens challenged and not treated); white bars represent SAB0.3 [chickens challenged and treated with 0.03% of an in-feed sorbic acid-based blend (SAB; Vetagro SpA, Reggio Emilia, Italy)]; gray bars represent SAB1 (chickens challenged and treated with 0.1% of the in-feed SAB); horizontally striped bars represent SAB2 (chickens challenged and treated with 0.2% of the in-feed SAB); and vertically striped bars represent SAB5 (chickens challenged and treated with 0.5% of the in-feed SAB). For each time point, values with different letters (a, b) are significantly different ($P < 0.05$).

may exert different antibacterial actions, depending on their molecular weight, pK_a (i.e., acid dissociation constant measured logarithmically), and number and types of side chains.

Van Immerseel et al. (2004a,b, 2005) investigated the role of different OA in preventing *Salmonella* proliferation in vitro and in vivo. Among the different acids, medium-chain fatty acids revealed better in vitro anti-*Salmonella* properties than did other short-chain fatty acids, such as formic, acetic, propionic, and butyric acids, but evidence provided by large-scale studies is still lacking (Van Immerseel et al., 2004a). On the other hand, in vivo experiments showed that coated butyric acid, but not the powder form, was able to reduce cecal *Salmonella* colonization in specific-pathogen-free experimentally infected chickens. Nowadays, butyric acid is considered one of the most promising additives for *Salmonella*, and its efficacy is primarily due to a down-regulation of genes responsible of *Salmonella* invasiveness (the SPI-1 and *hilA*) rather than to a mere anion model-linked antimicrobial effect (Van Immerseel et al., 2005; Fernández-Rubio et al., 2009).

Sorbic acid is a straight-chain unsaturated fatty acid that was first isolated in 1859 from the oil of berries of the *Sorbus aucuparia* tree, and since the late 1940s, it has been used as an antifungal agent. Later, its antibacterial properties were also discovered, and since then, it has been used for a variety of industrial applications in food preservation because, compared with other acids such as benzoic or propionic acid, it is much more effective at a less acidic pH because of its relatively high pK_a value (4.76). In vitro studies demonstrated that sorbic acid at 0.025 to 0.1% was effective in inhibiting the growth of *Staphylococcus aureus* S-6 and *Salmonella* Enteritidis 13076 at pH 5.2 in trypticase soy broth, whereas 0.08% was enough to inhibit the growth of coliforms, yeasts, and molds in cottage cheese, and 0.3% delayed the outgrowth and gas formation by a *Clostridium* strain (Robach et al., 1981).

Our findings revealed that the minimal inhibitory concentration of the SAB against both *Salmonella* Hadar and *Salmonella* Enteritidis was 0.02%, which is a lower value than those found in the literature. The greater efficacy could be due to its synergy with the NIC included in the blend. In fact, this "multihurdle" technology, which consists of combining OA and NIC to counteract pathogen development, has been tested successfully in vitro against *Salmonella* Typhimurium (Nazer et al., 2005). On the other hand, little has been published on the combined use of OA and NIC in vivo (Piva et al., 2007a,b; Grilli et al., 2010), and specific strategies in the preslaughter phase of the food-supply chain have yet to be implemented.

The mechanism by which OA and NIC can be effective against *Salmonella* can be explained by 2 hypotheses. The first one is based on the antimicrobial properties of both OA and NIC and the synergy developing by their combined use. The proposed mechanism of action of this synergy is that the altered membrane permeability attributable to the action of NIC (Ultee et al., 2002) would eventually facilitate the passage of acids through the membrane. The second hypothesis relies on the antioxidant and anti-inflammatory properties of plant-derived compounds. In fact, it has recently been reported (Winter et al., 2010) that *Salmonella* uses inflammation products as a mean for outgrowth and to

outcompete other microbes in the intestine. Moreover, the reactive oxygen species that are generated during intestinal inflammation caused by *Salmonella* react with the thiosulfate present at the intestinal level and form tetrathionate, which acts like an electron acceptor to allow *Salmonella* to "breathe" in an anaerobic environment. Essential oils and their pure-derived NIC, such as carvacrol and its isomer thymol, have both antioxidant and anti-inflammatory properties (Alma et al., 2003; Landa et al., 2009) and, as such, can suppress inflammation at the intestinal level and indirectly inhibit the growth of *Salmonella*. Along with this, a direct antimicrobial effect is exerted by both NIC and sorbic acid. An inhibition of bacterial growth by sorbic acid or its salts could be exerted through different mechanisms, such as the alteration of cell membranes, the inhibition of transport systems and key enzymes, the creation of a proton flux into the cell, or a combination of these (Sofos et al., 1986), even though the one most accounted for is the inhibition of enzymes involved in the metabolism of carbohydrates (enolase and lactic dehydrogenase) in the trichloroacetic acid cycle or enzymes containing –SH groups, and catalases or peroxidases (Sofos et al., 1986).

Furthermore, the benefits of using sorbic acid as a gut microflora stabilizer were shown by Pirgozliev et al. (2007), who measured the efficacy of sorbic acid in decreasing intestinal coliforms and lactic acid bacteria and in reducing the concentration of sialic acid in the excreta. An increased sialic acid concentration is generally associated with health problems, bacterial infections, and gastrointestinal mucins, with sialic acid being one of the most abundant components of mucins. The lower the proportion of sialic acid excreted with feces, the lower the mucin production and intestinal turnover would be, thereby indicating a better intestinal homeostasis. Mucins confer protection against invasion from bacteria such as *Salmonella*, even though the role of mucins in *Salmonella* pathogenesis is still controversial (Vimal et al., 2000; Carson and Vazquez-Torres, 2007).

In this study, we did not measure the inflammatory response or the levels of intestinal mucins, but we did observe a general reduction in *Salmonella* at the intestinal level of challenged birds regardless of the strain used (Hadar vs. Enteritidis) or the breeding of the birds (specific pathogen free vs. commercial). The extent of the reduction in *Salmonella* was consistent in both experiments (2 $\log_{10}$ cfu), but the dose of the additive necessary to allow such a reduction was different between the 2 trials. In the first one, *Salmonella* Hadar was significantly reduced at SAB inclusion levels ranging from 0.03 to 0.5%, whereas in the trial with *Salmonella* Enteritidis, there was a marked reduction at inclusion levels of 0.2 and 0.5%. The reason for this discrepancy could be because different serovars may have different sensitivities. Even if in vitro conditions are not 100% predictive of what happens in vivo, this hypothesis is not supported by our in vitro results because both serovars were sensitive to the same concentration of SAB (0.02%). Another hypothesis is that *Salmonella* Hadar colonization in the first challenge was established to a lesser extent than colonization in the second one with *Salmonella* Enteritidis (2 vs. 5 $\log_{10}$ cfu/g of cecal contents in control birds). In fact, in the first trial, the colonization peak was reached after 10 d postinfection at 3.2 $\log_{10}$ cfu/g, whereas in the second study, it was at its maximum level at 7 d, with values of 7 $\log_{10}$ cfu/g; therefore, the concentration of the blend needed to effectively reduce *Salmonella* Enteritidis counts could have been higher. In support of this view, it must be considered that in the trial with *Salmonella* Enteritidis, there was no relevant reduction of cecal colonization over time in birds belonging to the control group, whereas the reduction was marked in birds fed the higher doses of the blend (Figure 1).

The common outcome of the 2 trials was that no effect was observed during the first days postinfection because of the inclusion of the blend, whereas a pronounced reduction in *Salmonella* was established in the medium-term period (within 20 to 24 d). The inclusion of the blend did not prevent bacterial translocation to the spleen and liver, nor did it allow a complete intestinal clearing of *Salmonella*, even though, at the end of the second trial, 30% of the treated birds reverted to negative for *Salmonella* Enteritidis. These results are in accordance with those observed by Van Immerseel et al. (2005) and demonstrate that even though a significant reduction in the cecal *Salmonella* count was achieved, the fecal shedding at low concentrations was unavoidable. Nevertheless, a 2 $\log_{10}$ cfu reduction in intestinal colonization could eventually allow less chance of carcass contamination at the slaughterhouse and, consequently, less risk of foodborne illness.

In conclusion, to our knowledge, this is the first study in which an in-feed microencapsulated blend of both OA and NIC was investigated in a *Salmonella* challenge, and the results obtained are promising. Further investigations will help clarify the mechanism of action of those ingredients and will focus on in-field validation of these preliminary results.

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