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Can a combination of vaccination, probiotic and organic acid treatment in layer hens protect against early life exposure to *Salmonella* Typhimurium and challenge at sexual maturity?

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ABSTRACT

Day old layer chicks were challenged with *Salmonella* Typhimurium using a seeder bird technique. Treatment groups were untreated control, administration of a probiotic in drinking water weekly, vaccination by intramuscular injection of a live *aro*-A deletion mutant vaccine at 10 weeks of age (woa) followed by an oral dose at 16 woa, probiotic administration plus vaccination, vaccination plus the administration of an organic acid preparation in feed from 16 woa and a combination of probiotic, vaccine and organic acid. Faecal shedding was monitored by culture at 1, 2, 3, 4, 8, 12, 15, 17, 20, 21, 23 and 25 woa and in dust from settle plates by PCR at intervals from 8 woa. Birds from each group were separated at 17 and 18 woa and challenged orally with 10^6 CFU of *S.* Typhimurium. Both untreated and probiotic groups shed *Salmonella* until 56 days. *Salmonella* was also detected in dust from 8 until 12 woa but little after this. After vaccination, from sexual maturity (18 woa) all groups except those that were vaccinated with and without probiotic re-excreted *Salmonella*. The probiotic alone was ineffective against this re-excretion and all groups receiving organic acids shed *Salmonella*. At 17 woa, unchallenged controls were fully susceptible to caecal colonization, however all other groups showed reduced susceptibility, including the untreated challenged group. However, at 18 woa (sexual maturity) only the groups that were vaccinated with or without probiotic showed reduced susceptibility to colonization. The organic acid treated groups (including the vaccinated group) did not show a difference to the untreated controls. *S.* Typhimurium demonstrated an ability to re-emerge at sexual maturity, similar to other serovars. The vaccine assisted in limiting the re-excretion at sexual maturity and decreased susceptibility to subsequent challenge. Use of a probiotic augmented the vaccine's protective capacity.

1. Introduction

Non-typhoidal serovars of *Salmonella enterica* subspecies *enterica* remain a major concern in human salmonellosis cases where eggs and egg products are often incriminated as sources of food borne infection. Young chicks are prone to be intestinally colonised easily by early exposure to salmonellae [1–3]. It is also generally expected that such early infections will be followed by substantial excretion of the organisms in faeces for several weeks, after which the salmonellae become undetectable [3]. The immunological control of *Salmonella* infections of the gastrointestinal tract in chickens is believed to predominantly rely on the cell mediated immunity (CMI) component [4,5]. Wigley *et al.* [6] demonstrated that CMI in the hen is suppressed at sexual maturity allowing latent infection with *Salmonella* Pullorum to re-emerge at this time. Johnston *et al.* [7] showed this effect to be true also for *S.* Enteritidis. Similar situations with other serovars, particularly *S.* Typhimurium can be regarded as highly likely. Although CMI is regarded as the main method of immunity development against intracellular parasites like salmonellae [5], inactivated vaccines, which are thought to provoke only a humoral mediated immune (HMI) response, have been very successful in controlling *S.* Enteritidis infections in Europe and USA [8]. Australian work in broiler breeder operations [9] has demonstrated that if a certain level of humoral antibody

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is achieved following inactivated vaccination, establishment and maintenance of *S.* Typhimurium after point of lay can be prevented in the field. The literature often notes that salmonellae generally appear in flocks in early lay [10–12] and this is often assumed to be due to exposure to the organism when the birds are moved to their laying quarters [10]. However, when the hen reaches sexual maturity, the documented suppression of CMI may lead to re-expression of the salmonellae as a major source of infection of the point of lay flock [6,7]. Although *S.* Typhimurium is not generally regarded as causing contamination of eggs during their formation, this has been described as possible at the onset of lay [12]. Vaccines are not 100% effective in controlling colonization of the chicken gut by salmonellae [13]. There is a growing interest in the use of microbial products (probiotics) to assist in control of salmonellae in poultry. Many studies have suggested the capability of probiotics to suppress *Salmonella* in broiler chickens [14,15], however such studies have solely evaluated the individual products. Only a few studies have evaluated laying hens which may have additional requirements to maintain effective protection rates against the organism throughout the suppressed CMI system during sexual maturity [6,7,13,16,17]. Organic acid combinations, usually short chain fatty acids such as propionic, butyric and formic acids, have been employed in attempts to either inhibit salmonellae in poultry feeds or to modify the gut milieu such that it is inhospitable to salmonellae. There are few independent studies that compare these multitudinous products independently [18]. The present experiment aimed at evaluating the pattern of expression of *S.* Typhimurium following an early exposure. The hypothesis investigated was that birds that are infected in their first few days of life will shed the organism actively for several weeks. Thereafter the organism will not be detectable for some time but will re-emerge at sexual maturity, associated with the decline in CMI. Under this hypothesis we were interested in evaluating the effects of a vaccination protocol to ameliorate this effect and whether the addition of a probiotic and an organic acid product to feed could augment the vaccination effects.

2. Materials and methods

2.1. Animal Ethics

All work was carried out under the approval and supervision of Birling Animal Ethics Committee (approval number 1064/07/16AU) for birds held in the Zootechny facility and by The University of Sydney's Animal Ethics Committee (approval number 2017/1152) for birds held at the University Poultry Unit, Camden campus.

2.2. Experimental animals and treatments

Two hundred and ten 1-day-old Hyline brown egg layers were obtained and placed in cleaned and disinfected pens at the Zootechny facility at 30 birds per pen. Birds received the same basic ration of steam-pelleted pullet starter and grower rations (produced at Uni of Sydney feed mill). Each pen was treated as follows: Infection was applied by the presence of five "seeder" birds which were inoculated with a field strain of *S.* Typhimurium PT 135 orally (10^6 CFU per bird) at day 0. These seeders were held in an elevated cage and supplied with untreated feed for the first 5 days. The seeders were identified and released into the main pen throughout rearing. Cloacal swabs from the seeders were collected and cultured for the presence of salmonellae on day 5. All groups except those intended to remain uninfected (group 'A') had seeders. The following treatments groups were used with one pen per group:

A. Controls – unchallenged
B. Controls – no treatment – challenged from d 0
C. Vaccination program of live *S.* Typhimurium vaccine by intramuscular injection at 10 weeks of age followed by an oral does at 16 weeks of age. Challenged from d 0
D. As for C but received organic acids (SiloHealth™ at 1.5 kg/tonne) in feed from 16 weeks. Challenged from d 0
E. Probiotic (Poultry Star®) in drinking water over days 1 through 7 and then once weekly during rearing. Challenged from d 0
F. As for C but with probiotic in drinking water as for E. Challenged from d 0
G. As for F but also received organic acids (SiloHealth™) in feed from 16 weeks onwards. Challenged from d 0

The experimental design is somewhat complex, with certain treatments beginning at different ages. Up to 10 weeks, prior to any vaccination, there were effectively only three groups (A, B and E) This is depicted by age in Table 1.

The location of each pen and the placement of settle plates for dust collection are shown in Fig. 1.

2.3. Vaccine and administration

A live *aro*-A deletion mutant vaccine (Vaxsafe ST batch no. STM171541A, expiry 6 June 2020; Bioproperties Pty Limited, Ringwood Victoria) was used for both vaccination procedures. A 1000-dose vial of the live vaccine was diluted into 250 mL of sterile phosphate buffered saline (PBS). Intramuscular injections of 0.25 mL per bird (providing one label dose per bird, specified as a minimum of 10^7 CFU per bird) were administered using a 22 G × 25 mm needle into the superficial pectoral muscle at 10 weeks of age. The same dilution was prepared and given orally using a stepper pipette (Thermo Scientific, Cat. No. GH9902 4540 set to wheel position 5 using a 2.5 mL tip) to deliver 0.25 mL per bird into the crop at 16 weeks of age. The prepared vaccines were submitted for plate counts using serial dilutions after the vaccination procedures. Results showed a CFU titre of 9.3×10^7, equivalent to approximately 2.3×10^7 CFU administered intramuscularly per bird in 0.25 mL at 10 weeks of age; and the oral preparation given at 16 weeks was determined to contain 1.0×10^8 CFU per mL, providing 2.5×10^7 CFU per oral dose.

2.4. Selection of probiotic and organic acid products

Multispecies probiotics are reputably more effective than monospecies probiotics [19]. The probiotic selected for use in this study has a label claim to inhibit bacteria including *Clostridium perfringens, E. coli, Salmonella* (including serovars Enteritidis, Typhimurium, and Choleraesuis) and *Campylobacter jejuni* (Poultrystar®, batch no. 3504456A12, exp 30 October 2019; Biomin, USA), with different strains targeting different areas of the gastrointestinal tract (crop, jejunum, caecum, ileum). The product contains a combination of lactic acid producing bacteria (*Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis* and *Lactobacillus reuteri*) isolated from poultry sources. It also contains prebiotics (fructooligosaccharides) to further promote the growth of the probiotic organisms [20]. Its label usage recommendations require application of 20 g/1000 birds/ day via drinking water.

Short chain fatty acids (SCFA) are attributed with ability to inhibit some pathogenic bacteria [21]. Of the SCFAs, butyric acid is regarded as the most effective in inhibition of *Salmonella* in the chicken intestine [22,23]. Besides a direct bactericidal activity, butyric acid is claimed to have the ability to downregulate virulence genes of *S.* Enteritidis [24,25]. Butyrate also promotes lactic

Table 1
Experimental design by age explaining treatment regime.

Age (weeks): Treatment from day 0	Pen No.	1 Challenge by seeders day 0	10 Treatment from 10 weeks	16 Treatment from 16 weeks
A Unchallenged controls	23	No	A Nil	A Nil
B Challenged control	19	Yes	B Nil	B Nil
Challenged	15	Yes	C Vaccine	C Vaccine
Challenged	14	Yes	D Vaccine	D Vaccine + Organic acid
Probiotic + challenge	13	Yes	E Probiotic only	E Probiotic only
Probiotic + challenge	18	Yes	F Vaccine + Probiotic	F Vaccine + Probiotic
Probiotic + challenge	17	Yes	G Vaccine + Probiotic	G Vaccine + Probiotic + Organic Acid

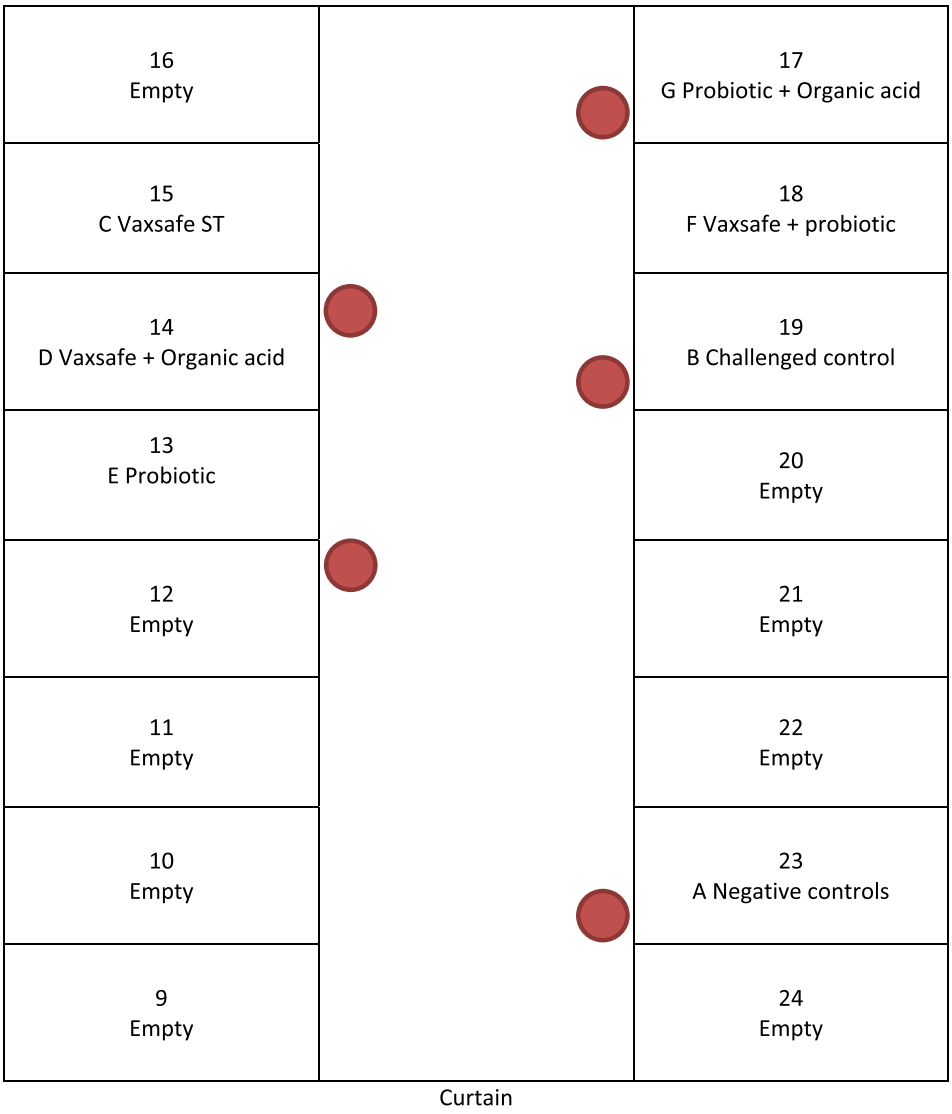

Fig. 1. Pen arrangement and settle plate locations (red circles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microflora which are purported to provide competitive exclusion against salmonellae [26]. However free butyrate is rapidly absorbed in the upper intestine which would limit its ability to affect *Salmonella* residing in the caeca. The use of butyrate glycerides however is reputed to allow the release of the active butyrate molecules lower in the gut following exposure to lipase (Sampungna *et al*., 1967 – cited by Bedford *et al*., 2018 [27]). It is also contended that medium chain fatty acids (MCFA) have greater bactericidal action against *Salmonella* spp. than SCFA [28,29]. SILOhealth 104 (SILO S.P.A., Florence, Italy) was chosen as a broad-spectrum organic acid product with claims for reduction of *Salmonella* infection and invasion in chicken intestinal cells. It contains mono- and di-glycerides of butyrate along with other short and medium chain fatty acids (propionic, caprylic, capric and lauric glycerides) [27].

2.5. Microbiological techniques

All microbiological testing was performed at a NATA accredited laboratory (Birling Avian Laboratories, Bringelly, NSW, Australia) in accordance with the Australian Standard AS 5013.10–2009 (equivalent to ISO6579:2002). The samples (cloacal swabs, faeces or

whole caeca) were initially emulsified in 2 mL for cloacal swabs or 1:10 w/v for faeces or caeca in buffered peptone water (BPW, Oxoid Thermo Fisher, CM509, Hampshire, UK) and then enriched and further cultured as described in the Standard. This involved incubation at 37 °C, followed by inoculation into modified semi-solid Rappaport-Vassiliadis medium (MSRV) with novobiocin (Oxoid Thermo Fisher SR0161E) for further incubation. Cultures were then streaked onto Hektoen and xylose-lysine-deoxycholate (XLD) media (bioMérieux, Brisbane, Queensland) and again incubated. Suspect isolates were presumptively confirmed using validated commercial chromogenic agar chromID™ *Salmonella* Agar (bioMérieux, Brisbane, Queensland).

Typical presumptive salmonellae were confirmed serologically with poly-O and poly-H antisera (Pro-Labs Diagnostic, Refs TL6002 and TKL6001, Ontario, Canada) by the slide agglutination technique after sub-culture on a nutrient agar slope.

The *Salmonella* enumeration method used a miniaturised Most Probable Number (MPN) [30] technique, conducted at Birling Avian Laboratories. The technique was used on cloacal swabs, faecal samples and caecal collections. Briefly, the sample was weighed and suspended in a known quantity of BPW 10^{-1} (at an approximate ratio of 1:10), vortexed, allowed to elute for 10–20 min and then a 1 mL sample pipetted into a 1.2 mL microtitre tube containing 900 μL of BPW. This was then serially decimally diluted eight times and covered. These were all incubated at 37 °C overnight and then 0.1 mL from each tube transferred into 500 μL semi-solid MSRV vials and incubated at 42 °C for 48 h. Resulting cultures were then directly plated onto ChromID plates and incubated overnight at 37 °C, and the positive colonies were enumerated.

Typical mauve (positive) colonies were then streaked onto nutrient agar and incubated (37 °C/24 h). Resultant colonies were then confirmed serologically with O5 antisera to confirm suspect *S.* Typhimurium identity. The vaccine strain is a gene deletion mutant (*aro*-A) and grows with a different colony morphology (non-H_2S production) on XLD and Hektoen agars [31] and is readily differentiated from wild-type *S.* Typhimurium strains.

The limit of detection for the MPN technique is 10 organisms per g. Where *S.* Typhimurium was detected by the standard method but did not reach a positive MPN limit, the MPN was arbitrarily assigned a value of 5 CFU/g for purposes of this analysis.

Where wild-type *S.* Typhimurium was detected from faeces or drag swabs at 18 and 20 weeks in the pens or from caecal culture following artificial challenge, representative colonies were streaked onto nutrient agar slopes and submitted to the Australian *Salmonella* reference laboratory (IMVS, SA Pathology, Adelaide, South Australia) for phage type identification.

2.6. Salmonella PCR technique

Settle plates (plastic picnic plates – see Fig. 1) were set up at 1.5 m heights outside five pens to collect dust. Dust samples from settle plates were collected at intervals from 8 weeks of age. Plates were wiped clean with an alcohol wipe after each collection. After collection, samples were stored at −20 °C then transported to the University of New England for PCR for salmonellae DNA detection. Dust samples were homogenised by vortexing and DNA was extracted from 5 mg of dust using the ISOLATE II Genomic DNA kit according to the manufacturer's instructions with modification (Ahaduzzaman, et al., 2019) and eluted in a final volume of 100 μL. Extracted DNA was tested for *Salmonella* by a qPCR using primers (forward: 5'–AACGTGTT TCCGTGCGTAAT–3′ and reverse: 5'–TCCATCAAATTAGCGGAGGC–3′) and TaqMan probe (5'-FAM-TGGAAGCGCTCGCATTGTGG-BH Q-1-3′) targeting the invA gene [32]. Each 25 μL of real-time PCR reaction contained 0.5 μL of each primer (0.5 mM), 0.5 μL of probe (10 mM), 5 μL of template DNA (1:10 dilution in molecular water), 12.5 μL of 2 × master mix, and 6 μL of nuclease-free water. PCR conditions were 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Absolute quantification of *Salmonella* was achieved using a standard curve based on *S.* Typhimurium DNA copies. The results were analysed by the Rotor-Gene Q version 2.3.1.49 software and reported as *Salmonella* $\log_{10}$ DNA copies per gram of dust.

2.7. Procedures and measurements

The pen containing Group A birds (unchallenged controls) was separated from the challenged pens by 6 m and two fans were installed behind this pen to direct air flow away from it. Settle plates were installed outside five pens in the shed and dust collected from these plates assayed for the presence of *S.* Typhimurium at intervals throughout the experiment by PCR as described above.

Cloacal swabs from 6 birds per pen (the same identified birds each time) were obtained at 1, 2, 3, 4, 8, 12, 15 and 17 weeks of age and 6 separate fresh faecal samples were collected at 20, 21, 23 and 25 weeks of age. These were assayed for *S.* Typhimurium by enumeration culture technique. Cloacal swabs after point of lay did not show appreciable faecal presence, possibly due to presence of an egg in the uterus at the time and an empty rectum, so individual fresh faecal samples were collected instead (a crate was placed in the pen lined with plastic – freshly voided faeces were collected from the plastic).

Drag swabs were collected in each pen at 4, 8, 12 and 16 weeks of age using two tampons per pen soaked in sterile buffered peptone water (BPW). These were cultured for the presence of salmonellae using the Australian Standard method.

Minimal serology was preformed to evaluate a response to challenge and to vaccination within each group. Blood samples were collected from the same identified birds (2 per pen) at 10, 12, 16, 18 and 27 weeks of age and submitted for determination of specific serum antibody against *S.* Typhimurium using a commercial ELISA kit (X-OvO, Dumferline, Scotland).

At 17 weeks, 16 birds per pen were removed and transported to University of Sydney and challenged orally with a field strain of *S.* Typhimurium PT 108 orally (10^6 CFU per bird, based on successful colonization rates in control birds achieved in previous experiments [13,16]). Eight birds from each pen were challenged at 17 weeks of age and a further eight at 18 weeks of age in a separate room. The birds were euthanized 10 days post challenge and their caeca removed aseptically and assayed for *S.* Typhimurium by enumeration culture (MPN).

2.8. Statistical analyses

The proportion of birds for which *S.* Typhimurium was isolated from the caeca was compared, between each vaccinated group and the unvaccinated control group using contingency table analysis (Pearson χ^2 or Fisher's exact test if an expected cell value was <5), performed using the StatCalc function of EpiInfo™ (CDC, 2000). Quantitative salmonellae levels (Most Probable Number) were compared after transformation to $\log_{10}$ value analysis of caecal MPN/g + 1 to provide zero values where appropriate by ANOVA and means were separated using Tukey's HSD test. The quantitative serology results using the *S.* Typhimurium ELISA were analysed using ANOVA and means were separated by Tukey's HSD test. All analyses were conducted using a computerised statistics package, Statistica™ (StatSoft Inc, 2001, Tulsa, OK, USA).

3. Results

3.1. Birds challenged at day old and maintained in pens

Table 2 shows results of drag swabs collected at intervals throughout the rearing and early laying phase. All treatment groups except for the unchallenged control (Group A) had *S.* Typhimurium PT 135 detected from the litter up to 12 weeks of age. *S.* Typhimurium could be detected from litter drag swabs in the untreated challenged control group (Group B) pen also at 16, 18 and 23 weeks and this was similar in the groups receiving Organic acids (Group D at 16 and 18 weeks and group G at 16 and 23 weeks). *S.* Typhimurium could not be detected in the vaccinated group (Group C) between 16 and 20 weeks but a positive result was returned at 23 weeks in this group. The probiotic treated group (Group E) showed intermittent detection between 16 and 23 weeks while the vaccinated plus probiotic group (Group F) had negative results from 18 to 23 weeks.

Table 3 displays the $\log_{10}$ copies of *Salmonella* DNA detected by PCR in dust collected on settle plates placed in the aisle of the shed outside five of the pens, including that of the unchallenged control group (Group A – pen 23). It shows a moderate level of detection ($\log_{10}$ 3–4 per g dust) up to 12 weeks of age followed by no detection in most plates until a transitory rise was seen at 15–16 weeks. *Salmonella* was not detected again from 18 weeks of age. This is somewhat similar to the drag swab results. One of these plates was near the unchallenged control group pen (Pen 23) and showed possible exposure to that group. Culture of the dust at 9 weeks (using AS 5013.10–2009 method) was positive indicating the organism was viable in this medium, and this was identified as *S.* Typhimurium PT135, the challenge strain.

Fig. 2 shows the proportion of cloacal swabs or faecal samples with presence of *S.* Typhimurium detected at each sampling time. *Salmonella* was not detected at any age from cloacal swabs or faeces from Group A (unchallenged controls – this group is not shown in Fig. 2) until a single faecal sample at 21 weeks. Infection of the trial birds was very efficient from the seeder bird method with between 60 and 80% of samples positive in the first two weeks in the challenged groups. As expected, the birds continued to shed *S.* Typhimurium for up to at least 56 days. *S.* Typhimurium was not detected in any cultured samples after 56 days and the proportion of birds with detectable *S.* Typhimurium in cloacal swabs declined with time (prior to vaccination at 10 weeks of age, the only treatments to have been instigated were the controls and the probiotic treatment; vaccination and organic acid provision did not begin until later). The provision of the probiotic during this time had no effect on the prevalence of *S.* Typhimurium detection. With one exception (group B: challenged untreated control), *S.* Typhimurium was not detected in cloacal swabs from any treatment group from 12 to 18 weeks of age. One cloacal sample from the group which was vaccinated and received both the probiotic and the organic acids gave a positive detection of *S.* Typhimurium PT 135 at 15 weeks.

Taken in context with the drag swabs and dust results, it appears that the organism is shed actively in faeces for up to 8 weeks but is still detectable in litter for up to 18 weeks. It can be detected in airborne dust in the environment for up to 12 weeks and showed the ability to reappear in dust approaching sexual maturity.

Egg production from the birds (sexual maturity) began at 18 weeks of age. Table 3 shows an analysis of faecal *S.* Typhimurium detection for each group over the weeks following sexual maturity. Between 20 and 25 weeks of age, positive detections were found in faecal samples in the challenged control group (Group B), the group receiving the probiotic alone (Group E) and the two groups receiving the organic acids (Groups D and G). The group which had vaccine (Group C) did not have *S.* Typhimurium detected in their faeces in any sample during this time and the vaccinated group which continued receiving the probiotic (Group F) did not have a positive detection after 20 weeks of age. Interestingly, some samples in the unchallenged control group (Group A) revealed positive *S.* Typhimurium PT 135 detection in faeces at 23 weeks of age. Stratified analysis of these results showed significantly less *S.* Typhimurium detection in the vaccinated and vaccinated plus probiotic groups (Table 4).

All positive samples shown in Table 4 were detected by the Australian Standard method (AS 5013.10–2009) but all were below the sensitivity of the MPN test (i.e. <10 cells per g of sample).

All *S.* Typhimurium detected from drag swabs and cloacal or faecal samples (Fig. 1 and Table 4) proved to be consistent with PT 135 (the strain given to the seeder birds at day 1).

Mean $\log_{10}$ ELISA titres of anti- *S.* Typhimurium antibody (X-OvO ELISA) for each group prior to and following vaccination times are shown in Fig. 3 against the positive ELISA cut off value. Prior to vaccination at 10 weeks of age, all groups exhibited serum titre levels well below the positive cut off value. Two weeks after vaccination, serum ELISA titres in the vaccinated groups (i.e. groups C, D, F and G) rose significantly compared to the unvaccinated groups. Interestingly, those groups that were vaccinated and were treated with the probiotic had significantly higher serum titres than the other vaccinated groups (i.e. vaccine alone or combined with organic acids) as can be seen in Table 5. At 13 weeks of age the serum titres had declined markedly and only the vaccinated groups that had also received probiotic (i.e. groups F and G) remained above the positive cut off level of the test. All groups' titres were below the positive cut off level by 16 weeks. Following the oral vaccination at 16 weeks a rise in titre for the vaccinated groups was predicted, however all groups, including the non-challenged group showed an increased titre above the positive cut off at 18 weeks of age and titres continued to rise up to 27 weeks of age.

Table 2
Drag swab results by age for each treatment group.

Treatment	Drag swab results[a] (*S.* Typhimurium) at ages (weeks)						
	4	8	12	16	18	20	23
A Untreated Unchallenged	Neg	Neg	Neg	Neg	Pos	Neg	Neg
B Untreated Challenged	Pos	Pos	Pos	Pos	Pos	Neg	Pos
C Vaccinated	Pos	Pos	Pos	Neg	Neg	Neg	Pos
D Vaccinated + Organic acid	Pos	Pos	Pos	Pos	Pos	Pos	Neg
E Probiotic	Pos	Pos	Pos	Neg	Pos	Neg	Pos
F Vaccinated + Probiotic	Pos	Pos	Pos	Pos	Neg	Neg	Neg
G Vacc + Probiotic + Organic acid	Pos	Pos	Pos	Pos	Neg	Pos	Pos

[a] Neg = negative isolation; Pos = ST detected from swab.

Table 3
PCR detection of *Salmonella* in dust samples from settle plates positioned outside of several pens of birds during the experiment. Pens 12–19 were pens which were challenged with *S.* Typhimurium from 1 day of age, Pen 23 was the unchallenged control group (A) separated by 6 m from the nearest challenged pen.

Bird age (weeks)	Log_{10} *Salmonella* DNA copies/g of settle dust				
	Pen 12	Pen 14	Pen 17	Pen 19	Pen 23 Unchallenged pen
8.5–9.5	3.71	3.61	4.00	3.57	3.16
12	3.62	3.44	3.62	3.60	3.45
14	0.00	0.00	0.00	0.00	0.00
15	2.96	0.00	0.00	0.00	0.00
16	3.10	2.87	0.00	0.00	0.00
18	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00

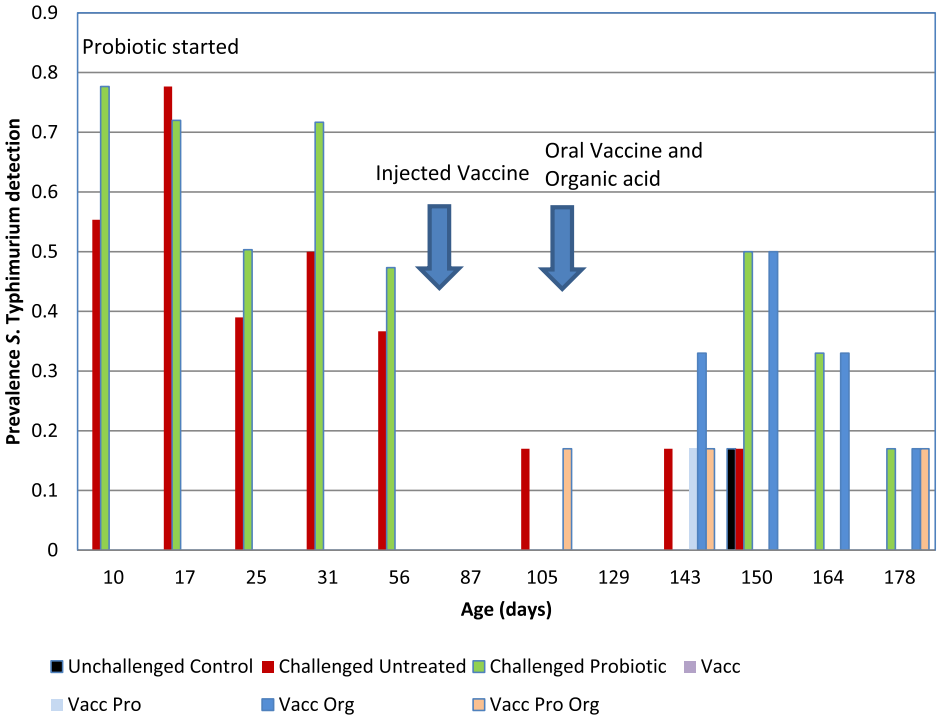

Fig. 2. Prevalence of S. Typhimurium in cloacal swabs or faecal samples at various ages (days) following exposure from seeder chicks at 1 day of age. Probiotic applied over days 1–7 and thence weekly in drinking water; organic acids supplied in feed from 16 weeks to appropriate treatment groups.

Table 4
Stratified analysis for S. Typhimurium detection from faecal samples over weeks 20–25 by treatment group.

Group	Number positive (n/6) at each sampling age (weeks) post sexual maturity				P[1]
	20	21	23	25	
A Uninfected untreated	0	0	1	0	a,b
B Infected untreated	1	0	1	0	a,b
C Vaccinated	0	0	0	0	b
D Vaccinated + organic acid	2	3	2	1	a
E Probiotic	0	3	2	1	a
F Vaccinated + probiotic	1	0	0	0	b
G Vaccinated + probiotic + organic acid	1	0	0	1	a,b

[a,b]Stratified prevalence with different postscripts differ significantly ($P < 0.05$).
Analysed using the StatCalc function of Epi Info™ version 7..
[1] Significant differences determined by Mantel-Haenszel stratified analysis using week as the strata. Corrected χ^2 values used.

3.2. Birds removed from pens and challenged individually

Table 6 shows the proportion of birds showing positive detection of *S.* Typhimurium in their caeca 10 days post challenge with *S.* Typhimurium PT 108 at an oral dose of 10^6 CFU per bird.

Ten days after challenge at 17 weeks of age, 100% of the unexposed control birds (Group A) had detectable *S.* Typhimurium in their caeca. The untreated controls which had been exposed previously to *S.* Typhimurium (Group B) revealed no detection of caecal *S.* Typhimurium at this time, indicating that they were naturally

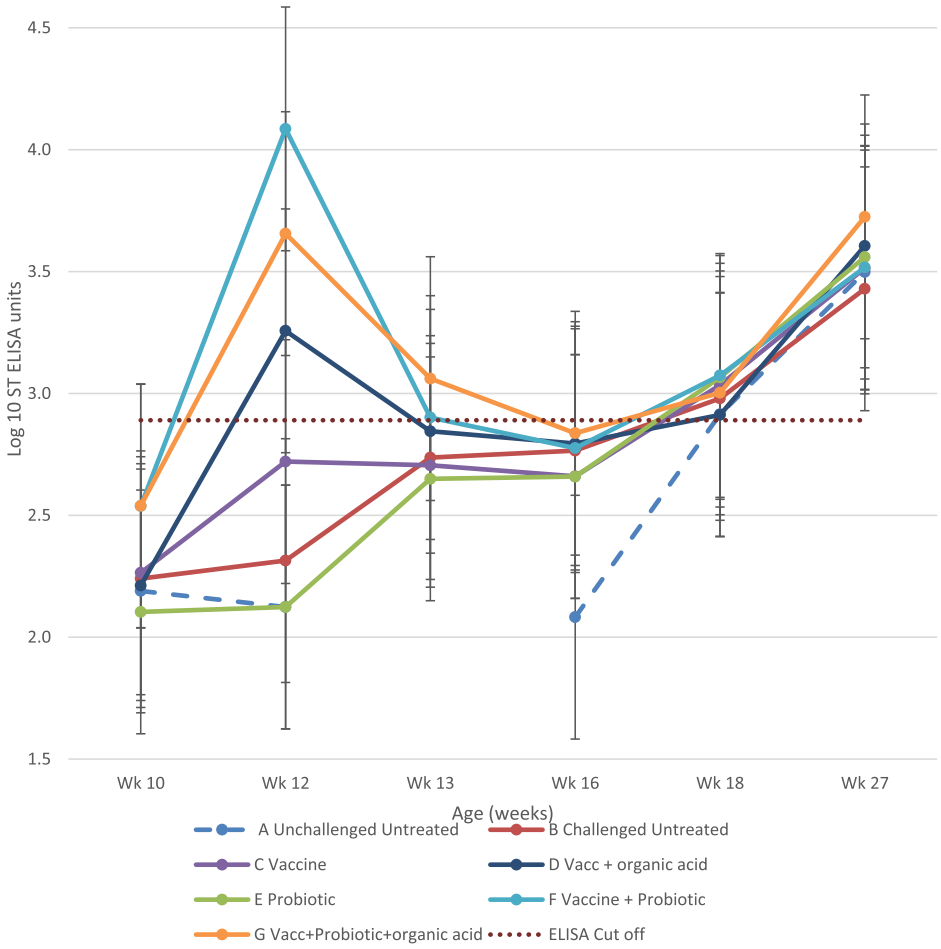

Fig. 3. Serology results for groups prior to and following the first vaccination (at 10 weeks) using X-OvO ST ELISA. Results are shown is mean $\log_{10}$ titre in ELISA units. The positive cut off for the ELISA is Log_{10} 2.89 (dotted line).

Table 5
Serum antibody titres against ST (Log10 ELISA units) at 12 weeks of age (2 weeks post injectable vaccination). Treatments grouped by initial vaccination and probiotic status.

Treatments applied						
Group[1]	Challenged	Vaccine	Probiotic	$\log_{10}$ ELISA titre Wk 12	95% confidence limits	
A Non challenged control	No	No	No	2.124[c]	−0.155	4.403
B Challenged control	Yes	No	No	2.219[c]	1.984	2.571
E Probiotic	Yes	No	Yes	2.123[c]	1.820	2.407
C & D Vaccinated	Yes	Yes	No	2.989[b]	2.406	2.821
F & G Vaccinated + probiotic	Yes	Yes	Yes	3.871[a]	3.000	3.412
	P=			*0.009*		

[a,b,c]Means with different superscripts differ (P < 0.05).
[1] Treatment groups by initial groups and probiotic status (note: prior to 16 weeks of age, organic acids had not been administered to any group).

protected. All of the treated exposed groups also had significantly lower prevalence of *S.* Typhimurium detection in caeca than the uninfected control group. However, when birds were challenged one week later, coinciding with the onset of sexual maturity, only the vaccinated plus probiotic group (Group F) showed a significantly lower *S.* Typhimurium detection in caeca than the unexposed control group. Significant increases in the proportion of positive *S.* Typhimurium caecal detections from 17 to 18 weeks age at challenge were seen in the untreated exposed control birds (Group B), the birds receiving only the probiotic (Group E) and the vaccinated group that was being treated with organic acids (Group D). An increase in the prevalence of *S.* Typhimurium detection in the vaccinated group also receiving probiotic and organic acid (Group G) was also observed although this did not reach statistical significance. The prevalence of *S.* Typhimurium detection in caeca following challenge did not increase during sexual maturity for vaccinated birds and vaccinated birds also receiving the probiotic (Groups C and F).

Table 6 shows the enumeration of *S.* Typhimurium isolated from the birds 10 days post challenge at 17 or 18 weeks of age. With the exception of the unexposed control group (Group A), mean numbers of *S.* Typhimurium detected were very low (generally near 1 $\log_{10}$ per g of caeca). Individual enumerations varied between MPN's of 0 and 10^4. (Table 7).

All previously exposed groups (B to G) had significantly lower *S.* Typhimurium enumeration compared to control group (Group A) at 17 weeks of age when challenged. As sexual maturity occurred, caecal numbers of S. *Typhimurium* tended to increase slightly in the

Table 6
Prevalence of positive detection of S. Typhimurium from caeca of birds removed and challenged orally with 10^6 CFU of ST PT108 at 17 or 18 weeks of age.

Treatment group	Number (and percentage) of birds (n = 8) with positive detection of S. *Typhimurium* in caeca following challenge at each week		Age effect by treatment[1]
	Challenged at 17 weeks	Challenged at 18 weeks	Fisher's exact 2-tailed test
A Untreated unchallenged	8 (100%)[a]	7 (87.5%)[a]	*1.00*
B Untreated challenged	0 (0%)[c]	5 (62.5%)[a,b]	*0.026*
C Vaccinated	3 (37.5%)[b,c]	3 (37.5%)[a,b]	*1.00*
D Vaccinated + organic acid	0 (0%)[c]	5 (62.5%)[a,b]	*0.026*
E Probiotic	3 (37.5%)[b,c]	8 (100%)[a]	*0.026*
F Vaccinated + probiotic	4 (50%)[a,b]	2 (25%)[b]	*0.61*
G Vacc + probiotic + organic acid	2 (25%)[b,c]	6 (75%)[a,b]	*0.13*
Treatment Pearson χ^2 *P=*	*0.00044*	*0.023*	

[a, b, c] Proportions within a column with different superscripts differ (Fisher's exact 2-tailed test, P < 0.05).
[1]Comparison of results of the same treatment at 17 and 18 weeks across a row.

previously exposed groups that were not vaccinated or that were receiving organic acids.

All *S.* Typhimurium isolated from challenged birds were consistent with *S.* Typhimurium PT 108, which was the strain used for the artificial challenge. The early exposure strain (PT 135) was not detected from these challenged birds in the challenge experiment.

4. Discussion

Challenge studies with *S.* Typhimurium in chickens show markedly variable results in effective infection [3]. *Salmonella* infection in chickens is usually assumed to be via the faecal-oral route, however exposure through the respiratory tract may be more efficient [3,33] and this may explain the success of the seeder bird approach seen here.

This study highlights the importance of sexual maturity in the expression and resistance to challenge of birds to *S.* Typhimurium infection and the role that effective vaccination plays at this stage in the hen's life. Caecal colonization is notably expedited at sexual maturity in hens [34]. Birds exposed naturally in early life shed the *S.* Typhimurium PT135 organism for up to 56 days, after which it became temporarily undetectable. Persistence in shedding of *S.* Typhimurium and *S.* Enteritidis by birds infected early in life for up to 8–9 weeks of age have been documented [1,35]. However, the same *S.* Typhimurium PT135 reappeared in faeces at and following sexual maturity except in birds that had been vaccinated using the described protocol.

Table 7
Log10 Most Probable Number (MPN) for *S.* Typhimurium CFU/g caeca detected after oral challenge with 10^6 CFU *S.* Typhimurium PT108 at 17 or 18 weeks of age.

Treatment group	Log_{10} MPN/g caeca 10 days post challenge with *S.* Typhimurium at each age		Age effect by treatment[1]
	Challenged at 17 weeks	Challenged at 18 weeks	ANOVA by week and group, P=
A Untreated Unchallenged	3.558[a]	2.643[a]	*0.098*
B Untreated Challenged	0.000[b]	0.691[b]	*0.209*
C Vaccinated	0.956[b]	0.431[b]	*0.340*
D Vaccinated + Organic acid	0.000[b]	1.254[a,b]	*0.024*
E Probiotic	0.837[b]	1.708[a,b]	*0.115*
F Vaccinated + Probiotic	0.847[b]	0.265[b]	*0.290*
G Vacc + Probiotic + Organic acid	0.543[b]	1.103[b]	*0.309*
Treatment χ^2 *P=*	*0.000002*	*0.000350*	

[a,b]Means within a column with different superscripts differ (ANOVA, P < 0.05).
[1] Comparison of results of the same treatment at 17 and 18 weeks across a row.

As described by Wigley *et al.* (2005) [6] for *S.* Pullorum and by Johnston *et al.* (2012) [7] for *S.* Enteritidis, the temporary suppression of cell mediated immune function (CMI) associated with sexual maturity in the hen allows a re-emergence of these serovars at that stage. This phenomenon is analogous to the *peri*-parturient relaxation of resistance (PPRR) which is well described in ruminants [36]. PPRR also involves a suppression of CMI which affords an upsurge in helminths in early lactation in ewes [36]. This CMI inhibition affords multiplication and excretion of pathogens and, in the hen, is probably responsible for many pathogens emerging from latency at point of lay (e.g. Chicken Anaemia virus, Avian Encephalomyelitis virus, Egg Drop Syndrome 1976 virus as well as salmonellae). But as HMI is not affected, this upsurge in pathogens will serve to stimulate antibody production and lead to higher inclusion of maternal antibody in the egg yolk. The present study provides evidence that this CMI suppression phenomenon will also allow a re-emergence of a previous infection with *S.* Typhimurium. The hen is also rendered more susceptible if challenged with *S.* Typhimurium at this time. This appears to override the protection given by natural exposure to pathogenic *S.* Typhimurium during rearing which could be assumed to be predominantly a cell mediated occurrence. Neither this natural exposure nor oral vaccination with the *aro*-A deletion mutant vaccine evoked a humoral antibody response in the hen in the present study. Beal *et al.* (2004) [1] concluded that the ability to clear *Salmonella* from the intestinal tract related to the competence of the CMI response. These authors also determined that re-challenge of birds at 15 weeks of age was less protected by an early natural exposure than if the birds were primarily exposed a few weeks later. At sexual maturity however, CMI suppression allows increased growth of gut-dwelling microbes, including salmonellae, and this may stimulate humoral antibodies in the absence of cell mediated controls [6]. This rise in serum antibody in all groups seen at sexual maturity in the present study can only be attributed to the resurgence of the natural infection at this time.

If some HMI antibody response is existing in the bird however, in this case because of the injected vaccine, the ability of the gut-dwelling salmonellae to proliferate may be limited [9,13]. The benefit of the use of the probiotic in this study may have been due primarily to an enhancement of the immune (antibody) response to this injected vaccination. A similar enhancement of humoral immunity after parenteral administration of a live *S.* Enteritidis vaccine with this particular probiotic, by stimulating bile antibody, has been reported [37].

The second, oral vaccine application appears also to be of vital importance in achieving *S.* Typhimurium protection. Given after the injected vaccine dose, this may have triggered an anamnestic antibody response which enhanced the vaccination effect. Also, being given 2 weeks prior to sexual maturity, it may have provoked an invasion-inhibition effect [38] against either the resurgence of pre-existing *S.* Typhimurium infection and the oral challenge dose at 18 weeks of age. Methner *et al.* (2010) [38] point out that this effect is short lived, more effective against homologous serovars of *Salmonella* and is not associated with a host response. Speculatively, it could be concluded that the use of the organic acids may have inhibited this effect of the oral vaccination given at 16 weeks of age and hence decreased the protective effect of the vaccination protocol. This may have been slightly ameliorated if the birds were also receiving the probiotic at this time.

The birds which were vaccinated and received a weekly dose of a probiotic in water appeared to have the best protection from both re-emergence at sexual maturity from a previous infection with *S.*

Typhimurium or from an artificial oral exposure of *S.* Typhimurium. This type of improved protection from the combination of probiotic and attenuated vaccine has been shown previously [38]. Another study indicated that protective effects of a combination of a probiotic and a live *Salmonella* vaccine may simply be additive [39].

The sudden detection of *S.* Typhimurium in the unchallenged control group at sexual maturity highlights the importance of this physiological stage of life. Although never detected in this group during rearing, the group was undoubtedly exposed to *S.* Typhimurium at a low level from quite early in the study. Settle plates were positioned in the aisles of the facility and dust was regularly tested. *S.* Typhimurium was detected in the dust from the settle plate outside the unchallenged control pen throughout the study and it could be easily assumed that some exposure within the pen had occurred, albeit at a very low level and perhaps only a in a very small number of birds. This may mimic the field situation where only a few birds may carry very low levels of infection but allowing them to shed reasonable numbers as the flock begins lay. This could lead to a rapid uptake and spread of the infection through a CMI-suppressed flock, allowing long term establishment of the organism in the birds and their environment.

5. Conclusions

When pullets are challenged with *S.* Typhimurium early in life, they will shed the organism in their faeces for up to 8 weeks of age. Shedding then becomes undetectable until the flock reaches the onset of egg laying, when shedding may occur again from a small number of birds at a low level. This is most probably related to the suppression of cell mediated immunity at sexual maturity. The use of the probiotic used in this study via drinking water weekly did not decrease this shedding pattern. When the birds under this challenge are vaccinated with a live *S.* Typhimurium vaccine by injection at 10 weeks, followed by an oral vaccination at 16 weeks, this *Salmonella* shedding pattern is inhibited. The use of the probiotic did appear to enhance the immune response to the injected vaccine and this combination gave the most successful inhibition of *S.* Typhimurium colonization of the caeca when these birds were orally challenged at 17 and 18 weeks (i.e. during sexual maturity in this case).

Birds that experienced a natural-type *S.* Typhimurium challenge using seeder birds during rearing appeared to be protected against further challenge pre-maturity (at 17 weeks), however this protection disappeared one week later, as egg production began. The vaccination protocol was protective against colonization at this immunosuppressed stage and the combination with the probiotic gave the best protective outcome.

The use of the organic acid product in feed from 16 weeks lead to enhanced shedding of *S.* Typhimurium from the infected birds during the resurgence of infection at maturity. The organic acid fed groups were also less protected during challenge at sexual maturity. It is possible that the organic acids may have interfered with the oral vaccination at 16 weeks and thus prevented sufficient vaccinal protection to develop.

CRediT authorship contribution statement

Peter J. Groves: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing - original draft. **Sarah L. Williamson:** Investigation, Resources, Validation, Writing - original draft, Writing - review & editing. **Md. Ahaduzzaman:** Methodology, Resources, Validation, Data curation. **Madeline Diamond:** Investigation, Writing - original draft, Data curation. **Melanie Ngo:** Investigation, Writing - original draft, Data curation. **Anita Han:** Investigation, Writing - original draft, Data curation. **Sue M. Sharpe:** Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing, Validation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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