Assessment of the anti-*Salmonella* activity of commercial formulations of organic acid products

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Abstract

Organic acid products are widely used in the United Kingdom poultry industry as feed or water additives. Claims for *Salmonella* control are made for some of these products, but there are few studies comparing the anti-*Salmonella* effect of the various products in tests that reflect field application. The present studies examined the effects of thirteen commercial blends (four water and nine feed products) on *Salmonella* Enteritidis and Typhimurium strains. Initial screening, in nutrient broth, of all products with all strains, revealed little variation between strains in respect of maximum inhibitory and bactericidal dilutions of each product. However, between the products there was wide and significant variation in the maximum inhibitory and bactericidal dilutions, spanning a 700-fold range in the case of bactericidal dilutions for feed-associated products.

Further tests were performed, examining reductions in inoculated *Salmonella* numbers in various matrices (water, feed, soiled litter, crop and caecal contents) following the addition of the products at recommended inclusion rates. One product, incorporating formaldehyde in addition to organic acid, was consistently most active in all matrices, exceeding reductions associated with other products by one to three log units at most time points. Many products showed only modest anti-*Salmonella* activity, amounting to zero or one log unit above negative controls at many time points, and the most active products were not the same in all matrices. Tap water appeared to enhance the ability of products to reduce *Salmonella*, in comparison with bottled mineral or river water.

Introduction

Organic acid (OA) products are used extensively in the poultry sector, typically as blends of acids and their salts, but sometimes with other constituents also. They are marketed as additives for drinking water or feed, with claims that include: improving feed or water hygiene, improving digestive health and efficiency, and combating specific pathogens such as *Salmonella* and *Campylobacter*. Of various acidic organic products, the carboxylic acids are attractive as antimicrobial compounds in food-producing animals to reduce the need for preventive medication or treatment with antibiotics, as they are a feature of normal metabolism and gut environment and thus do not generally pose an issue of residues in food.

The antibacterial activity of carboxylic OAs is believed to derive from their ability to cross bacterial membranes and, once in the cytoplasm, to dissociate into protons and organic anions and interfere with pH homeostasis (proton effect) and other important cell features including membrane structure, osmolarity and macromolecule synthesis (anion effects) (Cherrington *et al.*, 1990; Russell, 1992; Ricke, 2003; van Immerseel *et al.*, 2006). OA molecules differ in carbon chain lengths and in the number and nature of attached acid and other chemical groups. Studies over several years have yielded conflicting findings regarding the relative anti-*Salmonella* potencies of different OAs, possibly because of the many different conditions (for example moisture, pH, and bacterial physiological states) that may affect measurements (Wales *et al.*, 2010).

Because efficient penetration of bacterial cells occurs when OA molecules are in an electrically neutral, undissociated state, their activity is considerably enhanced in acidic environments, when most weak acid molecules are undissociated (Ricke, 2003). Inclusion rates (generally two to three percent) that depress pH sufficiently for this effect to occur in feed cause unacceptable problems including unpalatability and corrosion, and are generally reserved for treating feed ingredients prior to compounding (Pinchasov & Jensen, 1989; Adams, 1991). There is some evidence that poultry may show side effects of certain OAs at lower inclusion rates than other

livestock (EFSA, 2011). Thus, OAs and their salts are added at low inclusion rates (generally less than one percent) to feed and water for poultry, and much of the antibacterial effect may be exerted in the first low pH environment encountered, i.e. the foregut. This theoretically can also protect birds ingesting *Salmonella* from other sources whilst they are consuming OA-treated feed or water.

In addition to the above effects, certain short- and medium-chain OAs will inhibit the invasion of epithelial cells by *Salmonella*, even when present below minimum inhibitory concentrations for the organism. Butyric acid in particular has been found to suppress expression of *Salmonella* virulence proteins (van Immerseel *et al.*, 2006) and to induce defence peptides in poultry (Sunkara *et al.*, 2011). Feeding low concentrations (0.1% to 0.2%) of butyric acid, stabilised to maximise large intestinal concentrations to poultry had a suppressive effect on caecal colonisation and/or the level of excretion of *Salmonella* following oral exposure (van Immerseel *et al.*, 2004, 2005; Fernandez-Rubio *et al.*, 2009). This type of effect is quite specific to the acid being used, as stabilised acetic and formic acids appear to promote *Salmonella* virulence at low concentrations (van Immerseel *et al.*, 2004).

Much of the antimicrobial effect of OA products is claimed to arise from synergism between elements in the blend. However, data regarding the efficacy against *Salmonella* of various commercial formulations, if available, is based on non-standardised tests that may not replicate conditions of use in the field. There is a need to develop a validated assay procedure to guide decisions regarding the efficacy of such products. The main aim of the present *in vitro* tests was to screen a number of commercial preparations for comparative efficacy, first in the medium (water or feed matrix) for which they are marketed, and then in simulated post-ingestion and post-excretion environments of crop contents, caecal contents and litter.

Materials and methods

Selection of strains and products. *Salmonella* strains (Table 1) included representative field strains of current or emerging significance for UK egg production, plus two vaccine strains. The field strains were six recent (years 2006 to 2010) isolates from various laying farms, two isolated after on-farm treatment of feed and water, respectively, with an OA product. One other strain showed greater invasion in an *in vitro* organ culture test. Two field strains were *S. Typhimurium*-like monophasic serovars. The vaccine strains were cell wall-deficient and were suspected to be more highly sensitive to the effects of OAs.

The OA products tested (Table 2) were selected on the basis of their frequency of use in the UK poultry industry, as reported by poultry veterinarians, and interest in particular new products.

Bacteriological procedures. Stationary phase broth culture for inoculation of matrices was prepared by aerobic incubation of *Salmonella* Enteritidis (SE) overnight at 37 °C in Nutrient Broth No. 2 (Oxoid Ltd.), then at room temperature for a further 24 h. The culture was serially diluted in quarter-strength Ringer's solution to create a hundred-fold dilution for inoculation. Each culture preparation was enumerated by spread-plating.

Enumeration of surviving *Salmonella* from matrix tests was accomplished using a modified most probable number based semi-quantitative technique, as previously described (Wales *et al.*, 2006). Briefly, an initial five- or ten-fold dilution of the recovered material was made in buffered peptone water (BPW; Oxoid Ltd.) and a sequential dilution series through seven decimal stages was created immediately in BPW. For the water matrix the initial sample aliquot was 1 ml but for the composite matrices the size was 5 g, to ensure a representative sample and good dispersal. An initial ten-fold dilution of the 5 g sample proved difficult, given the container sizes readily available, so for later experiments a five-fold initial dilution was used. Dilutions were pre-enriched by incubation overnight and then 0.1 ml was plated

onto modified semi-solid Rappaport-Vassiliadis (MSRV; Oxoid Ltd.) enrichment agar. Spreading growth on MSRV was subcultured onto Rambach (CHROMagar) indicator agar.

Initial screening of strains and products for maximum inhibitory and maximum bactericidal dilutions.

Maximum inhibitory dilution (MID). For each *Salmonella* strain to be screened, dilution series of each OA product in Nutrient Broth No.2 were prepared in 96-well microtitre plates. For starting dilutions, 1 g of powder or 1 ml of liquid product was added to 24 ml sterile distilled water. Powders were dissolved in cold or warm water, depending on their solubility. Every microtitre well was prepared with 75 µl of broth then, for each product, 75 µl of the 1:25 starting dilution was added to the first well in a row. A doubling dilution series from 1:50 to 1:25,600 was created by sequential transfer of 75 µl between 10 adjacent wells. Two control wells at the end of each row of 12 contained Nutrient Broth only. An aliquot (7.5 µl) of a broth culture of the test *Salmonella* strain (37 °C, in Nutrient Broth no. 2, incubated aerobically overnight without shaking) diluted 1:100 in Nutrient Broth no. 2 was added to every well except the twelfth in each row. Plates were incubated (37 °C, 18 h) and bacterial growth in each well was then determined by the presence of visible turbidity. The highest dilution without visible turbidity was recorded as the MID.

Maximum bactericidal dilution (MBD). The MBD was then determined by taking a 10 µl aliquot from each well showing no turbidity and adding it to 190 µl of Nutrient Broth no.2 in a corresponding well on a new microtitre plate, then incubating the new plate and examining it for bacterial growth as previously. The highest original dilution showing no growth after the second dilution and incubation steps was recorded as the MBD. One to four tests were done per product with each *Salmonella* strain.

Performance of products in matrices. In matrix tests, a single *Salmonella* Enteritidis (SE) strain (S9549/07) was used and products were applied at manufacturers' maximum recommended concentrations. Products marketed for water were tested

in water matrices and in caecal and crop contents; those marketed for feed were tested in all matrices except water. For negative controls, products were either omitted (water matrices) or replaced by an equivalent volume or mass (depending on the physical form of the product) of sterile water (other matrices). Quarter-strength Ringer's solution was added to some matrices in order to facilitate mixing and dispersal of products and SE inoculum.

Water matrix. Each product was dissolved and mixed thoroughly in 100 ml tap water or river water and the pH was measured with a handheld meter. Stationary-phase broth culture (1 ml) was added to each flask, with thorough mixing. The test mixes were held at room temperature (18 °C). Aliquots (1 ml) were withdrawn after 1, 2 and 4 hours and subjected to enumeration of surviving *Salmonella*. Follow-up studies were performed using the product that was associated with the most rapid and substantial reduction in *Salmonella* numbers in the initial tests. Differing temperatures (room temperature or waterbath at 18 °C or 4 °C) and water types (tap water or 'Fairbourne Springs' supermarket bottled still mineral water) were used in these later studies. The mineral water was chosen as an example of unchlorinated water with minimal suspended solids. Its source and typical chemical content of the mineral water used is provided as a supplementary file.

Feed matrix. Mixed grain from a local supplier was ground using a kitchen blender until it resembled layer mash. This formulation did not contain added fats, oils, proteins or antimicrobial additives as may be found in compounded feed. Each OA product was added to 20 g of feed in a sterile 110 ml tube, followed by diluted stationary-phase SE culture (0.1 ml) with tumbling of the tubes on a rotator arm for 10 minutes after each step. The tubes were held at room temperature (20-22 °C) and 5 g samples were taken after 24 hours and seven days. These were dispersed in 45 ml BPW by soaking for two to three minutes followed by vortex mixing and the resulting suspensions were processed for enumeration.

Litter matrix. Soiled litter from a specified pathogen-free flock at the Animal Health and Veterinary Laboratories Agency (AHVLA) Weybridge site was vortex-mixed with

quarter-strength Ringer's solution in a 1:2 ratio. OA preparations were added to 10 g samples of this, followed by 0.1 ml of stationary-phase SE culture. The preparations were vortex-mixed, held at room temperature (20 to 22 °C), and 5 g aliquots were taken after 4 and 8 hours. These were each mixed with 20 ml BPW and dilution series were then prepared for enumeration of *Salmonella*.

Caecal and crop matrices. The contents of both anatomical structures were collected from slaughtered small-scale commercial broilers and spent AHVLA layer hens. Caecal contents were used fresh, whilst crop contents were stored at -80 °C then thawed before use. Both were mixed with quarter-strength Ringer's solution (crop at 1:1 ratio, caecum at 1:2). OA preparations were added to 20 g aliquots in tubes, which were incubated for 10 min at 41.5 °C in a waterbath before 0.1 ml stationary-phase SE culture was added. The pH of some crop content mixes was measured with a handheld meter and all preparations were vortex-mixed then incubated at 41.5 °C. After various time intervals, 5 g aliquots were taken, mixed with 20 ml BPW and prepared for *Salmonella* enumeration.

Statistical analysis of maximum inhibitory and maximum bactericidal dilutions. The data were transformed according to the formula $y = \log_2(x/25)$ so that the transformed dilution values of 50, 100, 200 etc were 1, 2, 3, etc. Dilutions of <50 were recorded as zero and these were regarded as lying in the interval 1 to 50. In order to take account of the interval data, the interval regression model in STATA ('intreg') was used to fit main effects models, with strain and product as categorical variables. When either effect was significant at $p \leq 0.05$ the individual strains/products were compared with one another by Tukey's HSD test at a significance level of $p = 0.05$, with adjustments made for the unequal variances of the predicted means. The predicted means were then transformed back to the original scale, i.e. $x = 25(2^y)$.

Results

Maximum inhibitory and bactericidal dilutions for *Salmonella* strains. The eight strains tested did not differ significantly in the predicted mean inhibitory or bactericidal dilutions of products, except in the case of mean bactericidal dilutions amongst products marketed for feed, where a significant difference ($p = 0.024$) was seen only between the most and least resistant strains.

Maximum inhibitory and bactericidal dilutions of organic acid products. Significant differences were seen amongst the four water-treatment products for both MID and MBD values (Table 3), with a wider range of predicted mean dilutions for the latter. One product (B) was most potent for both MID and MBD; similarly one product (A) was least potent in both tests. For the nine feed treatments, a wide range of predicted mean dilutions was seen for both MBD (7 to 4936) and MID (64 to 23,777; Table 3). The three least potent (N, O, K) and the three most potent (J, H, M) preparations occupied the same positions in the rank order for both MID and MBD. Significant differences in mean dilutions were seen, except among the lower-potency preparations.

Matrices

Viable *Salmonella* counts in inocula for the matrices ranged between 1.1 and 1.8 $\times 10^7$ cfu.ml⁻¹.

Water matrix. There was an initial *Salmonella* concentration in the test flasks of approximately 1×10^5 cfu.ml⁻¹. Four out of five products effected rapid reductions in viable *Salmonella* concentrations in tap water, but had substantially less effect in river water, despite starting pH values being similar to or lower than those in tap water (Table 4). The remaining water product (B) was associated with higher starting pH than any of the others, and was associated with much lesser reductions in *Salmonella* in tap water, but performed similarly to three other products in river water.

In follow-up studies with product D, starting pH values were in the ranges 3.4 to 3.6 for OA preparations, 7.1 to 7.7 for tap-water controls and 8.0 for the bottled water control. A lower temperature (4 °C versus 18 °C) was associated with a slower rate of inactivation of *Salmonella* (Figure 1). The tap-water controls showed substantial anti-*Salmonella* activity, especially when in a stable, warmer (18 °C waterbath) environment. Bottled water was associated with substantially less inactivation of *Salmonella* than was tap water (Figure 2) despite the initial pH values of both media, after addition of acid product, being very similar.

Feed and litter matrices. In initial experiments with feed matrix, reductions in *Salmonella* of between zero and three log units were observed after 24 h for 10 of the 11 products (Table 5). A maximum of one further log unit reduction was seen after six more days with these products. The remaining product (M) was associated with the most marked reduction by 24 h, exceeding the 3 log unit quantification limit of the assay. Further studies were performed with the three products (E, F and M) that had been associated with a two log unit or more reduction by 24 h in the initial test. These additional tests involved two repetitions, each with two subsamples processed on each sampling occasion. Reductions in *Salmonella* were similar to the previous experiments (data not shown), although there was also a measurable reduction in *Salmonella* (up to two log units by seven days) in the negative control preparations.

Results from tests using soiled litter are summarised in Table 5. Two products (L and M) were associated with greater reductions in *Salmonella* numbers than the other products.

Crop and caecal content matrices. In crop content, the most effective products (D, F, M) were associated with a reduction of at least five log units after an hour (Table 6, run a). In negative controls, *Salmonella* declined at a lesser rate, which was nonetheless substantial compared with control preparations in other matrices. With some products (for example I), a modest reduction in four hours was followed by much more substantial reduction (six log units or more) by eight hours. Four of the

most effective products again performed similarly in replicate experiments (Table 6, run b). In these, the pH of the crop contents was 4.7 to 4.8 before addition of product and 4.2, 4.0, 4.5 and 3.3 after addition of products D, E, F and M, respectively.

In incubated caecal content (Table 6), two of 14 products were associated with a *Salmonella* reduction of six log units or more by nine hours. With one (F), a progressive reduction was observed over time; with the other (M), a reduction of greater than six log units was evident by one hour. Other products were similar to the negative control in respect of *Salmonella* reductions.

Discussion

The efficacy that is seen in the field with OA products is rather variable and sometimes disappointing (Davies & Carrique-Mas, 2010; Wales *et al.*, 2010). The present studies aimed to explore the usefulness of tests to evaluate and compare OA products in conditions that mimic application in the field. *In vivo* effects of low concentrations of specific OAs (for example butyric acid) at epithelial surfaces in the large intestine were outside the scope of the study.

In an attempt to survey a wide range of products with the resources available, repetitions were generally only performed on those products that showed comparatively high efficacy in initial experiments. Given that some matrices were by their nature complex and non-uniform, it is notable that the findings were generally consistent, showing few aberrant results in sequential samples or repetitions.

The 'masking' of viable *Salmonella* cells owing to OAs depressing the pH of media in the culture-enumeration process, has been demonstrated (Carrique-Mas *et al.*, 2007), wherein it was concluded that a formaldehyde/OA product demonstrated little masking in comparison with OA-only products. This effect potentially could have helped overstate the apparent efficacy of OAs in the present study. However, beyond an initial dilution of 1:5 or 1:10 in BPW, residual OA in the decimal dilution

series would be so low as to be unlikely to have any practical pH effect on the media. Therefore masking wouldn't be likely to have helped overstate the effect of the preparations associated with low or moderate reductions (up to around 3 log units) in *Salmonella* counts.

Screening of eight stationary-phase *Salmonella* strains, varied in respect of serovar, virulence, source and recent exposure to OAs in the field, for inhibition and killing by the OA products revealed a modest spread of susceptibilities. Where there was most variation between strains (bactericidal activity of feed products), this amounted to little more than a four-fold range. Therefore, a single strain could be selected for further tests with some confidence that findings would be broadly applicable to other field strains.

By contrast, much greater variations in MID and MBD were observed between products. Only in the case of MID for water products did this spread appear to correlate with the manufacturers' recommended maximum concentrations. In the other cases these recommended working concentrations did not prove to be a reliable indicator of absolute or relative potency against *Salmonella* suspensions in culture medium. The most marked example is MBDs of feed products, where a 700-fold range of predicted mean dilutions was seen, among products with a four-fold range of recommended working concentrations. Given that most of the products tested were not marketed for application to liquid media, and in many cases not specifically for *Salmonella* control, a wide range of potencies in an *in vitro Salmonella* suspension test is not surprising.

The bactericidal action of the products marketed for use in water proved to be greatly affected by the type of water in which they were dissolved. At recommended concentrations, their effect was attenuated in river water compared with tap water, an effect that might be associated with the influence of organic and mineral particulate matter, including the action of free-living bacteria, in the river water (Sawaya *et al.*, 2008). However, when the most effective product (D) was compared

in tap and bottled mineral water, a substantially higher, and pH-independent, anti-*Salmonella* activity was again seen with the tap water.

It is possible that there is significant synergy between acidifiers and residual chlorine in tap water, and the noticeable but variable anti-*Salmonella* effect observed in unmodified (control) tap water may be a pointer to a chlorine effect. Whatever the cause, the environmental anti-*Salmonella* effect of water acidifiers may be quite sensitive to the composition of water used, and on-farm trials may be required to establish efficacy in any particular application. Low temperature also reduced *Salmonella* killing, something which may be significant in colder seasons.

The control of *Salmonella* by OA products applied to drinking water or feed may be mediated to some extent by effects in the foregut, where low pH and endogenous (secreted and fermentative) acids add to, or synergise with, the bactericidal effect of exogenous products. The pH and retention times of feed in the chicken crop appear to be highly variable and heavily influenced by feed composition and feeding schedule (Svihus, 2010). Products in feed or water that can rapidly reduce the pH of the crop, and which are associated with rapid killing of ingested *Salmonella*, may be particularly well suited to protecting birds against ingested environmental *Salmonella*. In this regard, although heavy reductions in *Salmonella* numbers were seen eventually with all products, some products were only associated with modest reductions in counts after four hour's exposure in crop contents. Therefore, the eventual reductions seen after eight hours may in some cases have owed more to endogenous activity of the crop contents than to the added products, as control mixes of crop contents with water were associated with four log unit reductions and more by five to eight hours.

The feed matrix study found reductions in *Salmonella* of around one log unit after seven days. The products associated with greater reductions showed more substantial reductions much sooner, by 24 hours after incorporation. Examination of several replicate runs with these more active products confirmed their relative efficacies and demonstrated again that most reduction was seen up to 24 h after

application in this model system. The incorporation rates used are not typically associated with a marked depression of pH in feed matrices (Wales *et al.*, 2010), and therefore it is perhaps unsurprising that the anti-*Salmonella* effect was, in most cases, modest compared with the crop contents. It is also logical that a product (M) that has a non-acid bactericide (formaldehyde) as a major component should demonstrate comparatively high efficacy in this matrix.

However, one product that comprises medium-chain fatty acids (F) was, in both feed matrix trials, clearly associated with a more rapid and substantial reduction in *Salmonella* than the other acid-based products. This suggests that there is a significant bactericidal mechanism (or mechanisms) associated with this blend that does not depend on a low pH. Indeed in caecal contents, another matrix that does not typically have a low pH, this same product again showed a comparatively high level of anti-*Salmonella* activity. It may be that such activity is rather matrix-specific, as the same product did not (in common with most products) show a high level of activity in poultry litter, another non-acid matrix.

The consistency seen in results where repetitions were performed indicates that the fact that products did not appear to perform consistently from matrix to matrix was not a consequence of variation inherent in the experimental procedures. Moreover, one product (containing formaldehyde) was consistently associated with high levels of *Salmonella* inactivation in all matrices. It is therefore reasonable to surmise that there is indeed substantial, and unpredictable, variation between the anti-*Salmonella* effects of OA products when applied at recommended inclusion rates to different matrices. This may be for reasons that can be inferred from the composition of the product, but in some cases the effect cannot at present be explained. Superior performance may be quite matrix-specific, and this phenomenon extends even to the source of water used in drinking systems.

Inconsistent effects of OA products on *Salmonella* in differing matrices may contribute to variable, and often disappointing, *Salmonella* control when these products are applied in the field. In addition to differences in the prevailing

challenges to *Salmonella* control on any particular premises, realistic expectations of the contribution OA products can make to *Salmonella* control need to take account of this identified variability.

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Tables

Table 1: Details of *Salmonella* strains screened for susceptibility to organic acid products *in vitro*

Farm (strain)	Serovar ^a	Phage Type	Comments
A (S0814-10)	4,12:i:-	193	Monophasic S. Typhimurium-like
B (S1764-10)	4,12:i:-	193	Monophasic S. Typhimurium-like
C (S1887-06)	Enteritidis	4	SE invasive strain
D (S2922-08)	Enteritidis	4	Twelve days after use of acid product
E (S9549-07)	Enteritidis	4	Before use of acid product
E (S3513-08)	Enteritidis	4	Ten days after use of acid product
(Sm24/Rif12/Ssq)	Enteritidis		AviPro <i>Salmonella</i> vaccine strain
(Nal2/Rif9/Rtt)	Typhimurium		AviPro <i>Salmonella</i> vaccine strain

^a Serotyped at Animal Health and Veterinary Laboratories Agency *Salmonella* unit

Table 2: Details of commercial organic acid products used

Product	Intended use	Maximum concentration^a	Form used in present study. Further information / manufacturer's description
A	Drinking water	0.05%	Liquid. Concentrated acidifier: formic, propionic, sorbic, lactic and citric acids.
B	Drinking water	0.2%	Liquid. Propionic acid plus copper sulphate, terpenes and ammonia.
C	Drinking water	0.3%	Liquid. Formic, acetic and sorbic acids, zinc and cupric acetates, ammonium formate.
D	Drinking water	0.3%	Liquid. Synergistic blend of free and buffered organic acids.
E	Feed	0.45%	Solid. Multipurpose feed acidification mixture for swine and poultry feed.
F	Feed	0.3%	Liquid. Medium chain fatty acid blend for feed.
G	Feed	0.6%	Solid. Sodium formate and formic acid.
H	Feed	0.2%	Solid. Detergent, organic acids and their ammonium salts.
I	Feed	0.3%	Liquid. Organic acids, butyrate and mannobiose.
J	Feed	0.3%	Liquid. Propionic, formic and lactic acids, ammonium formate, flavour and emulsifiers.
K	Feed	0.6%	Solid. Formic acid, Ammonium formate, Ammonium Propionate
L	Feed or drinking water	0.8% (feed) 0.2% (water)	Liquid. Formic acid, Ammonium formate, Ammonium Propionate
M	Feed	0.3%	Liquid. Mixture of formalin, propionic acid, terpenes and surfactant.
N	Feed	0.3% (active ingredients) ^b	Blend of organic acids and flavours microencapsulated with a lipid matrix.
O	Feed	0.1% (active ingredients) ^b	Blend of organic acids and their salts microencapsulated with a lipid matrix.

^a As recommended by manufacturer; v/v (liquids) or w/v (solids).

^b Product is encapsulated; active ingredients were tested as a free powder.

Table 3: Results of maximum inhibitory dilution and maximum bactericidal dilution determinations using eight test strains of *Salmonella*

	Maximum inhibitory dilution (MID)				Maximum bactericidal dilution (MBD)			
	Maximum recommended concentration (%)	Predicted mean dilution ^a	95% confidence interval ^b	Multiple comparisons ^c	Maximum recommended concentration (%)	Predicted mean dilution ^a	95% confidence interval ^b	Multiple comparisons ^c
Water products								
B	0.2	637	512 – 793	α	0.2	7	1 – 44	α
D	0.3	1131	892 – 1435	β	0.3	69	40 – 119	α β
C	0.3	1131	919 – 1393	β	0.3	154	94 – 253	β
A	0.05	1497	1240 – 1807	β	0.05	1131	689 – 1857	γ
Feed products								
N	0.3	64	51 – 80	A	0.3	7	2 – 30	A
O	0.1	64	51 – 80	A	0.1	7	2 – 30	A
K	0.6	71	56 – 89	A	0.6	7	2 – 30	A
F	0.3	88	72 – 107	A	0.45	47	24 – 92	A, B
I	0.3	372	303 – 457	B	0.3	56	31 – 100	A B
L	0.8	400	327 – 489	B C	0.8	68	37 – 127	A B C
E	0.45	636	511 – 793	C	0.6	119	66 – 213	B C D
G	0.6	1131	894 – 1432	D	0.3	255	142 – 460	D
J	0.3	1381	1118 – 1705	D	0.3	336	188 – 603	D
H	0.2	2506	2003 – 3136	E	0.2	1600	892 – 2869	E
M	0.3	23777	19370 – 29186	F	0.3	4936	2755 – 8842	E

^a Highest dilution of product associated with inhibition (MID) or abolition (MBD) of bacterial growth.

^b Not adjusted for multiple comparisons.

^c Products in the same column with a letter in common do not differ significantly at p=0.05 by Tukeys HSD test. Water products were not compared with feed products.

Table 4: Reductions in *S. Enteritidis* over time with exposure to organic acid preparations in tap and river water

Product	Tap water				River water			
	pH ^a	Log ₁₀ reduction ^b			pH ^a	Log ₁₀ reduction ^b		
		1hr	2hr	4hr		1hr	2hr	4hr
A	3.9	>5	>5	>5	3.3	1	1	0
B	5.1	2	3	3	4.8	1	1	2
C	3.9	>5	>5	>5	3.8	1	1	2
D	3.4	>5	>5	>5	3.5	2	2	4
L	4.9	3	5	>5	4.5	1	0	1

^a pH after addition to water of product at manufacturer's maximum recommended concentration, as given in Table 2.

^b Values are net reductions, after subtraction of any reduction seen in controls without added product.

Table 5: Reduction in *S. Enteritidis* numbers over time with exposure to organic acid preparations mixed with feed or poultry litter

Product ^a	<i>Salmonella</i> log ₁₀ reduction ^b			
	In feed matrix		In litter matrix	
	after 24h	after 7d	after 4h	after 8h
E	2	2	1	1
F	3	3	1	1
G	1	1	0	0
H	1	2	1	0
I	0	1	1	0
J	0	1	0	0
K	0	1	1	1
L	1	1	2	6
M	> 3	> 3	3	6
N	0	0	1	0
O	1	1	0	0

^a Products used at concentrations given in Table 2.

^b Values are net reductions, after subtraction of any reduction seen in controls without added product.

Table 6: Reductions in *S. Enteritidis* over time with exposure to organic acid preparations mixed with poultry caecal and crop contents

Test preparation ^a	Run/sub-sample	Salmonella log ₁₀ reduction after specified time							
		Caecal contents			Crop contents				
		1h	4h	9h	1h	2h	4h	5h	8h
Water products									
A	a		1	1	1		5		>5
C	a		1	1	4		>5		>5
D	a		0	1	5		>5		>5
	b/1				>5	>5		>5	
	b/2				>5	>5		>5	
	b/3				>5	>5		>5	
Feed products									
E	a	1	0	1	3		4		>6
	b/1				>5	>5		>5	
	b/2				>5	>5		>5	
	b/3				4	5		>5	
F	a	2	4	6	>6		>6		>6
	b/1				>5	>5		>5	
	b/2				>5	>5		>5	
	b/3				>5	>5		>5	
G	a	1	1	2	3		>6		>6
H	a	0	1	2	2		>6		>6
I	a	0	1	2	2		3		>6
J	a	2	1	2	1		>6		>6
K	a	1	0	1	1		1		5
L	a	2	0	2	2		>6		>6
M	a	>6	>6	>6	>6		>6		>6
	b/1				>5	>5		>5	
	b/2				>5	>5		>5	
	b/3				>5	>5		>5	
N	a	1	1	2	1		2		>6
O	a	1	1	2	1		2		6
Water (control) ^b									
	a	0	1	2	0		1		4
	b/1				0	2		4	
	b/2				1	1		>5	
	b/3				0	3		5	

^a Products used at concentrations given in Table 2.

^b Organic acid product substituted by 0.3% v/v water.

Figure legends

Figure 1: Effect on inoculated *Salmonella* of organic acid product D (0.3%) in tap water.

Figure 2: Effect on inoculated *Salmonella* of organic acid product D (0.3%) in tap and bottled water, at room temperature (14-15 °C).