Chemical treatment of animal feed and water for the control of *Salmonella*

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Abstract

The control of *Salmonella* in animal feedstuffs is important, principally to protect the human food chain from contamination by *Salmonella* derived from infected animals. The transmission of *Salmonella* from animal feeds to animals, and onwards to human food products, has been convincingly documented. This is especially important for chicken breeding and laying flocks and pigs, in view of the consequences of recent or imminent control legislation in the European Union. Animal feed ingredients, particularly animal and plant-derived protein meals, are frequently contaminated with *Salmonella* either from source or from processing plant, and recontamination in compounding mills is an additional problem. Several complementary strategies have been used to control this feed contamination, and these include a range of chemical treatments. The principal agents used are: organic acids and their salts, formaldehyde, and bacterial membrane disruptors such as terpenes and essential oils. Experimental agents include chlorate compounds. Many products use blends of agents from the same or different chemical groups to achieve synergistic or combination effects. The present review draws upon published and company data to describe the various modes of action and efficacies of different chemical agents delivered in feed or in drinking water against *Salmonella* occurring in feed or in livestock environments. Reasons for the failure of protection are explored, along with problems in usage such as corrosion and reduced palatability. Given the wide array of products available with contrasting modes of action, the need for standardised tests of efficacy is also discussed.

Keywords: Animal feed, Salmonella, organic acid, formaldehyde, chlorate, terpene, essential oil

Introduction

Human non-typhoidal salmonellosis remains a significant issue in public health. In 2007 there were over 155,000 reported cases in the European Union (EU) and associated countries (European Food Safety Authority, 2009), and several times this number are probably unreported (Mead *et al.*, 1999; Wheeler *et al.*, 1999; European Food Safety Authority, 2006b). Food is the major route of transmission of non-typhoidal salmonellas to humans (Mead *et al.*, 1999; Crump *et al.*, 2002), and animal food products (especially poultry, pig and bovine meat, eggs and dairy) are the vehicles primarily implicated (Enternet surveillance hub, 2006; European Food Safety Authority, 2009). Confirmed foodborne outbreaks of human salmonellosis in the EU show a heavy predominance of serovars Enteritidis and Typhimurium.

Ingredients for animal feedstuffs are commonly contaminated with *Salmonella* (Hacking *et al.*, 1978; Kidd *et al.*, 2002; Jones and Richardson, 2004; Dargatz *et al.*, 2005; European Food Safety Authority, 2006a; Veterinary Laboratories Agency, 2006). Cereal and vegetable ingredients may come in contact with wildlife excreta and agricultural effluents during growth, harvesting, storage and transport (Bains and MacKenzie, 1974; Bauer and Hormansdorfer, 1996; Beuchat and Ryu, 1997). Animal by-products (fishmeal and, where permitted, feather meal and meat and bone meal) may be heavily contaminated from source (Nesse *et al.*, 2003), and processing of this material into its final form often provides opportunities for *Salmonella* to survive and recontaminate the product (Gabis, 1991). Vegetable protein sources are commonly derived from processed oilseeds (e.g. soya and rape), and these meals are particularly prone to contamination by salmonellas that are endemic in the processing plants (Morita *et al.*, 2003; European Food Safety Authority, 2006a).

Salmonella is commonly also found in compounded feeds, including those that have undergone heat treatment (Hacking *et al.*, 1978; Cox *et al.*, 1983; Veldman *et al.*, 1995). Recent national data from EU countries (European Food Safety Authority, 2006a) shows that in most countries 0% – 1.5% of compounded poultry feed samples are *Salmonella*-positive, although higher frequencies are seen in some countries.. Similar contamination rates are reported for pig and cattle feeds.

When most probable number estimates of *Salmonella* levels are carried out on finished feeds they are usually very low (Taylor and McCoy, 1969) but it is not certain whether it is individual organisms which are being counted or microcolonies attached to small feed particles. Microcolonies will manifest as single colony-forming units in quantitative culture, yet will present several times this number of viable *Salmonella* cells to the host upon ingestion, equivalent to an experimental broth culture dose with a higher nominal count. Salmonellas present in feed may also be protected by fatty material and cause infection with very low numbers (Jones *et al.*, 1982). The infective dose is lower for animals under stress (such as poultry at the onset of lay), those suffering intercurrent disease, and very young animals where the infective dose can be below 1 cfu/g (Schleifer *et al.*, 1984; Hinton, 1988). *Salmonella* present in low numbers in feed may multiply in warm, moist conditions such as feed bins and ad-lib feed hoppers.

Studies have shown strong links between *Salmonella* contamination of feedstuffs or feed mills and infections with the same serovars of groups in chickens, turkeys, pigs and cattle (Newell *et al.*, 1959; Boyer *et al.*, 1962; Glickman *et al.*, 1981; Jones *et al.*, 1991; Primm, 1998;

Davies *et al.*, 2001; Davis *et al.*, 2003; Nayak *et al.*, 2003; Österberg *et al.*, 2006)..When a serovar or strain that is well-adapted for persistence is introduced via feed, its establishment in breeder and then production flocks can be rapid and widespread (Shapcott, 1985).

It has been amply demonstrated that *Salmonella* strains, including *S.* Typhimurium, from broiler feed sources can correlate with those found in birds and on derived broiler meat (Pennington *et al.*, 1968; Semple *et al.*, 1968; MacKenzie and Bains, 1976; Shapcott, 1985; Humphrey and Lanning, 1988; Davies *et al.*, 2001; Corry *et al.*, 2002; Bucher *et al.*, 2007). Similar evidence exists for turkey feed serovars in birds and subsequently in slaughter and processing facilities (Bryan *et al.*, 1968). *Salmonella* contamination from animal feed may also pass more directly into the human food chain via eggs or milk (Knox *et al.*, 1963) or more indirectly through breeding flocks (Jones *et al.*, 1991).

The need for controls on sources of *Salmonella* in farmed animals has been heightened by recent legislation in the European Union, where the European Food Safety Authority is in the process of defining and adopting verifiable and enforceable targets for the herd or flock-level prevalence of *Salmonella* among important farmed species. Regulation (EC) No. 1003/2005 requires that five serovars of public health importance are present in no more than 1% of breeding hen flocks of over 250 birds by the end of 2009. Regulation (EC) No. 1168/2006 stipulates annual minimum percentage reductions in the prevalence of laying flocks infected with either *S.* Enteritidis or *S.* Typhimurium for each member state. Work to establish regulations concerning *Salmonella* in broilers, turkeys and pigs is in progress, with a target level for contamination in pig production soon to be set that is likely to have a significant impact on preventive actions at farm level.

The principal approaches to reducing and eliminating *Salmonella* in animal feedstuffs centre upon monitoring and control of contamination in ingredients, process control and monitoring, often using the Hazard Analysis/Critical Control Point (HACCP) model, thermal treatments during feed manufacture, and chemical treatments applied at one or more stages of manufacture and storage. These approaches are, to varying extents, complementary, and all have their associated costs and technical weaknesses. For these reasons, manufacturers and users of animal feeds will typically employ a range of tactics, including chemical treatments, in attempts to suppress, eliminate or prevent *Salmonella* contamination. The principal chemical agents used are organic acids and formaldehyde, although blended products may additionally employ surfactants, bacterial membrane-disrupting compounds and other elements.

Chemical treatment of feed may exert its effect before it is consumed, and/or upon ingestion when the feed is moistened by the animal's alimentary secretions and encounters the pH conditions and endogenous acids in the crop, rumen, stomach and intestines, according to species (Cherrington *et al.*, 1991). The antimicrobial effects may be rapid or slow and progressive, and a particular advantage of chemical treatments is that the antimicrobial capability may persist during storage, thereby helping to protect the feed against recontamination. However, persistence of the chemical can also be a disadvantage if it interferes with microbiological testing and the detection of any residual organisms that are still viable, a phenomenon referred to as 'masking'. Some treatments may principally be aimed at suppressing existing endemic *Salmonella* infections, often using agents that are mainly or entirely active *in vivo*, delivered via feed or drinking water. This is becoming a more active field with the recent restrictions and bans on antibiotic growth promoters in many countries.

The present review summarises and discusses the literature on chemical treatments for *Salmonella* as applied to feed and water. These treatments vary in composition, application and aims, and their efficacy is subject to many factors encountered in feed, feedmills and on farms. The review aims to present and explain the essentials of usage, effects and limitations of these agents, in a field where information has tended to be fragmented and incomplete and where, in consequence, costly treatments can fail to perform as anticipated.

Organic acids and derivatives

Suitability for use in animal feedstuffs

Organic acids and their salts are relatively stable in feed and some of them occur naturally in living organisms, especially in the alimentary tract. They are selected for use in animal feeds because they are generally metabolised by recipient animals, or if stabilised they may pass through unabsorbed, and therefore leave no residues in foods of animal origin. Individual acids vary in their effect on *Salmonella* but, in general, medium-chain fatty acids are more effective than short-chain fatty acids (van Immerseel *et al.*, 2002). Within the EU, such substances are classed as preservatives, which are defined in Regulation (EC) No. 1831/2003 as substances that protect feed against deterioration caused by micro-organisms or their metabolites.

Mode of action and relative efficacy. Organic acids are weak acids, meaning that at acid pH they are only partly dissociated into charged organic anions and protons. Their acid dissociation constants (pK_a values) correspond to the pH values at which they are 50% dissociated in aqueous solution, and these are within the range 3 to 5 for the short- and medium-chain fatty acids that are of greatest interest in the present context. In solutions with low pH values, more of the acid molecules will be undissociated and, because in this state they carry no net charge, they can pass through the lipid membranes of bacterial cells (Ricke, 2003). Once in the bacterial cytoplasm, a high proportion of the acid molecules will dissociate at the near-neutral pH found in this environment, releasing protons and organic acid anions. There is evidence that the antibacterial effect of organic acids is due primarily to their ability to disrupt cellular pH gradients and intracellular regulation of pH, so that vital metabolic processes are also disrupted (Cherrington *et al.*, 1990; van Immerseel *et al.*, 2006). In addition to the disruption of bacterial intracellular pH, direct toxic effects of organic anions in areas including membrane structure, osmolarity and macromolecule synthesis have been hypothesised (Cherrington *et al.*, 1990; Russell, 1992; Ricke, 2003; van Immerseel *et al.*, 2006). *Salmonella* may become relatively tolerant to acid pH with exposure, reducing the susceptibility of the organism to strong (mineral) acids, which penetrate the cell less well than organic acids at comparable pH values. However, acid-adapted cells may still be vulnerable to the toxic effects of organic acids (Baik *et al.*, 1996). In relation to the colonisation of livestock, acids may interfere with the expression of virulence genes, thereby reducing the ability of *Salmonella* to penetrate the intestine and survive inside macrophages, although prior exposure to acids can avoid these effects by habituation of the organisms (Kwon and Ricke, 1998; de Jonge *et al.*, 2003; Greenacre *et al.*, 2006; El-Sharoud and Niven, 2007).

The direct toxic effects of organic acid anions may be the principal reason why the overall inhibitory effects vary between acids. Determination of minimum inhibitory concentrations

(Diebold and Eidelsburger, 2006) showed that effects of acids on *S.* Typhimurium could be ranked as follows: formic > propionic > lactic. However, the influence of pH was not considered. Another study (Martin and Maris, 2005) demonstrated that formic acid was more potent than acetic, propionic, lactic or citric acids, while earlier work (Khan and Katamay, 1969), using an agar disc-diffusion assay, showed that the most effective acids against *Salmonella* were the four- and five-carbon (C4 and C5) compounds butyric and valeric acids, and acids with a carbon chain length greater than six were the least inhibitory. More recent studies on growth suppression of *S.* Enteritidis in nutrient broth at pH 6 (van Immerseel *et al.*, 2003, 2004a, 2006) have shown that C6 to C12 fatty acids have greater potency than C1 to C4 acids at equivalent molar concentrations. According to Skrivanova *et al.* (2006), who tested a range of acids at concentrations up to 0.5%, only caprylic acid (C8) was inhibitory to *Salmonella* at pH 6. Data reviewed by van Immerseel *et al.* (2006) suggest that factors such as chain-length, side-chain composition, pK_a values and hydrophobicity can all affect antimicrobial activity.

Among *E. coli*, the pattern of resistance to differing organic acids at low pH has been shown to be highly strain-dependent (Buchanan and Edelson, 1999), a finding which may apply to other *Enterobacteriaceae* and which perhaps accounts in some measure for the inconsistent rankings of chemical agents between investigators. The susceptibility of *Salmonella* to acids also shows strain variation, illustrated by Berk *et al.* (2005) for strains of *S.* Typhimurium DT104 in relation to inorganic acids. Following exposure at pH 2.5 for 2 h, the percentage survival of different strains varied from < 0.01% to > 10%, i.e. greater than 1000-fold. Strains known to be highly virulent for humans tended to be more acid-resistant. The situation is further complicated by interactions with the food matrix in which the acid is incorporated. A range of enteropathogens, including *Salmonella*, were substantially protected against low pH challenge when inoculated onto the surface of foods composed of protein (precipitated egg white) or protein plus fat (beef), and a localised pH-buffering effect was hypothesised (Waterman and Small, 1998). Hansen *et al.* (1995) showed that salmonellas in naturally-contaminated processed cottonseed matrix s were more resistant to formic or formic plus propionic acids than they were in the corresponding form of rapeseed, although the relative contributions of strain and food matrix to this phenomenon were not determined.

Efficacy is also influenced by the initial level of feed contamination and whether the target organism occurs naturally or is added artificially, the latter effect probably reflecting the degree of integration of the organism within the feed matrix and its physiological state. The presence or absence of other micro-organisms can be significant, as prior sterilisation of chemically-treated feed considerably enhanced the elimination of a subsequent inoculum of *Salmonella* (Ricke, 2005). Other factors affecting the apparent efficacy of organic acids are the conditions of pH and moisture under which effects are measured, i.e. more acidic environments enhance the antimicrobial effects of organic acids, while increasing moisture alters organic acid activity and concentrations, but also *Salmonella* growth potential. There is also the possibility of a 'masking' effect in bacteriological tests, whereby the pH value of the recovery medium is modified to a point where growth of any surviving *Salmonella* is inhibited (Carrique-Mas *et al.*, 2007). Results can also be affected by the time between application of the treatment preparation and measurement of its effects, and whether the measured outcome involves ingestion of the treated feed.

All the above factors contribute to variations in observed outcomes between (and sometimes within) studies, but in many cases certain potentially-relevant factors have been either not

measured or not reported. The methodology and media employed for isolating and enumerating *Salmonella* will also contribute to inter-study variations in observed efficacy.

For ease of application and safe handling of treated feed, organic acids may be used in the form of stabilised preparations, salts or appropriate mixtures of salts and straight acids. When added thus, it is likely that the organic ions will exert much of their effect only when the feed is ingested, i.e. in the dissolved state and associated with protons in a low pH environment.

The effects of organic acids on bacterial virulence and acid tolerance has led some to express concern that the use of such compounds in feed may enhance the pathogenicity to humans of surviving bacteria (de Jonge *et al.*, 2003; Fratamico, 2003; Theron and Lues, 2007; Álvarez-Ordóñez *et al.*, 2008, 2009). However, at present this remains a theoretical possibility and it is not clear to what extent exposure to added acids might increase the virulence or survival of bacteria, or select for pre-existing minority populations of resistant organisms, over and above any effect associated with endogenous acids in the alimentary tract of animals. Furthermore, to pose a threat to human consumers any increased tolerance or virulence associated with acids in animal feed or water would need to persist through slaughter, food processing, storage and cooking stages, i.e. probably representing a genetic selection rather than a phenotypic adaptive response. In a study of *Shigella*, (*Enterobacteriaceae*), highly acid-resistant survivors of a simulated gastric environment exhibited acid survival kinetics when re-cultured that were similar to that of the less acid-resistant parental strain (Gorden and Small, 1993). Another study showed that induced acid tolerance may actually reduce virulence characteristics of *Salmonella* strains (Karatzas *et al.*, 2008). Thus, at present there is little or no evidence that acid exposure in livestock feed or water might lead to a persistent enhancement of bacterial virulence.

Acid treatment of feed. The treatment of feedstuffs may be done before or after compounding into finished feed. Preparations may comprise straight acids applied via spray nozzles or adsorbed onto inert powder carriers, or powdered blends of acids and acid salts. As already discussed, organic acids may achieve the goal of *Salmonella* reductions in one or both of two modes: toxicity *ex vivo* associated with relatively high concentrations in feed (significantly depressing pH) or toxicity exerted *in vivo* after ingestion. The latter mode operates even when the pH of the treated feed remains near neutral prior to ingestion. For *in vivo* effects, the local environment created by the host and its microflora, in which chemical treatments may act, varies substantially between regions of the alimentary tract, and also between livestock species.

In studies of young and mature chickens the crop and gizzard were moderately acidic (pH generally around or below 5), the caecum was mildly acidic (pH around 5.7), and the small intestine and colon were near neutral with pH values between 6.4 and 7.6 (Hume *et al.*, 1993; Thompson and Hinton, 1997; Al-Natour and Alshawabkeh, 2005). Thus, ingested free organic acids are likely to be present in the highest luminal concentrations in the foregut (crop and gizzard), where the pH conditions are most conducive to their lethal effects. By contrast, endogenous organic acids (short-chain and lactic) are most concentrated in the fermentative environment of the hindgut, where their lethal effects are likely to be attenuated by the prevailing pH. Pigs lack a crop, therefore ingested acids pass directly to the usually highly acid environment of the stomach, where their microbicidal effect should be enhanced. Similar to chickens, the porcine small and large intestines have higher organic acid concentrations but pH values around neutral, although the caecum is mildly acidic (Högberg and Lindberg,

2004). Ruminants differ substantially from monogastric species, having a fermenting foregut with a high volatile organic acid concentration but a mildly acidic to neutral pH (Briggs *et al.*, 1957; Church, 1979; Kleen *et al.*, 2003). Similar conditions exist in the ruminant hindgut (Lewis and Dehority, 1985).

The toxicity of particular organic anions appears to be a feature in addition to pH effects, and combinations of organic acids and/or their salts can be additive or synergistic. Mixtures of organic acid salts, with or without organic acids and other elements, are commonly marketed. These have widely differing pH values and appear to have highly variable antibacterial effects, from one product to another, in dry feed. The organic acid derivative potassium diformate has been licensed as a non-antibiotic in-feed growth promoter in pig production, and it has also shown potential as an anti-*Salmonella* agent (Dennis and Blanchard, 2004; Papenbrock *et al.*, 2005).

When used in ingredients prior to compounding, acids tend to be used at relatively high concentrations to achieve significant reductions in potentially heavy contamination of ingredients. The most marked antibacterial effects before the ingestion of feed are seen when application rates are high enough to substantially depress feed pH, probably to less than 5. These inclusion rates (usually 2 – 3%) are really only suitable for ingredients, as they cause palatability problems in finished feed and corrosion of steel feeding equipment (Pinchasov and Jensen, 1989; Adams, 1991). The eventual concentrations in the compounded feed of acids added at the ingredient stage are obviously dependent on inclusion rates in the final blend, plus any losses in processing, for example during heat treatment. Thus, the principal effect aimed for is a rapid *ex vivo Salmonella* kill in the affected ingredient batch.

In an examination by Hansen *et al.* (1995) of naturally *Salmonella*-contaminated high-protein oilseed residues, the application of formic acid or formic plus propionic acids was associated with an apparent 2 – 3 $\log_{10}$ unit reduction in contamination within 24 h, but only when the feedstuff pH was below 5, i.e. at inclusion rates of $\geq 2\%$. Little additional kill effect was seen beyond 24 h. Propionic acid alone was less effective than formic acid at equivalent concentrations. A blend of formic and propionic acids applied at 1.5% w/w consistently rendered artificial contamination of 10^2 cfu/g *Salmonella* in fishmeal undetectable by sensitive culture after 24 h or more (Carrique-Mas *et al.*, 2007), although some masking was demonstrated. The same treatment was generally ineffective for the elimination of higher level contamination (10^3 or 10^4 cfu/g). Al-Natour and Alshawabkeh (2005) reported similar findings, wherein 1.5% formic acid in dry feed reduced pH to around 5 and reduced inoculated *S.* Gallinarum levels by about 1.5 $\log_{10}$ units more than in untreated controls over seven days.

Notably less effect *ex vivo* has been seen at lower inclusion levels of short-chain organic acids, or with organic acid salts or buffered mixes of acids and their salts. In artificially contaminated dry feed over seven days, de Albuquerque *et al.* (1998) reported that a formic/propionic acid mix at $\leq 0.6\%$ did not measurably reduce *Salmonella* counts, whereas Al-Natour and Alshawabkeh (2005) found a modest reduction of 0.9 $\log_{10}$ units more than controls in feed treated with 0.5% formic acid. Similarly, a formic/propionic acid mix added at 0.50 – 0.68% w/w to poultry feed contaminated with *Salmonella* (initially adsorbed onto desiccated coconut to promote dispersal within the feed) had modest effects on *Salmonella* isolation rates over three weeks (Hinton and Linton, 1988). A propionic acid/phosphoric acid/isopropyl alcohol blend at 0.2% did not reduce the level of artificial *Salmonella* contamination of chick feed over 10 days (Duncan and Adams, 1972), and Smyser and

Snoeyenbos (1979) reported that low concentrations of organic acids or their salts (≤ 0.3% w/w in dry meal, including acetic acid and propionate salts) did not measurably inhibit *Salmonella* growth in highly moisturised meat and bone meal with pH values of 5.3 - 6.6.

Buffered propionic acid (pH 6.8) was associated with only modest *Salmonella* reductions of up to 1 $\log_{10}$ unit after seven days in artificially *S.* Typhimurium-contaminated poultry mash, even with high inclusion levels of up to 10% (Ha *et al.*, 1998). Park *et al.* (2003) reported that sodium acetate or propionate (1% w/w) did not significantly affect the rate of decline (one $\log_{10}$ unit over nine days) of *S.* Typhimurium contamination introduced to sterile poultry mash. However, a buffered organic acid mix applied at 0.3% (liquid) to 0.5% (powder) to artificially contaminated poultry meal was associated with a reduction in *Salmonella* counts by 24 h that was >1 $\log_{10}$ unit higher than an untreated control (Hall, 1988). This product applied to naturally contaminated meat and bone meal at between 0.25 and 2% was also associated with reductions of between 1 and 2 $\log_{10}$ units in most probable number counts of *Salmonella* by seven days (Pumfrey and Nelson, 1991). A progressive decline was seen over time with the lower application rates, whilst most effect was seen by 24 h with a 2% concentration. Other organic acid-plus-salt preparations (0.1 – 0.3% inclusion rates) were associated with a 1 – 2 $\log_{10}$ unit reduction after 24 h in counts of *S.* Typhimurium previously inoculated into poultry feed (Moustafa *et al.*, 2002).

Thompson and Hinton (1997) attempted to model the actions of ingested organic acids *in vivo* in the foregut of the chicken. They replicated *in vitro* the pH (around 4.5) and levels of lactic, propionic and formic acid found in the crops of mature layer hens given feed treated with 0%, 0.68% or 1.2% of a product containing 68% formic acid plus 20% propionic acid. All the acid mixes had similar bactericidal effects against inoculated *S.* Enteritidis, but there was an additional sublethal injury, manifested as a reduction in counts on selective media, seen in the presence of formic plus propionic acid. Measurements *in vivo* did not demonstrate any depression of crop pH when the product was in the feed, and showed that formic and propionic acid had largely been absorbed by the time ingesta reached the gizzard. Another study (Hume *et al.*, 1993) reported the crop pH of young chicks to be in the range 4.6 – 5.3, and to be unaffected by propionic acid at 0.5% in the feed. By contrast, Al-Natour and Alshawabkeh (2005) reported that 0.5 to 1.5% formic acid in the diet modestly, but consistently, depressed the pH of the crop and the small and large intestines by up to 0.3 (intestines) or 0.4 (crop) units.

Experiments involving live animals (generally poultry) fall broadly into those where *Salmonella* is presented as a bolus dose, those where it is artificially inoculated into feed, and those where the feed is naturally contaminated. Feed with 0.7% acetic and 5.7% lactic acids was not protective of young chicks against the excretion of *S.* Enteritidis administered by an oral bolus of 10^2 – 10^6 cfu per bird (Heres *et al.*, 2004). Several other studies have shown that organic acids and/or salts in feed generally fail to protect poultry from colonisation by *Salmonella* when it is administered as single or multiple high doses (generally 10^4 – 10^5 cfu) by the oral route (Izat *et al.*, 1990a; Hinton *et al.*, 1991; McHan and Shotts, 1992; Hume *et al.*, 1993; Waldroup *et al.*, 1995; Allen, 1997; Al-Natour and Alshawabkeh, 2005). Notable exceptions, i.e. effective protection, have been reported when inclusion rates have exceeded 2%, associated with depression of crop and caecal pH values (Al-Tarazi and Alshawabkeh, 2003), and when the microbial dose has been lower, at $10^{2.5}$ cfu (Allen, 1997). Relative protection may be seen: reductions in caecal counts and the proportion of positive cloacal swabs were observed with 0.3% caproic acid in chick feed plus an oral dose of 10^3 cfu *S.* Enteritidis at six days of age (van Immerseel *et al.*, 2004a). Buffered propionic acid at 0.4 –

0.8% w/w in feed was associated with a reduction in *Salmonella* counts in carcass rinses from broilers exposed to 10^5 – 10^6 cfu/ml *S.* Typhimurium in drinking water (Izat *et al.*, 1990b). A significant reduction in broiler chick caecal *Salmonella* counts was observed following feed treatment from hatch with 1% formic or propionic acid and an oral challenge of 10^6 cfu *S.* Typhimurium at two days of age (McHan and Shotts, 1992). Preparations of acids that are stabilised to foregut absorption (and thus may be directly active in the hindgut) can be more effective, as discussed below.

Experiments using artificial contamination of feed allow the continuous exposure of chicks or older birds to known levels of *Salmonella*. A mix of formic and propionic acids, added after *Salmonella* contamination, protected most young chicks from colonisation by *S.* Kedougou in feed contaminated via a desiccated coconut carrier, but only when inclusion levels were at ≥ 0.5% and contamination did not exceed 500 cfu/g (Hinton and Linton, 1988). The effect of the chemicals on the incidence of *Salmonella*-positive feed samples was much less marked. A similar contamination protocol was used in a series of experiments reported by Allen (1997), using *S.* Kedougou and *S.* Typhimurium. One percent inclusion of a formic/propionic acid product provided complete protection against colonisation of young chicks by 50 cfu/g *S.* Kedougou, but this concentration offered only modest protection against 200 cfu/g *S.* Kedougou or 15 cfu/g *S.* Typhimurium, demonstrating a serovar difference. Reducing the concentration of the product to 0.68% or 0.3% provided only partial protection for chicks from 50 or 5 cfu/g respectively of *S.* Kedougou. Adding 1% to heavily-contaminated (10^3 cfu/g *S.* Kedougou) fishmeal did not protect chicks eating starter ration into which it was incorporated. Formic acid at 0.5 to 1.5% in feed was associated with reduced counts of *S.* Gallinarum (introduced continually in the feed) throughout the alimentary tract of young broilers (Al-Natour and Alshawabkeh, 2005).

Exposure of experimental animals to naturally-contaminated feed generally involves a challenge dose that is unknown, probably variable, and possibly intermittent. When naturally-contaminated feed was treated with formic acid and fed a week later to broiler chicks from one day of age, protection from colonisation was prevented if the acid inclusion level was more than 0.4% (Hinton *et al.*, 1985). Hinton and Linton (1988) found that formic acid at 0.6% (but not 0.3%) inclusion completely protected chicks from natural feed contamination by *Salmonella* from one day to seven weeks of age. Protection was dependent on use of the acid from day one. In a field study where *Salmonella* was endemic in broiler breeders (Humphrey and Lanning, 1988), the introduction of formic acid at 0.5% in feed was associated with a significant reduction in the incidence of *Salmonella*-positive samples from the flock environment and from newly-hatched broiler chicks. Pigs exposed naturally and artificially to *Salmonella* in feed, and to 0.9% formic acid also in feed, showed a significant reduction in isolations of *Salmonella* from internal lymph nodes at slaughter, when compared with those on untreated feed (Vanderwal, 1979).

Organic acid treatment of feed can reduce the colonisation of chicks via non-feed routes, i.e. following environmental exposure to *Salmonella* via seeder birds or by naturally or artificially-contaminated litter and surfaces. A formic/propionic acid product at 0.68% inclusion proved protective of chicks exposed to the lower end of a range of surface contamination, but not to higher levels, nor to litter contaminated with 10^3 – 10^5 cfu/g (Allen, 1997). Calcium formate at 0.72% in feed did not protect chicks from colonisation via an unquantified challenge from feed and previously used litter, neither did feed treatment at 0.36% protect from colonisation via seeder birds inoculated with 10^7-10^8 cfu *S.* Typhimurium (Izat *et al.*, 1990a).

A novel approach to the administration of organic acids in feed has been the use of short-chain fatty acids stabilised on microbeads to provide activity in the chicken large intestine (van Immerseel *et al.*, 2004b, 2005). Hatchlings were relatively protected from colonisation by orally-administered *S.* Enteritidis by stabilised butyric acid at just 0.16% in feed, whereas similarly-presented formic acid and acetic acid actually increased colonisation compared with controls, possibly due to local effects on the large intestinal epithelium. When free and stabilised sodium butyrate, singly and in a 1:1 combination at low inclusion rates of around 0.063% were fed from hatching, it was the combination of free plus stabilised acid treatment that proved most protective for chicks against an oral bolus of 10^6 cfu *S.* Enteritidis given at five days of age. The microencapsulated preparation was also protective, but free sodium butyrate at a similar inclusion rate was not. Stabilised sodium butyrate at around 0.08% inclusion in feed reduced the excretion of *S.* Enteritidis by broilers during exposure via seeder birds throughout the rearing period, but there was no difference between treatment and control groups in the rates of caecal colonisation by six weeks of age.

Acid treatment of drinking water. The use of organic acids in drinking water rather than feed has the advantage of allowing animals to be treated during periods of feed withdrawal (particularly pre-slaughter) when susceptibility to infection with *Salmonella* and other pathogens is likely to be increased (Ramirez *et al.*, 1997; Byrd *et al.*, 1998; Corrier *et al.*, 1999), whilst potentially retaining some or all of the acids' *in vivo* activity against ingested *Salmonella*. In addition, it may destroy or reduce any vegetative pathogens in the water itself. Acids in water may also be used, strategically or throughout rearing, to suppress endemic *Salmonella* infections in pigs or poultry. A drawback in many drinking water systems is corrosion affecting galvanised pipes, joints and nipple drinkers.

In an *in vitro* examination of organic acid preparations, tested over a range of concentrations, Allen (1997) found that a formic/propionic acid product reduced *Salmonella* in water to undetectable levels within 4 h when added at concentrations of 0.15% or above. However, the product when given at this level in water failed to influence the colonisation of chicks challenged artificially via the feed with moderate numbers (around 50 cfu/g feed) of the pathogen. Al-Chalaby *et al.* (1985) reported that an existing natural *Salmonella* carrier state did not prove susceptible to treatment with a commercial aqueous acid treatment, despite *Salmonella* being eliminated from the water in the drinkers.

Byrd *et al.* (2001) dosed broiler chickens twice orally with 10^8 cfu of *S.* Typhimurium and then added 0.5% acetic, formic or lactic acid to the drinking water during an eight-hour period without feed. Both formic and lactic acids produced a significant reduction in the proportion of positive crops and on the numbers of *Salmonella* present. However, in common with treated feed studies involving concentrated oral boluses of *Salmonella*, there was no effect on prevalence or levels of caecal carriage. In a further experiment involving naturally-infected commercial broilers, 0.44% lactic acid in drinking water significantly ($P \leq 0.001$) reduced the proportion of *Salmonella*-contaminated crops post-slaughter, as well as the prevalence of positive carcass-rinse samples. One publication (Parker *et al.*, 2006), by a company marketing organic acid blends for use in drinking water, reported that an undefined acid blend at 0.04 – 0.08% in water during the first two weeks and the final week of broiler rearing did not prevent intestinal colonisation in the face of oral challenge of hatchlings by 10^7 cfu *S.* Heidelberg. However, the treatment did reduce the incidence and degree of colonisation of in-contact birds and of environmental contamination by *Salmonella* after seven weeks, indicating some

activity against a lower-level horizontal challenge. Using another proprietary blend (Parker *et al.*, 2007), differences in caecal colonisation of in-contact birds were observed within 2 weeks, although consistent differences in crop isolations were not seen between treatment and control groups.

The study of Parker *et al.* (2006) reported that water acidification (≤ 0.08% acid blend) led to a significant improvement in feed conversion, but had no effect on bird weight-gain or mortality. A formic/propionic acid product at ≥ 0.2% did reduce weight gain in chicks (Allen, 1997). Chaveerach *et al.* (2004) reported that treatment of drinking water with (unspecified) organic acids had no effect on broiler weight-gain and caused no visible histological changes in gut epithelium.

Letellier *et al.* (2000) added 0.02% formic acid to the drinking water of early-weaned piglets and challenged them with *S.* Typhimurium two weeks later. Reductions in *Salmonella* colonisation of mesenteric lymph nodes were not observed with this treatment. Of three *Salmonella*-positive finishing pig herds treated with a buffered acid mix at around 0.2% in drinking water (pH 3.5 – 3.9), one showed a marked reduction in *Salmonella* serological titres in comparison with untreated control groups, and the others showed a statistical trend towards reduced serological responses (van der Wolf *et al.*, 2001). In another study, a product comprising organic acids plus ammonium formate was used (0.2% in drinking water) in 10 pig herds with high *Salmonella* titres, indicating high endemic *Salmonella* challenge (van der Heijden *et al.*). This treatment was followed by a reduction in titres, which paralleled that in 10 herds treated with feed acidification. This reduction in titres was not observed in five high-titre herds using improved hygienic practices alone. *Salmonella* was not eliminated from the herds over a two-year acid treatment period. Over a shorter period, an unspecified acid preparation administered to finishing pigs for the last two weeks prior to slaughter reduced drinking water pH to 3.6 – 4.0, but had no effect on *Salmonella* shedding (De Busser *et al.*, 2009).

In reviewing the use of organic acids in feed or water given to weaned and fattening pigs, Giannenas (2006) noted that, aside from anti-*Salmonella* effects, improvements in growth-rate and feed efficiency could be anticipated, but in practice the results were highly variable. Improvements in daily weight gain associated with a blend of organic acids in water appeared to be specific to later growth phases, and was associated with reduced feed efficiency (Walsh *et al.*, 2007). The same author observed that combined acid-treatment of feed and drinking water had negative effects on growth performance via reduced feed intake.

Formaldehyde

Formaldehyde has a high level of disinfectant activity against most bacteria, effected by irreversible cross-linking of proteins, and has been shown to be the most effective compound to use for disinfection of poultry houses which have been contaminated by *Salmonella* (Davies and Wray, 1995). It is less likely to be inactivated by organic matter than most disinfectant classes, but requires several hours to achieve its full effect. Despite the absence of conclusive evidence of toxicity in humans who regularly work with formaldehyde, this substance has come to be regarded as hazardous (Arts *et al.*, 2006). However, it does not appear to cause adverse effects or tissue residues in animals given feed or rations which have been treated (Duncan and Adams, 1972; Vanderwal, 1979; Buckley and Fisher, 1984; Bugarski *et al.*, 1990; McAllister *et al.*, 1992; Allen, 1993). In the EU, it is permitted as a feed

processing aid, pending a review of the use of biocides and preservatives in foods and feedingstocks.

The long-term protective effect of formaldehyde may be limited to some extent by evaporation after mixing, unless feed is held in closed bins (David *et al.*, 1972; Khan *et al.*, 2003). For this reason some commercial formaldehyde-based products also contain acids, such as propionic acid, and other antimicrobial compounds such as bacterial membrane-disrupting terpenes (Trombetta *et al.*, 2005; Carrique-Mas *et al.*, 2007). This produces a synergistic combination allowing lower levels of formaldehyde and acids to be used which minimises fuming, operator hazard and corrosiveness. The antibacterial effects of formaldehyde are not considered to rely to any significant extent on processes occurring after ingestion by the animal. Given the mode of microbicidal action, and in contrast to organic acids, the apparent efficacy of formaldehyde is not likely to be substantially enhanced by low pH or an intestinal environment. However, other variables known to affect acid susceptibility, such as strain variation, moisture, matrix structure and composition, and natural versus artificial contamination, are likely to result in variations in observed efficacy between and within studies.

Formalin (37% w/v formaldehyde) at $\geq 0.12\%$ reduced counts of *Salmonella* in moistened meat and bone meal, by contrast to bacterial growth observed in the presence of various organic and inorganic acids at up to 0.3% (Smyser and Snoeyenbos, 1979). The bactericidal effect was gradual at 0.12% and rapid at 0.2%. It was also reported that at very low moisture (2%), 1% formalin had little effect on *Salmonella* in meat and bone meal. Formaldehyde, applied as fumigant gas to chick feed (five minutes' exposure with mixing), reduced artificial *Salmonella* contamination to low levels immediately after treatment and to undetectable levels 12 h later (Duncan and Adams, 1972). Relatively concentrated formaldehyde (formalin 0.5 – 1% v/w) was rapidly effective in rendering inoculated *S.* Typhimurium undetectable in feed (Moustafa *et al.*, 2002).

Allen (1997) reported that 1% of a combined formaldehyde/propionic acid/terpene product (giving an inclusion rate of around 0.3% w/w formaldehyde) was an effective treatment for fishmeal artificially contaminated with 10^3 cfu/g *Salmonella*, such that a starter ration prepared from it did not infect chicks. At inclusion levels of 0.25 – 0.70%, formaldehyde was associated with 1 to ≥ 2 $\log_{10}$ unit reductions in natural *Salmonella* contamination of oilseed meals within 48 h (Hansen *et al.*, 1995). When a formaldehyde/propionic acid product was added at 1 – 2% to artificially-contaminated animal protein meals, contamination of 10^2-10^4 cfu/g was eliminated within 24h (Carrique-Mas *et al.*, 2007). Unmasking treatment of pre-enrichment broths using histidine suggested that masking had occurred in up to half of samples with a high (10^3 or 10^4 cfu/g) initial *Salmonella* count, but not at lower contamination levels. Results were not different 72 h after application. A field study using a formaldehyde/ propionic acid/ terpene product at 0.27% w/w in layer feed found productivity benefits but no change in environmental microbial populations (Anderson *et al.*, 2002).

Other chemical treatments

Essential oils are plant-derived mixtures of terpenes, terpenoids and low molecular-weight organic compounds of several classes, which exert antimicrobial effects probably by a range of mechanisms that includes the disruption of bacterial membranes (Trombetta *et al.*, 2005; Benchaar *et al.*, 2008). Essential oils, or constituents such as terpenes, are sometimes used in

combination with other agents (organic acids +/- formaldehyde) to improve the microbicidal effect of these agents, particularly at near-neutral pH before feed consumption.

Chlorate salts have recently been investigated for their ability to effect suppression *in vivo* of enteric facultative anaerobes, including *Salmonella* and *E. coli*, via a bacterial respiratory nitrate reductase, expressed in anaerobic conditions, which converts chlorate to cytotoxic chlorite. Susceptible bacteria expressing the enzyme, which can be induced by exposure to nitrate and some organic nitro compounds, are selectively killed (Anderson *et al.*, 2005). Experiments have concentrated on the use of chlorate preparations for the suppression of *Salmonella* and *E. coli* in the intestinal flora in the period leading up to slaughter, as the effects are rapid. Significant reductions in crop and caecal colonisation (reduced incidence and 1 – 3 $\log_{10}$ unit lower counts) by orally-inoculated *Salmonella* Typhimurium and Enteritidis serovars have been reported after using chlorate preparations in feed or water for broilers and turkeys (Byrd *et al.*, 2003, 2008; Burnham *et al.*, 2004; Anderson *et al.*, 2005; McReynolds *et al.*, 2005). Among pigs, similar significant reductions in large intestinal counts of both inoculated *Salmonella* and *E. coli* of the natural flora have been reported from weaners and finishers treated with chlorate preparations (Anderson *et al.*, 2004, 2005, 2006). Pre-treatment with oral nitrate or certain nitro compounds appears to potentiate the effect of chlorate (Burnham *et al.*, 2004; Anderson *et al.*, 2005, 2006). Ruminants also show reductions in large intestinal *E. coli*, of the order of 1 – 3 $\log_{10}$ units within 24 – 48 h of chlorate administration, but the additional benefits of nitrogen compound pre-treatment are uncertain, possibly because of their metabolism in the rumen (Anderson *et al.*, 2005; Gutierrez-Bañuelos *et al.*, 2007). Chlorate is currently an experimental additive, as it does not have authorisation for use in food-producing animals.

Summary, based upon the cited publications plus unpublished industry data

High concentrations of organic acids can be used as a processing aid in the treatment of contaminated ingredients, with the aim of reducing or eliminating *Salmonella* contamination before consumption of the feedstuff. Additional benefits of using acids in this mode include the suppression of *Salmonella* contamination in feedmill, farm storage and feeding equipment through which the material passes, and some residual antimicrobial effect in the finished feed, depending on the inclusion rate of the treated ingredient. Formaldehyde, used alone or in concert with other agents, can be used in the same mode. The treatment of compounded feed, or of grain fed as straights, with high concentrations of organic acids (generally $\geq 2\%$) runs into problems of palatability, corrosion and cost.

Single acids, acid blends and buffered blends including acid salts can be used at lower concentrations (generally up to 1%) in finished feeds, relying to some extent on actions *in vivo* for their full antimicrobial effect. One approach to increasing the effect of organic acids before ingestion has been to incorporate compounds which disrupt bacterial cell membranes, which appears to have the effect of reducing the bacterial barrier to the entry of organic acids at around neutral pH. The observed effects of buffered mixes in dry feeds may be equivalent to or poorer than a similar concentration of a straight acid, notwithstanding potential handling benefits of buffered products. With recommended inclusion rates and artificial *Salmonella* contamination, reported reductions from <1 up to 2 $\log_{10}$ units, over 24 h to several days are typical of such blends. Evidence as to the time-frame of organic acid anti-*Salmonella* effects in feed before ingestion is conflicting: in some studies little additional reduction in bacterial counts was seen beyond 24 h post-application, whereas in others, incremental effects were

seen over 48 h to several days. The inclusion rate may be significant, whereby high concentrations achieve most of the bacterial kill within 24 h.

Whereas several studies have examined the protection against *Salmonella* colonisation afforded to individuals by organic acids, others have focussed on the effects of organic acids in feed or water upon groups of poultry or pigs where *Salmonella* is already endemic. In these situations, organic acids in straight acid or salt formulations appear to have a progressive, subtotal suppressive effect on environmental contamination and excretion of *Salmonella*, and are associated with reduced humoral immune responses of animals to the pathogen.

Formaldehyde appears to act rapidly *ex vivo* at all but marginal application rates, and a 24 h measurement point is often used. In artificially-contaminated feed, $10^3 – 10^4$ cfu/g *Salmonella* was not detectable 24 h after treatment with formaldehyde in a blended product, and commercial studies suggest that concentrations of either 0.1% w/w alone, or of 0.06% w/w with 0.02% propionic acid plus terpenes and surfactant, have been associated with $2 – 5$ $\log_{10}$ unit reductions of naturally-occurring *Salmonella* in animal protein and compound feeds. Some feed matrices (particularly certain oilseed meals) and/or *Salmonella* strains can appear relatively resistant, showing lesser reductions in some studies.

Some commercial studies indicate that liquid formulations can be more efficacious or more potent than powdered preparations of the same chemical mix, suggesting that dispersal and penetration of chemicals are significant factors affecting antibacterial action. The form (powdered versus liquid) and stage of application (ingredients, blending before milling, mixing after milling, surface application to pellets) will clearly influence the effects of chemical treatment. Masking of viable *Salmonella* has been demonstrated with organic acids and formaldehyde, and this may lead to overestimation of the antimicrobial efficacy of these agents in unconsumed feed. However, masked organisms may have been rendered more susceptible to lethal injury after ingestion, so the implications of masking in terms of risk of infection to the animal remain unclear.

The persistence of protection by organic acids may extend for several weeks and is potentially useful, although it has to be regarded as limited by both time and the level of challenge. Formaldehyde is generally considered to have a rapid but not prolonged antimicrobial effect once applied, and formaldehyde-containing products with a claim for persistent effects also contain other antimicrobial agents. In one commercial study, the effect of a blended acid treatment on *Enterobacteriaceae* counts in feed reduced with transfer from mill to farm. There are many factors, including recontamination challenge and moisture levels, which may contribute to this diminution of effect.

The antibacterial effects of organic acids are markedly enhanced in a moist or wet low-pH (< 5) environment, such as the crop, monogastric stomach or the abomasum of pre-ruminant cattle and sheep. There may additionally be sublethal injury inflicted under these conditions that renders *Salmonella* more susceptible to other body defences. Typical inclusion rates of organic acids for compounded feeds do not appear consistently to depress the foregut pH in chickens, but this is in any event low enough to create conditions for potent organic acid antibacterial actions, and this may be the primary mechanism for the protective effect of organic acids in feed against *Salmonella*. By contrast, the pH of rumen contents is normally above 5.5, and it infrequently drops below 5.0 in healthy animals (Briggs *et al.*, 1957; Kleen *et al.*, 2003; Grove-White, 2004). In addition, endogenous volatile fatty acids are efficiently absorbed across the rumen wall, so it seems likely that exogenous ingested organic acids

would not exert a useful antimicrobial activity in the pH conditions of the rumen of ruminating cattle or sheep, nor would they reach the abomasum in significant quantities in such animals.

Modest inclusion rates of organic acids in feed (typically < 1% w/w) appear protective against typical natural *Salmonella* contamination of feed when used before exposure. However, the level of contamination at which this protection is overcome varies widely, and there is evidence that serovar, feed matrix and mode of contamination (artificial versus natural) will all influence this. Oilseed meals and animal protein concentrates in particular may contain *Salmonella* cells protected by lipids or protein, or in dense microfoci.

It appears to be easy to overcome feed- or water-administered organic acids by oral dosing of chicks with *Salmonella* at doses of 10^4 cfu or above, which is consistent with protection generated in the foregut. Once this is overcome by a bolus dose, colonisation of the hindgut can proceed. Accordingly, when the organic acid dose is increased to a level that depresses crop pH or the organic acid is delivered in a stabilised form that is active in the hindgut, oral boluses of *Salmonella* are less successful at establishing colonisation of chicks. There is also evidence that caecal *Salmonella* concentrations in young chicks may be suppressed by non-stabilised organic acids in feed, although a mechanism for this is unclear. There are no published animal challenge studies with a quantified initial contamination rate and defined formaldehyde treatment of feed.

Organic acids and chlorate have both been proposed as potentially of value in short-term treatments aimed at reducing *Salmonella* excretion and transmission at times of stress, such as pre-slaughter, and delivered via feed or water.

Conclusions

The decontamination of feed ingredients and of compound feedstuffs using chemical agents needs to take account of likely initial contamination rates, opportunities for recontamination in storage and transfer, and the susceptibility of the target livestock to *Salmonella* infection. In addition, the mode of action of the agents will alter the judgement of when to use an agent and how best to assess efficacy. Organic acids, their salts, formaldehyde and membrane-disrupting compounds, singly or in combination may provide adequate protection against new *Salmonella* challenges in feed, and can act progressively to reduce endemic *Salmonella* problems, provided the challenge level is not too great. Treatments at recommended application rates may fail if application is uneven, if particularly resistant *Salmonella* strains are present, or if existing environmental contamination exceeds the protection threshold. 'Problem' batches of ingredients, particularly protein concentrates, are best identified in advance and treated intensively before incorporation into compound feed. The success of chemical treatment may not necessarily be measured in the prevention or elimination of a *Salmonella* problem, but in a progressive diminution of endemic infections in a herd or flock.

Some agents can usefully be assessed for efficacy by studies in dry feed, and assessments of ingredient decontamination following high chemical application rates may be reliable, with careful methodology. However, the results of many examinations can show substantial variation in results owing to factors both known (e.g. masking, strain variation, natural vs. artificial contamination, effects of background flora) and unknown. The most complete assessment of efficacy for any chemical treatment would involve controlled challenge studies plus field studies, involving realistic feed handling and the appropriate type and age of

livestock. Few of these have been performed. There is also a need for the development of one or more reference methods for evaluating the efficacy of such products, so that informed decisions can be made on selection of suitable products for a particular mode of application from the large number of such preparations available.

Finally, as with any treatment, chemical treatments must be applied consistently. Errors in application caused by mechanical failure, blocked nozzles, electrostatic or sedimentation effects or miscalculation of dose rates can lead to contaminated material entering the feed supply. If chemical treatment is used with material that is known to be contaminated, its effectiveness should ideally be checked before the batch is used. Over-reliance on a single method will predispose pathogen control to periodic failure, so chemical treatments are best used as one element in a package of feed safety measures such as careful testing and selection of ingredients, mill hygiene and control of recontamination.

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