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Salmonella enteritidis' vaccine produces *in vitro* and *in vivo* protection against colonization

Abstract

Salmonella enteric can be considered as one of the most important causes of food- poisoning with poultry thought to be the main source. Although *S. Typhimurium*, *S. Enteritidis* and the vast majority of other *Salmonella* serovars generally produce little systemic disease in adult chickens, they are able to colonize the alimentary tract of poultry. The two caeca are the main sites of the colonization of *Salmonellae* in chickens, and the bacteria can be easily harvested from the caeca for analysis. Bacterial proteins analyses utilizing SDS-PAGE showed differences between in *vitro* and *in vivo* that out of about 40 protein bands of *in vitro* preparation only a few (3-5) bands can be visualized from *in vivo* preparations. We suggested that some avian proteases might be responsible. Accordingly, and to investigate the hypothesis that an inactivated vaccine harvested from chickens is thought to be more protective than bacteria grown in broth culture, the immunogenicity of inactivated vaccines, either proteins or formalin killed bacteria (*S. Enteritidis*) harvested from chicken intestine and those from broth culture (*in vitro*), were compared using bacterial proteins or formalin killed bacteria as an orally inºCulated vaccine candidate in chicken. The results demonstrated that sonicated proteins extracts in general have a stronger effect as vaccines against *S. Enteritidis* colonization in chickens than formalin killed bacteria. In contrast, the *in vitro* sonicated proteins obtained from a nutrient broth culture had a much better protective vaccine effect than the *in vivo* sonicated proteins preparations harvested from bacteria grown in chickens.

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Introduction

Many bacterial pathogens such as *Clostridium, StaphylºCºCcus*, *Campylobacter* and many other bacterial strains are capable of causing food-poisoning, and *Salmonella enterica* can be considered as one of the most important causes with poultry thought to be the main source. Although *S. Typhimurium*, *S. Enteritidis* and the vast majority of other *Salmonella* serovars generally produce little systemic disease in adult chickens, they are able to colonize the alimentary tract of poultry, resulting in contamination of poultry carcasses and entry into the human food chain. However, there is a great demand to control food-poisoning salmonellosis at both breeder and layer levels at the national and global level in order to produce *Salmonelle*-free poultry products, due to the current correlation between *S. Enteritidis* PT4 and poultry products. Salmonellosis costs the European Union a minimum of 500-900 million Euros annually. Salmonellosis in food animals is a major target for reduction of human infection by the European Union. Legislation has been introduced to monitor the most important *Salmonellae* serovars. The major *Salmonellae* serovars of public health consequence are *S. Typhimurium* and *S.* Enteritidis (causing 15% and 60% respectively of all cases in Europe in 2002).

Elazomi and Dhawi studied newly-hatched chickens infected with *S. Enteritidis*. They analyzed proteins of *S. Enteritidis* in the caeca of 1-day old checks (*in vivo*) together with a comparison with nutrient broth medium (*in vitro*) in order to detect changes in the pattern of protein expression during infection. The preliminary exploratory study of individual bands identified major proteins (flagellin of *S. Enteritidis* and *Typhimurium fliC*) and mixtures of proteins including 60kDa chaperonin *groEL* and glyceraldehyde-3-phosphate dehydrogenase *gap A*. Some proteins may be expressed equally both *in vivo* and *in vitro* (e.g. fimbrial , flagellar, outer membrane protein, metabolic , regulatory, and LPS-synthesis encoded genes). These proteins are predicted to play a major role in colonization. Chicken caecal colonization by paratyphoid *Salmonella* (e.g. Enteritidis, Typhimurium and others) has been linked to the physical attachment by fimbriae[1] motility,[2] type three secretion system (T3SS) of *Salmonella* Pathogenicity Islands "SPI-1 and SPI-2",[3] bacterial cell wall component lip polysaccharide "LPS"[4,5] and outer membrane proteins "OMPs".[6] This comparison showed differences between the two profiles and indicated that it is difficult to make a reasonable comparison as out of about 40 protein bands of *in vitro* preparation only a few (3-5) bands can be visualized from *in vivo* preparations, the reason behind that thought to be the degradation of *in vivo* protein with some avian proteases.

Then we hypothesized that inactivated vaccines prepared from bacteria grown *in vivo* in chickens will give better protection than a vaccine prepared from bacteria cultured in vitro because they will be expressing antigens normally expressed during infection/ colonisation. Accordingly, and to investigate the hypothesis that an inactivated vaccine harvested from chickens is thought to be more protective than bacteria grown in broth culture, the immunogenicity of inactivated vaccines, either proteins or formalin killed bacteria (*S. Enteritidis*) harvested from chicken intestine and those from broth culture (*in vitro*), were compared using bacterial proteins or formalin killed bacteria as an orally inºCulated vaccine candidate in chicken.

Materials and methods

Preparation of formalin-killed bacterin from bacterial

Cells cultured *in vitro* in nutrient broth

A single colony of *S. Enteritidis* PT4 (antibiotic sensitive parent strain) was inºCulated into 10ml NB and incubated overnight at 37 °C and 1ml of this broth culture was transferred into 2 x 100ml NB in 250ml flasks and incubated for two hours at 37 °C in a shaking incubator at 200rpm. Each flask was then decanted to three 50ml Falcon tubes each containing 33.3ml, the tubes then centrifuged at 5000g for 30min at 20°C and the supernatants were discarded

without disturbing the pellets. Subsequently the pellet from each tube was re-suspended with 3.33ml NB, then the content of three Falcon tubes arising from the same flask were mixed into one Falcon tube. The contents (10ml NB 109 bacterium/ml) which is equivalent to 108/0.1ml=3x108 bacterium/0.3ml = 5x108/0.05ml which used for chicken injection (i.m) to both breast sides.

Preparation of formalin-killed bacterin from bacterial

Cells cultured *in vivo* in chicken caeca

90 newly hatched chickens were in°Culated orally with *S. Enteritidis* PT4 (antibiotic-sensitive parent strain) culture (1x106 cell). On the following day the chickens were killed and the infected caecal contents were collected. Caecal contents from three of the chickens were collected separately for viable count estimations and purity checking. Caecal contents from the remaining birds were collected together in two Falcon tubes in dry ice (1 tube each day as the collection was made over two separate days (from 15 and 40 birds respectively) and used for this vaccine preparation. The first tube that contained the caecal contents of 15 birds contained 0.936g and 2.5x1010 cfu/ml and tube two that contained the caecal contents of 40 birds contained 4.652g and 1x1011 cfu/ml; so the total weight was 5.5gm in 5.5ml. Therefore, the total bacterial number was 1.25x1011 bacteria. Both tubes were diluted together in 12.5ml PBS and mixed well and 3ml were collected from them and added to 27ml of NB for formalization. Each sample was diluted to thus contain 1x109cfu/ml and was stored at -80° C until used.

Formalization of *S. Enteritidis* killed vaccine

To each bottle of 10ml suspension of bacteria 0.2ml of 40% formaldehyde was added and vortexed for 30s and divided equally between 10 x 1ml sterile tubes (Eppendorff) and stored in a cold room at 4 °C until needed.

Preparation of sonicated protein vaccine

Whole cell proteins were prepared from *S. Enteritidis* bacteria harvested from chickens or from a NB culture.

Preparation of *in vivo S. Enteritidis* protein vaccine

A total of 100 newly hatched chickens were in°Culated orally within 18 h of hatching. Chickens were infected orally with 0.1 ml of a culture of the antibiotic-sensitive parent *S. Enteritidis* PT4, grown for 16 h in nutrient broth at 37ºC and diluted in sterile nutrient broth to contain 107 cfu/ml. After 16-18 hs post-infection chickens were killed one-by-one, and the caecal contents were harvested from both chicken caeca of each bird. The caecal contents of three randomly chosen chicks were transferred to three separate sterile universal tubes which were placed on ice to test for purity and viable bacterial number estimation on MacConkey agar and nutrient agar. The caecal contents of the remaining chickens were put in 50 ml Falcon tubes in dry ice and then were stored at -80°C until needed. Three chicks were left without in°Culation and their uninfected caecal contents were used to streak on MacConkey agar and nutrient agar plates and incubated for overnight at 37ºC to ensure that there were no contaminants with other bacteria. For the vaccine preparations the *S. Enteritidis*-infected caecal contents were diluted in nutrient broth and then centrifuged at 20,000xg for 5 min at 4ºC (Avanti®J-E Beckman centrifuge coulter), then the supernatant was discarded and the pellets were resuspended in NB, followed by sonication (Sonics VCX500) for 5 min immediately after adding the protease inhibitors (Sigma P8465). This sonicates was then centrifuged at 15,000 g for 10 min at 4°C. Subsequently, the supernatant was filtered using 0.45 µm filters and stored in 1ml aliquots in Eppendorff tubes at -20°C until required.

Preparation of *in vitro S. Enteritidis* protein vaccine

A single colony of the parental *S. Enteritidis* PT4 sensitive strain was picked and used to in°Culate 10ml NB in a universal bottle which was then incubated overnight at 37°C. On the following day 250ml flasks, each containing 100ml nutrient broth, were in°Culated with 1ml of the overnight broth culture of *S. Enteritidis* PT4 and incubated overnight at 37°C shaking incubator (150 rpm). The contents of these broth cultures were divided into four 50ml centrifuge tubes and centrifuged at 20,000 x g for 5 min at 4ºC (Avanti®J-E Beckman centrifuge coulter).The pellet from each tube was resuspended in 5ml NB and sonicated for 5 min (Sonics VCX500)) after adding the protease inhibitors (Sigma P8465), followed by centrifugation at 15,000xg for 10 min at 4°C and filtration as mentioned above. The proteins preparations were then stored at -20°C until required.

Vaccine Quality Control

Protein sonicates harvested from both *in vivo* and in vitro environment were streaked on MacConkey and nutrient agar plates which were incubated overnight at 37°C to chick for any *Salmonella* growth, this also applied for the formalized bacteria.

First vaccination experiment

A lot of 100 1-day commercial layer chickens obtained from Millennium Hatchery Hy-Line UK Ltd (Studley Warwickshire), were utilized in this experiment. On the day of arrival birds were divided into five groups each of 20 birds with each group being placed in separate rooms which were cleaned down with a mixture of water and disinfectant (Trigene Disinfectant 20L Clear from Scientific Laboratory Supplies Ltd (CLE1320). Followed by chemical fogging with Virkon disinfectant from Sigma (Z692158), this allowed to rest for more than 24h and rinsed off. Chicks were distributed between the rooms as follows (*in vivo* formalin-killed – Room I; *in vivo* sonicated proteins – Room II; *in vitro* formalin-killed – Room III; *in vitro* sonicated protein – Room IV; unimmunized – Room V). All chickens were kept on solid flooring covered with wood straw and were provided with food and sterile drinking water. Subsequently, all birds in all groups were in°Culated with 0.1 ml of neat Avigard gut microflora (Microbial Developments Limited, UK), then at the fifth day of age all chickens were in°Culated intramuscularly (i/m), into the breast muscle, with 0.05ml containing either formalin-killed bacterial cells or protein preparation. Chickens were also in°Culated orally with 0.1 ml of the corresponding vaccine for each group as shown in Table 1. The remaining group number five (control) was in°Culated with sterile NB. At three weeks of age the vaccination program was repeated with all birds in°Culated with 0.3 ml orally and 0.1 ml i/m using the corresponding vaccine for each group. All birds were challenged with 0.5 ml of NB culture (3 x 10^8 cells) of a nalidixic acid resistant (Nal^R) mutant of *S. Enteritidis* strain at week 5 of their age. Cloacal swabs were collected from all birds at 1st, 2nd, 3rd, 4th, 7th, 14th, 21st and 28th day post- challenge for a semi-quantitative estimation of bacterial shedding [7,8] of the challenge *S. Enteritidis* NalR by plating on BG agar supplemented with nalidixic acid (20µgm/ml-1) and novobioCin (1µgm/ml-1). On day 28 post-infection after collection of cloacal swabs all birds were slaughtered and their caecal contents were collected for a semi-quantitative *S. Enteritidis* NalR count estimation.

Second vaccination experiment

This experiment was different from the first experiment only in the route of challenge and types of sample collected. The birds and groups were identical to those in the first experiment. As in experiment 1

all birds in all groups were inºCulated with 0.1 ml of neat Avigard gut microflora (Microbial Developments Limited, UK), followed by intramuscular and oral vaccination as before as shown in Table 2. When birds were 3 weeks of age the vaccination program was repeated as before. Subsequently, all birds were challenged intravenously via the wing vein with 0.1ml (1x106 cells) of *S. Enteritidis* Nal^R at 5 weeks age. Five birds from each group were selected randomly and killed at 1, 4, 6 and 8 days post-challenge. Immediately after killing each bird was sprayed with 70% ethanol and then spleen and liver samples were collected in pre-labelled, pre-weighed sterile universal bottles using separate sterile scissors and forceps. During sample collection all organs sampled were observed for any clinical signs. The caecal contents for each bird were then collected separately in pre-weighed sterile universal bottles. The three bottles for each bird were kept on ice prior to reweighing and diluting in x 9 the weight of the sample in PBS. All tissue samples (liver and spleen) were kept on ice until they weighed and then proportional amounts (10xweight expressed as volume) of PBS (pH 7.2) were added into each tube. Each tissue portions was homogenized in a Griffiths tubes in PBS (pH 7.2) to obtain homogenous suspension[2] prior to dilution for counting. This together with ax9 dilution of the caecal contents were used for bacterial count estimations.

Table 1 Vaccine administration regime and sample collection for vaccination experiment one (oral challenge)

ay/Group	Group I *in vivo* formalin killed bacteria	Group II *in vivo* sonicated proteins	Group III *in vitro* formalin killed bacteria	Group IV *in vitro* sonicated proteins	Group V unvaccinated control
1	0.1ml Avigard orally	0.1 ml Avigard orally	0.1ml Avigard orally	0.1ml Avigard orally	0.1 ml Avigard orally
5	0.05ml *in vivo* killed bacteria i/m	0.05 ml *in vivo* protiens i/m	0.05ml *in vitro* killed bacteria i/m	0.05ml *in vitro* proteins i/m	0.05ml sterile NB i/m
	0.1ml *in vivo* killed bacteria orally	0.1 ml *in vivo* proteins orally	0.1 ml *in vitro* killed bacteria orally	0.1ml *in vitro* proteins orally	0.1 ml sterile NB orally
21	0.1ml *in vivo* killed bacteria i/m	0.1 ml *in vivo* proteins i/m	0.1 ml *in vitro* killed bacteria i/m	0.1ml *in vitro* proteins i/m	0.1 ml sterile NB i/m
	0.3ml *in vivo* killed bacteria orally	0.3 ml *in vivo* proteins orally	0.3ml *in vitro* killed bacteria orally	0.3ml *in vitro* proteins orally	0.3 ml sterile NB orally
35	All groups were challenged with 0.3ml contains ($5X10^8$) *S. Enteritidis* orally				
Cloacal swabs	Cloacal swabs were collected from each birds in all groups for *Salmonella* count at day 1,4,7, 14, 21 and 28 post challenge				
Caecal contents	All birds were killed at week nine of their age and caecal contents were collected from each bird in all groups for *Salmonella* count				

Table 2 Vaccine administration regime and sample collection for vaccination experiment two (intravenous challenge)

y/Group	Group I *In Vivo* formalin killed bacteria	Group II *In Vivo* sonicated proteins	Group III *In Vitro* formalin killed bacteria	Group IV *In Vitro* sonicated proteins	Group V unvaccinated control
1	0.1 ml Avigard orally	0.1 ml Avigard orally	0.1 ml Avigard orally	0.1 ml Avigard orally	0.1 ml Avigard orally
5	0.05 ml *in vivo* killed bacteria i.m	0.05 ml *in vivo* protiens i.m	0.05 ml *in vitro* killed bacteria i.m	0.05 ml *in vitro* protiens i.m	0.05ml sterile NB i.m
	0.1 ml *in vivo* killed bacteria orally	0.1 ml *in vivo* proteins orally	0.1 ml *in vitro* killed bacteria orally	0.1 ml *in vitro* proteins orally	0.1 ml sterile NB orally
21	0.1 ml *in vivo* killed bacteria i.m	0.1 ml *in vivo* proteins i.m	0.1 ml *in vitro* killed bacteria i.m	0.1 ml *in vitro* proteins i.m	0.1 ml sterile NB i.m
	0.3ml *in vivo* killed bacteria orally	0.3 ml *in vivo* proteins orally	0.3ml *in vitro* killed bacteria orally	0.3ml *in vitro* proteins orally	0.3 ml sterile NB orally
35	All birds in all groups Challenged intravenously with 0.1 ml containing ($1x10^6$) Live *S. Enteritidis*				
Post challenge sample collections	Five randomly selected birds from each group were killed at day 1, 4, 6 and 8 post infections, tissue portion of their spleen and liver plus caecal contents were collected for *Salmonella* count.				

Table 3 Effect of vaccinating with formalin-killed *S. Enteritidis* and whole-cell sonicated protein preparation on faecal excretion of *S. Enteritidis* NalR (challenge strain),results obtained from direct plates; plus results of BGA enriched plates shown number of *S. Enteritidis* NalR positive birds (positivity %), from cloacal and caecal sample collected at different time points post infections, chicks were orally inoculated

Percentage of chickens (20 birds per group) excreting *S. Enteritidis* nalr (challenge strain) from direct plates and number of positive birds (Positivity %) from enriched plates at different time points post-infection

Sample	Days PI	*In Vivo* Killed Bacteria		*In Vivo* Proteins		*In Vitro* Killed Bacteria		*In Vitro* Proteins		Unvaccinated	
		Direct	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched	Direct	Enriched
		≥ 50	≥ 1 Birds(No& %)	≥50	≥1 Birds(No& %)	≥ 50	≥ 1 Birds(No& %)	≥50	>1 Birds(No& %)	≥ 50	≥ 1 Birds(No& %)
Cloacal swabs	1	0%	0% 13 (66 %)	0%	10% 10(52%)	5%	33% 9 (43 %)	0%	16% 5(26%)	0%	20% 9 (45 %)
	4	0%	24% 9 (43 %)	14%	38% 16(81%)	0%	10% 11 (57 %)	0%	16% 5(26%)	0%	25% 10 (52 %)
	7	0%	29% 10 (52 %)	5%	5% 7(33%)	0%	5% 12 (62 %)	0%	0% 6(32%)	5%	5% 10 (50 %)
	14	0%	0% 6 (29 %)	0%	0% 3(14%)	5%	5% 4 (19 %)	0%	0% 0(0%)	5%	10% 3 (15 %)
	21	0%	0% 4 (19 %)	0%	0% 2(10%)	0%	0% 3 (14 %)	0%	0% 0(0%)	5%	15% 4 (20 %)
	28	0%	0% 3 (14 %)	0%	0% 2(10%)	0%	0% 2 (10 %)	0%	0% 0(0%)	0%	15% 4 (20 %)
Caecal content	28	0%	0% 0(0%)	0%	0% 0(0%)	0%	0% 0(0%)	0%	0% 0(0%)	5%	20% 7 (35 %)

Citation: Elazomi A, Rahman MEA, Dhawi A, et al. *Salmonella enteritidis'* vaccine produces *in vitro* and *in vivo* protection against colonization. *J Microbiol Exp.* 2017;4(3):11–12. DOI: 10.15406/jmen.2017.04.00112

Table 4 The protective effect of formalin-killed bacteria and protein preparation from *S. Enteritidis* harvested from chickens *in vivo* or nutrient broth *in vitro*measured by liver and spleen counts of chicks inoculated intravenously by the parent strain. Log_{10} mean viable counts of *Salmonella* per ml of homogenized liver tissue of 5 birds from each group/time poin

Days PI	*In Vivo* Killed Bacteria						*In Vivo* Proteins						*In Vitro* Killed Bacteria						*In Vitro* Proteins						Unvaccinated					
	Liver			Spleen			Liver			Spleen			Liver			Spleen			Liver			Spleen			Liver			Spleen		
	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P
1	3.9	0.1	0	5.1	0.04	0.23	3.8	0.1	0.07	5	0.03	0.2	4	0.1		4.6	0.2		4	0.1	0.03	4.7	0.1	0.7	3.4	0.08	-	4.8	0.15	-
4	3.6	0.1	0.08	5.3	0.15	0.5	3.5	1	0.08	5.2	0.1	0.1	3.2	0.1	0.18	5.2	0.1	0.1	3.1	0.1	0.2	5.1	0.1	0.2	2.3	0.5	-	4.7	0.19	-
6	2.6	0.1	0.04	4.9	0.13	0.1	2.6	0.2	0.08	4.8	0.1	0.08	3	0	0.04	4.9	0.2	0.1	3	0.1	0.02	4.8	0.1	0.1	0.9	0.6	-	3.1	0.48	-
8	2	0	0.1	4.5	0.6	0.09	2.06	0.06	0.8	4	1	0.8	2.2	0.1	0.4	4.9	0.3	0	1	0.2	0.12	4.1	0.2	0.7	2.3	0.7	-	4	0.13	-

Table 5 The effect of formalin-killed bacteria and protein preparation of *S. Enteritidis* harvested from *in vivo* and *in vitro* conditions on colonisation of chicken caeca with lactose fermentor bacteria when challenged intravenously by the parent strain *S.* Enteritidis NalR. (Average viable counts log_{10} per 1ml of caecal contents).

Days post infection	*In Vivo* Killed Bacteria			*In Vivo* Proteins			*In Vitro* Killed Bacteria			*In Vitro* Proteins			Unvaccinated		
	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P	Log_{10}	SE	P
1	2	0	0.3	2	0	0.07	4.7	0.2	0.04	3	0.2	0.6	2.7	0.7	
4	2.6	0.37	0	2.5	0.3	0.08	4.8	0.2	0	2.3	0.2	0.18	2	0	-
6	2.7	0.28	0.9	2.6	0.2	0.08	4.9	0.2	0.01	2.8	0.2	0.64	2.65	0.4	-
8	2.9	0.56	0.15	2.9	0.5	0.3	4	0.8	0.1	3.2	0.8	0.61	2.46	0.3	-

SE, Standard Error; P, P value.

Bacterial Culture and Count

Enumeration of bacteria in chicken faeces (Experiment I)

After collection of all swabs 2ml selenite broth (Oxoid, CM0395) were added to each tube, followed by brief vortexing. Once they were mixed, each swab was placed in a standard manner on brilliant green agar plate (BGA) supplemented with nalidixic acid (20µgm/ml^{-1}0) and novobi^{o}Cin (1µgm/ml^{-1}). BGA in a standard manner.[9] The in^{o}Culated plates and the selenite broths were incubated overnight at 37°C. Then the swabs were left into selenite broth tubes for overnight incubation at 37°C prior to plating on BGA, to encourage the growth of *Salmonellae* and inhibit the growth of other flora. Then the overnight incubated swabs were plated again on the antibiotic-containing BGA media and incubated overnight at 37°C. Plates in^{o}Culated directly were read and observed for *Salmonella* growth using a semi-quantitative estimation of faecal shedding and caecal colonization of *Salmonella* from infected chickens.[1,7,10-12] Next day the enrichment plates were also checked for *Salmonella* growth. Xylose Lysine Deoxycholate (XLD) media (Oxoid, CM0469) was used as a confirmatory test for any *Salmonella* growth. Suspect colonies were sub-cultured on this media and incubated overnight at 37°C, and the plates were checked for black colonies indicating *Salmonella* as a result of H_2S production, in addition to slide agglutination tests.

Bacterial enumeration in tissues samples (Experiment II)

The bacterial count of *S. Enteritidis* Nal^{R} in spleen, liver and caecal contents for the 5 birds of each group (at day 1, 4, 6 and 8 post challenge), were estimated by serial dilution and plating aliquots of dilutions.[9] Aliquots of each dilution were plated on BGA plates supplemented with nalidixic acid (20µgm/ml^{-1}) and novobi^{o}Cin (1 µgm / ml^{-1}) and incubated overnight at 37°C. Bacterial colonies were counted and the viable count converted into Log_{10} numbers. The Xylose lysine Deoxycholate medium XLD (Oxoid, CM0469) and slide agglutination tests were also used as confirmatory test to confirm any *Salmonella* growth.

Data Analysis

Analysis of data obtained from experiment I

Cloacal swabs were taken from each bird two days previous to challenge in^{o}Culation for culture to guarantee that the chicks are free from *Salmonellae*. Differences in percentage excretion rates between groups of birds were compared using χ^2, and this was considered as statistically significant if the P value was (<0.05).

Analysis of data obtained from Experiment II

As in experiment I Cloacal swabs were taken from each bird two days before being challenged for culture to guarantee that the chicks are free from *Salmonellae.* The bacterial counts of *S. Enteritidis* Nal^{R} (challenge) of the tissues (spleen and liver) and caeca in different groups on BGA plate, in different time points were recorded and the P value of each group compared to the control group were calculated using Student's unpaired t test (Microsoft Office 2007). A P value of (< 0.05) was considered as statistically significant.

Results

It was decided to carry out experimental *in vivo* infection using 1-day old chicks primarily to avoid the development of intestinal microflora, which would be likely to have a significant effect on interference in interpreting the patterns of protein expression in *S. Enteritidis* as well as to enable the bacterium of interest (*S. Enteritidis*) to multiply extremely well in the absence of competitive colonizers.[2,13] Using birds aged from 2-6 weeks is the best model to study *Salmonella* colonization of chicken, as their gut flora is mature (Barrow, personnel communication), but for studying *Salmonella* proteins this might give a false results due to cross contaminations of gut flora. The model used therefore, uses colonization during infection in newly-hatched chickens and which might ^{o}Ccur in a hatchery. Interestingly, a study of *Campylobacter jejuni* gene transcription using a similar model revealed a similarity between this model and birds that had already established their flora.[13] On the other hand, other researchers have used a different model (spectinomycin-treated mouse) with *E. coli*.[14,15] So there is good reason to suppose that the protein expression of *Salmonella* in newly hatched chickens may also be similar to that in old chicks with established flora. In addition, the caeca empty and fill several times a day, the phases of growth of the in^{o}Culated bacteria in individual birds may be different. However, it will be clear that major differences in transcription between *in vivo* and *in vitro* culture will be identified. A

protein analysis of *S. Enteritidis* in the caeca of 1-day old checks (*in vivo*) together with a comparison with nutrient broth medium (*in vitro*) was used to detect changes in the pattern of protein expression during infection and in particular to identify proteins that enable this strain to colonise the caeca. It was decided to use *S. Enteritidis* proteins extracted from stationary phase as the protein concentration of mid log phase was very low. The problems assºCiated with harvesting *in vitro* grown cultures also apply, particularly relating to a synchronicity of growth. The issues assºCiated with cultures of this sort are that (i) bacterial growth is asynchronous. However, bacterial growth *in vivo* will also be asynchronous, and (ii) the relevance of using a rich nutrient broth culture as a control can be questioned. However, there is no truly rational way of establishing a control for *in vivo* growth and previous work,[16] indicates that our approach is an appropriate approach. We compare the immunogenicity of bacteria (*S. Enteritidis*) harvested from the intestine with those grown *in vitro* in nutrient broth cultures. The preparations would include (i) formalin-killed whole bacteria harvested from the caeca, (ii) whole cellular proteins prepared from *in vivo*-cultured bacteria and (iii) *in vitro*-grown bacteria in NB (iv) whole cellular proteins prepared from *in vitro*-grown bacteria in NB all of which would be tested for their ability to protect against *Salmonella* colonization in chicken.

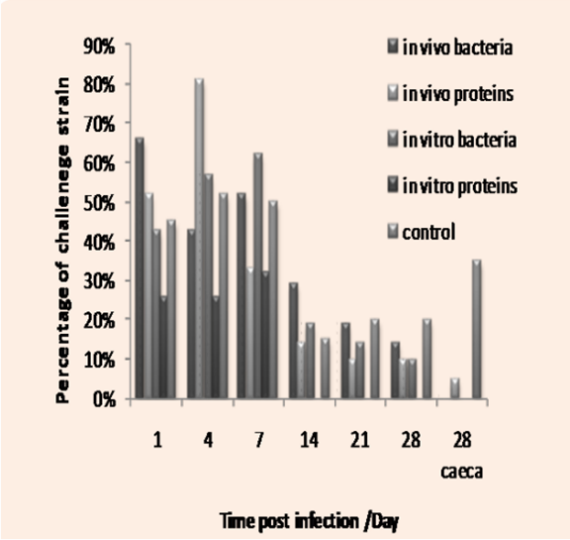

Figure 1 Faecal excretion of challenge *S. Enteritidis* strain following vaccination with formalin killed Salmonella harvested from chickens (*in vivo*) or nutrient broth (*in vitro*) plus Salmonella proteins produced from bacteria cultured either in chickens (*in vivo*) or in nutrient broth (*in vitro*) compared with unvaccinated control, this figure also shown that no growth of any Salmonella were detected in caecal contents of all treated groups 4 weeks post infection.

Quality control of the vaccine

No growth was detected after culturing the formalized *S. Enteritidis* PT4, as well as protein sonicates harvested from both *in vivo* and *in vitro* environments on MacConkey and nutrient agar plates for overnight at 37 °C.

Results for experiment I (orally challenged chicks)

No *Salmonella* organisms were isolated from the chickens on receipt. The percentage excretion rates of the challenge *Salmonella* strain in the different groups are shown in Table 3. When *Salmonella* was cultured by direct plating if the colony numbers present per plate was 1 or more this was designated as ≥1, while when they were 50 colonies or more this was designated as ≥50.[17] The bacteria cultured by enrichment followed by plating were shown as the percentage of positive swabs, which had been confirmed by XLD agar and slide agglutination tests as shown in Table 3.

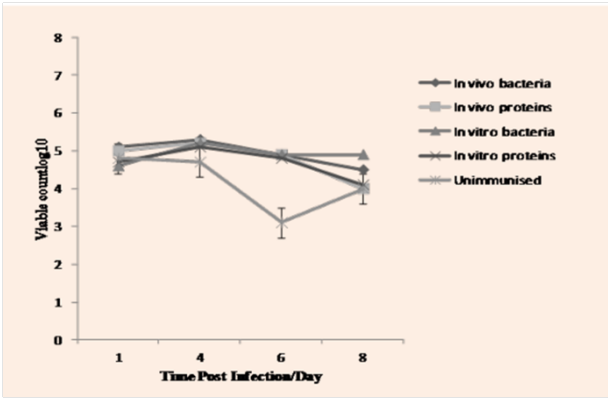

Figure 2 The number of *Salmonella* Log_{10} cfu/ml in chicken's spleen tissue in the groups of birds (each of 20 birds) treated with either *S. Enteritidis* whole cellular *in vivo* and *in vitro* sonicated proteins preparation or *in vivo* and *in vitro* formalin killed *S. Enteritidis* compared with unimmunised control post challenge with parent strain *(*S. *Enteritidis* NalR) inoculated intravenously.

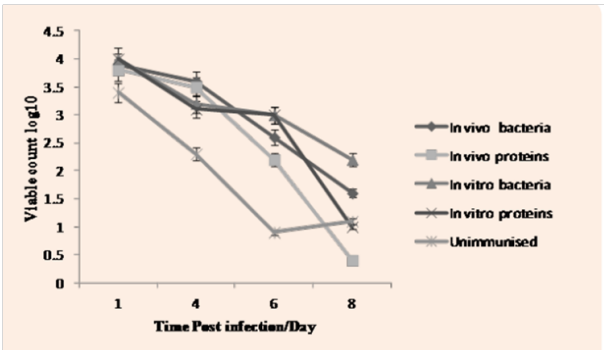

Figure 3 The number of Salmonella Log_{10} cfu/ml in liver tissue in the groups of birds treated with either S. Enteritidis whole cellular *in vivo* and *in vitro* sonicated proteins preparation or *in vivo* and *in vitro* formalin-killed *S. Enteritidis* compared with unimmunised control post challenge with parent strain (*S. Enteritidis* NalR) intravenously.

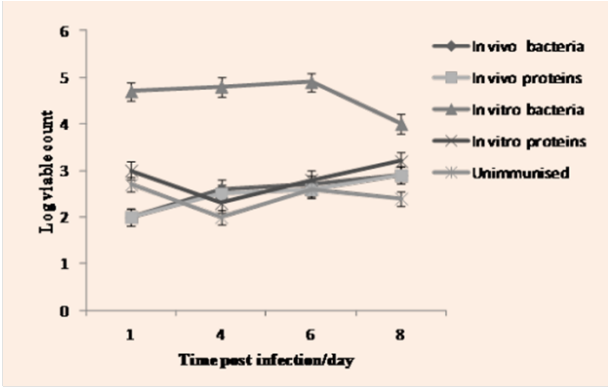

Figure 4 The number of lactose fermentor bacteria Log_{10}cfu/ml-1 in caecal contents in the five groups of birds treated with *in vivo* or *in vitro* protein preparations of *S.Enteritidis* plus formalin killed bacteria harvested from *in vivo* and *in vitro* conditions compared with unimmunised group at 1st, 4th, 6th and 8th day post intra-venous infection with challenge strain *S. Enteritidis* NalR.

Based on evaluation of the results of all samples collected (either caecal contents or cloacal swabs), the percentage of chickens positive for *S. Enteritidis* Nal^R challenge strain for the first day post-infection was 66% and 43% for the birds in group one and three which were treated with formalin killed bacteria harvested from *in*

vivo and *in vitro* environment respectively, whereas the percentage of faecal excretion of the challenge strain in group two and four which were treated with *in vivo* and *in vitro* sonicated proteins preparation respectively were 52% and 26%, compared with 45% in untreated control (group five). There were a variable change of these percentages at 4th day post infection as the percentage remained same (26%) in group four which had been treated with *in vitro* sonicated proteins while in group one, two and three the percentages were 43%, 81% and 57% respectively, with 52% in the control group. Subsequently, one week post infection the corresponding values were 52%, 33%, 62% and 32% in treated groups from one to four respectively and 50% in the unvaccinated control group. Then there was a noticeable decrease in the percentages of faecal excretion in all groups two weeks post infection as the percentages were 29%, 14%, 19%, 0% and 15% in groups from one to five respectively. Moreover, three weeks post challenge the percentage of positive birds' faecal excretion was 19 % for the *in vivo* killed vaccine group (group one) and 10% for the *in vivo* sonicated proteins (group two), and it was 14% for the *in vitro* killed vaccine group (group three), while there was no faecal excretion (0%) in group four which was vaccinated with *in vitro* protein preparation with no difference from 2nd week post infection compared with (20%) of unvaccinated control group. There were no noticeable difference in faecal excretion in week four post infection with percentages 14%, 10%,10%, and 20% in groups from 1 to five respectively. Figure 1 below illustrate the percentages of chickens' faecal exertion of *S. Enteritidis* Nal^R (challenge strain) at different time points (1st ,4th, 7th, 14th, 21st, and 28th) days post infection. In summary, both *in vitro* and *in vivo* protein preparations had a much greater immunization effect than that produced by killed bacteria. The *P* values were (χ^2=28.3. *P*<0.001) and (χ^2=16.77. *P*<0.001) for the *in vitro* and *in vivo* proteins treated groups respectively, which were considered as statistically significant. The *in vivo* protein preparation unexpectedly had a lower immunogenic effect than did the *in vitro* proteins preparation. From the caecal samples collected at week four post infection *Salmonella* was detected only in unimmunised control (group five), while no growth of any *Salmonella* were observed in all treated groups (<1x102cfu/ml) as shown in Table 3 above and Figure 1 below.

Results for experiment II (Intravenously challenged chicks)

The results presented in Table 4 shows the averages ofLog_{10} *Salmonellae* counts in liver and spleen of five chickens taken at different time points post-infection from the four groups of immunised birds plus the control group with the *P* values. No *Salmonellae* were detected in caecal contents of any bird from the group immunised with sonicated proteins, or groups treated with formalin-killed bacteria although some other bacterial growth such as *E. coli, Klebsiella* were observed as illustrated in Table 5 and Figure 4. *Salmonellae* challenge organisms were detected in the control group. Moreover, as shown in Figure 2 below when chickens were challenged intravenously with parent *S. Enteritidis* Nal^R, the viable counts (Log_{10}) of *Salmonellae* in the spleen on the 1st day post-infection were 5.1, 5, 4.6 and 4.7 cfu/ml in group I, II, III and IV which were vaccinated with *in vivo* killed bacteria, *in vivo* proteins, *in vitro* killed bacteria and *in vitro* proteins respectively, whereas the count in unimmunised control group (V) was 4.8 cfu/ml. Surprisingly, the count of *Salmonellae* in spleen tissues on the 4th day post-infection in all immunised groups (Log 5.3, 5.2, 5.2 and 5.1 cfu/ml) respectively, were all higher than unimmunised control group which was Log 4.7cfu/ml, with only very small variations of *Salmonellae* count on the 6th day post infection 4.9, 4.8, 4.9 and 4.8 cfu/ml for the groups immunised with *in vivo* killed bacteria, *in vivo* proteins, *in vitro* killed bacteria and *in vitro* proteins respectively, compared with 3.1 cfu/ml in control group. There were no big differences observed in chicken spleen colonization with *Salmonellae* on the 8th day post challenge in all treated groups as well as unimmunised control group with bacterial count log_{10} 4.5, 4.0, 4.9, 4.1 and 4.0 cfu/ml respectively. At 1 day post-infection the bacterial count in liver were Log 3.9 and 4.0 cfu/ml for the birds in group I and III, which were immunised with *in vivo* and *in vitro* formalin-killed bacteria respectively, whereas the *Salmonellae* count in groups two and four immunised with *in vivo* and *in vitro* proteins preparation were Log 3.8 and 4.0 cfu/ml. This result was unexpected as the counts in all vaccinated groups were again higher than that of unimmunised birds (Log 3.4 cfu/ml) as illustrated in Table 4 below. On this day the *P* value of *Salmonellae* in liver tissue in all vaccinated groups was $P < 0.00$, $P < 0.07$, $P < 0.01$ and $P < 0.03$ respectively compared to the unimmunised control. *Salmonella* countsin liver on 1st, 4th, 6th and 8th day post infection steadily decreased in all vaccinated and unimmunised birds as shown in Table 4 and Figure 3 below. Furthermore, the Log_{10} *Salmonella* counts in liver on the 4th day post infection were 3.6 and 3.2 in groups vaccinated with *in vivo* and *in vitro* killed bacteria respectively, while in both groups vaccinated with *in vivo* and *in vitro* proteins preparation the Log counts were 3.5 and 3.1 respectively compared with 2.3 in unimmunised birds, with *P* value ($P < 0.08$, $P < 0.08$, $P < 0.18$, $P < 0.2$) in the groups treated with *in vivo* killed bacteria, *in vivo* proteins, *in vitro* killed bacteria and *in vitro* proteins respectively. On day 6 post challenge the mean *Salmonellae* Log_{10} count was 2.6 in groups I and II which were inºCulated with either *in vivo* bacteria or *in vivo* proteins, while in groups III and IV, which were inºCulated with *in vitro* killed *Salmonellae* and *in vitro* proteins respectively, the *Salmonella*e Log_{10} count was 3.0 in both group. Consequently, however, on the last day of sample collection (the 8th day post infection), the mean Log_{10} *Salmonella*e count in liver was 2.0 and 2.2 for *in vivo* and *in vitro* killed bacteria respectively, 2.06 and 1.0 for *in vivo* and *in vitro* protein vaccines respectively and 2.3 for unimmunised birds as shown in Figure 3 below. In addition, bacterial counting was performed on caecal contents for all birds and with the exception of some lactose fermentor bacteria cultured from different group birds' caeca, no *Salmonella*e were detected (< Log 2 cfu/ml) in caecal contents of any bird.

However, 1 day post infection birds vaccinated with *in vitro* formalin killed showed the highest bacterial count (Log_{10} 4.7cfu/ml) of lactose fermentors in their caecal contents with *P* value (*P*<0.04), followed by the group immunised with *in vitro* proteins which produced counts of Log 3.0 cfu/ml with *P* value (*P* <0.6). On 4th day post-infection the birds immunised with *in vitro* bacteria again showed the highest count (Log 4.8 cfu/ml) followed by the groups immunised with *in vivo* bacteria and *in vivo* proteins which had mean counts of Log 2.6 cfu/ml and 2.5cfu/ml respectively as illustrated in Table 5 and Figure 4. Caecal contents that showed no bacterial growth were considered as (<Log 2cfu/ml) which means that the average of bacterial count is less than the limit of detection.

Discussion

Control of *Salmonella* infections in chickens is crucially important towards the aim of reduction of human food-poisoning salmonellosis. Legislation has been introduced by the European Union (Directive 2003/99/EC, Regulation 2160/2003) to monitor the most important *Salmonella* serovars with timetabled requirements for submission of action plans to control infections in major hosts, particularly pigs and poultry. As a part of this both live and inactivated vaccine are now used in many countries both in the EU and around the world. Nevertheless, live vaccines used in the EU are produced by chemical mutagenesis, and are antibiotic resistant.

In the present study four types of vaccine were produced from *S. Enteritidis* PT4. The vaccines were a sonicated protein preparation from (i) bacteria harvested from *in vivo*- and (ii) from *in vitro*-cultured bacteria, plus formalin-killed bacteria harvested from the (iii) *in vitro* and (iv) the *in vivo* environment. The hypothesis was that both types of vaccines (either killed bacteria or protein preparation) prepared from *Salmonellae* harvested directly from the chicken intestine would be more immuno-protective than those cultured *in vitro* in nutrient broth, as they were prepared from the same environment where protection would be required (gut). The protective effect of formalin-killed bacteria plus protein preparations was assessed for their effect in chickens against colonisation and systemic invasion of the homologous challenge strain. Caecal colonisation was assessed by cloacal swabbing with a semi-quantitative method of enumeration which has been used extensively for large groups of birds[10,11,18] Some authors,[19-21] have shown that killed *Salmonella* vaccines induce just partial immune protection in poultry. Newly hatched chickens have no established gut flora which is why Avigard gut flora was provided on day one to make available a gut flora that is naturally present in adult birds[22] and to reduce the level of coloisation to something more closely resembling that of the adult.

After vaccination Cloacal swabbing has demonstrated to be a useful semi-quantitative method for the faecal shedding of *Salmonellae* and estimation of caecal colonisation when chickens experimentally infected, as found previously.[10,11,18] However, when direct *Salmonella* counts were made from caecal contents and compared at the end of vaccination experiment I (orally challenged), there appeared little correlation with the semi-quantitative measures determined by Cloacal swabbing. This phenomenon is well known, and is probably assºCiated with intermittent caecal evacuation.[23] In the present work when birds were challenged orally to assess *Salmonella* caecal colonisation by Cloacal swabbing (Experiment I), the response was great enough to significantly prevent caecal colonisation completely in the groups of birds challenged orally with a virulent *S. Enteritidis* Nal^R strain when the vaccine was the protein preparation harvested from either the *in vivo* condition (in chickens) or *in vitro* (in nutrient broth culture). The results show that protection by both *in vitro* and *in vivo* proteins preparation were statistically significant ($P<0.001$) in their ability to protect against *Salmonella* colonization. However, the level of protective immunity induced by the *in vitro* protein preparation was higher than that induced by the *in vivo* preparation.

The good level of protection induced by the *in vitro* preparation is in agreement with the previous work conducted by Khan et al.,[6] who found that outer membrane proteins of *Salmonella* when inºCulated with adjuvant are effective against *S. Enteritidis* in chickens.[6] In contrast, the protection produced by formalin-killed *Salmonella* harvested from either *in vitro* or *in vivo* conditions when used as vaccine candidate in chickens was found to be statistically insignificant. This is in agreement with other researchers who found that proteins (OMPs) gave better protection than formalin-killed bacteria.[25] For other organisms, such as *S. Gallinarum*and *Pasteurella multºCida*, OMPs also give better protection than whole bacterins or sonicated cells.[25,26] Immunization with *Helicobacter* whole-cell extracts or purified components also effectively prevented *H. pylori* infection in mice.[27-30] Protein subunit antigens induce production of antibodies and can also induce cell-mediated immunity, resulting in long-lasting protection against bacterial infections.[31]

At 4th week post infection no *Salmonella*e were detected from caecal swabs from the vaccinated groups ($<1 \times 10^2$cfu/ml) compared with unimmunised group that show the percentage of *Salmonella*e positive to be 35%.However, these results showed that the better protection induced by the proteins from the *in vitro* cultured bacteria in comparison with the proteins from the *in vivo* harvest bacteria were unexpected, since it was anticipated that the *in vivo* preparation would have been at least as immunogenic as the *in vitro* preparation, as the protein concentrations of both *in vivo* and *in vitro* preparation were similar. However, the reason that the effectiveness of the *in vivo* preparation as vaccine was less than that of the *in vitro* preparation may have been the result of degradation of the proteins harvested from chicken caeca by avian proteases as discussed earlier (Chapter 4). The *in vivo* preparation would have contained a number of antigens that are expressed in the very earliest stages of infection and these may have been important. Moreover, certain genes that encode some important antigens such as LPS and flagella were down-regulated in the intestine of chickens.[17,32]

It is also known that genes assºCiated with invasion and early infection, including *Salmonella* Pathogenicity Island 1 (SPI1) genes are up-regulated in the intestine and during infection of epithelial cells but are largely down-regulated during infection of macrophage like cells.[33,34] In contrast, Jones et al.[35] have reported that in chicken *S. Typhimurium* SPI-1 system is involved in both systemic infection and gut colonization, but does not appear completely crucial for either infection prºCess, and in one day old chicks SPI-1 TTSS is not important in the initial stages of infection.[35] However, in *S. Pullorum* SPI-1 contributes to disease in poultry during infection.[36] In chickens the majority of *S. Typhimurium* genes involved in flagella production including *flgM*, *flgN*, *flgK*, *flgB*, *fliC*, *fljB*, *fliA* are down-regulated.[17] These are highly immunogenic and immuno-modulatory proteins and their down-regulation in the intestine suggests that a vaccine prepared from bacteria harvested from the intestine may, in fact, express lower levels of key immunogens. The altered expression of these antigens; play a key functional role in establishing infection.[37-39] It is been reported that flagella play a role in the pathogenesis in chickens[40,41] and mouse.[42,43] The result of this study is in agreement with previous work conducted by Toyota-Hanatani et al.,[44] suggesting that a part polypeptide in *S. Enteritidis* Fli-C (SEp 9) inhibits *S. Enteritidis* colonization in the intestine of chickens two weeks after challenge, similarly to commercial inactivated *S. Enteritidis* vaccine,[44] *S. Enteritidis* colonization inhibition by SEp 9 alone may have been comparable to that by commercial inactivated *S. Enteritidis* vaccine.

In *S. Typhimurium* the SPI-2 TTSS plays a role in both intestinal colonisation and systemic infection of chickens.[35] Furthermore, microarray work conducted within our group demonstrated that genes of *S. Enteritidis* Pathogenicity Island (SPI-1, SPI-2 and SPI-5) are up-regulated *in vivo* indicating a close assºCiation with the mucosa during colonization.[32] It is thus likely that the antigenic profile of *Salmonella* during the infection of antigen-presenting cells is very different from that of *Salmonellae* during intestinal colonisation, or that the proteins may have some immune-suppressive effects.[45] The immunogenicity of bacteria harvested from macrophage infections has not been assessed but given that the biology of *Salmonella* organisms is very different in the gut and in macrophages,[17,32,33] as candidate antigen presenting cells, this would be a worthwhile experiment to do. This may suggest that growth of bacteria in the gut to generate a more rational inactivated vaccine may be less important than culturing them in the conditions found within an antigen presenting cell.

As we ensured that the protein concentrations in the vaccine preparations prepared from the *in vivo* and *in vitro* cultures were similar and obtained from a similar number of bacteria and we can state that the *in vitro* bacteria did not produce larger amounts of

protein compared to the *in vivo* bacteria. So, the difference may lie in the levels of specific proteins expressed under the different conditions of culture. Thus, in contrast to expectations, *in vivo* harvested proteins were poorer immunogens than proteins harvested from *Salmonellae* grown in nutrient broth but were better than killed-bacterins.

Formalin killed bacteria as vaccines were therefore found to be poorly protective against faecal shedding. This may be as a result of *Salmonella* antigen damage during formalization or as a consequence of enzymatic degradation of *Salmonella in vivo* proteins by avian proteases as found in this study. However, the *in vitro*cultured formalin-killed bacteria had a slightly better effect than the *in vivo* bacteria but this was statically not significant. However, formalin killed *Salmonella* bacterins are used commercially in the field to control the losses acquired by *Salmonella* in calve in many countries.[46,47]

The caeca empty and fill several times a day and therefore, the phases of growth of the in°Culated bacteria in individual birds may be different and a considerable proportion of the cells may be in the stationary phase. In addition, the fall in temperature during the specific experiment before challenge might explain the poor immunity provided for groups treated with this vaccine. Inactivated vaccines can reduce *S. Enteritidis* faecal excretion and that effect may depend on their composition.[48] Other authors have suggested that several factors may account for this effect, such as adjuvant type and composition, *S. Enteritidis* strain and inactivation method.[49,50]

In this study (experiment II), when birds were challenged intravenously (systemic infection), to asses *Salmonella* systemic invasion of internal organs such as spleen and liver, no bacteria were observed in the caecal swabs collected from all birds. This observation is different from what has been reported previously where *S. Enteritidis* was shed in faeces after intravenous challenge.[24,51,52] The clearance of the challenge strain from internal organs in both vaccinated and unvaccinated birds were similar. During systemic infection following intravenous challenge the macrophage interaction with *Salmonella*e is the key in the progress of the systemic infection.[53] *Salmonella* clearance into gastrointestinal tract from the tissues is through gall bladder.[24] It is been previously reported that in chickens biliary antibodies are involved in *S. Typhimurium* clearance from the gut.[54] This observation correlated with the results of Woodward et al.[54] who reported that the *Salmonella* count in gall bladder is higher in unimmunised group compared with vaccinated birds.[55] However, other authors used a similar route of challenge and reported that bacterial shedding in the faces reached the highest number 1 - 2 weeks post infection,[52] which might explain the absence of *Salmonella* from the caecal sample at day 8.

In the present study a combination of inactivated vaccines and CE was used, with all groups of birds in oCulated with Avigard microflora product prior to exposure to the corresponding vaccine. Such a combination might be considered as a control measure to control infection by *Salmonella*. Experimentally, a combination of live and CE products was shown to provide a considerable degree of protection of chickens against *S. Typhimurium*.[56]

Protection of the chicken can oCcur from transfer of passive immunity to broiler and layers, so a killed vaccine might be useful and used under these circumstances. However, secretory IgA responses which are thought to play a central role in mucosal surface protection cannot be elicited by killed *Salmonellae* vaccine.[57,58]

Poultry immunisation against *Salmonella*e is considered as an important contributory measure to infection control. In chickens vaccination may reduce the severity and period of infection and help avoid re-infection,[59] which indirectly should reduce the number of human food-borne salmonellosis cases.[60] It has been shown that inactivated vaccines of *S. Enteritidis* can induce a good humoral immune response, and induce some reduction in intestinal colonization by *S. Enteritidis*.[57,61-63]

Previous studies indicated that humoral responses are less important than cell-mediated immunity in protection against *Salmonellae*.[64,65] Other published research agreed that cell mediated immunity determines the overall defense against systemic salmonellosis[64,66] probably even in the intestine.[67] Several studies have reported that live vaccines are more effective and provide greater protection than killed vaccines in protecting birds from *S. Enteritidis* infection.[61,68-70] Much earlier work indicated that compared with a live vaccine killed bacteria do not induce as strong a protective response in controlling fowl typhoid,[71] and also disease-free intestinal carriage of *Salmonella*[19,20] as indicated previously.

It is not clear if one of the reasons is antigen destruction during vaccine preparation or whether in a related way persistent presentation of the antigen on active multiplying bacterial cells is necessary for stimulation. Other researchers have tested *S. Enteritidis* inactivated vaccines (Bacterin with Modified Freund's adjuvant) and demonstrated a variable reduction in the *S. Enteritidis* faecal shedding; the best protection was obtained by bacterins with modified Freund's adjuvant, with the *S. Enteritidis* cells inactivated by 20% acetone.[50] An inactivated *S. Enteritidis* PT4 which comprised 10^{11} cfu/ml in 50% oil adjuvant was also developed by Timms et al.[24] which provided a significant protection against both intramuscular and intravenous challenge 5 and 8 weeks post challenge when it inoculated intramuscularly[24] although it must be remembered that this was parenteral challenge with a bias towards the vaccine. One other reason for reduced immunogenicity of killed *Salmonella* vaccines is that the presented antigens are expressed under the conditions of culture which in most cases is a rich medium *in vitro*.[72] Conversely, live attenuated vaccines may be antibiotic resistant and may be unsafe due to insufficient attenuation.[73,74] The most extensive currently used inactivated vaccine is produced by culturing *Salmonella* bacteria under conditions of iron restriction, on the basis that this will generate immunogenic surface proteins required for iron uptake.[52] However, other factors besides iron restriction are important during infection of macrophages,[33] or of the intestine.[32,75] Killed vaccines stimulate antibody production and express only the antigens present at the time of *in vitro* harvesting.[64,72] Killed vaccines may be eliminated and destroyed rapidly, with poor immunogenicity in unprimed hosts.[63,76] In contrast to crude undefined preparations perhaps the future route is through specified proteins known to be immunogenic such as the outer membrane proteins (OMP). Besides lipids and lip polysaccharides the outer membrane of Gram negative bacteria contain some proteins which represent integral proteins of the bacterial outer membrane.[77] *Salmonella* OMP play a vital role in the stimulation ofimmune response and may be used as effective vaccine candidates providing active immunity. A previous study used *S. Enteritidis* isolated from chicken meat showed that OMPs of molecular weight 14.4 - 24 KDa are immunogenic and might be used for vaccine preparation to control salmonellosis in human and animals.[77] Another study revealed that *S. Typhimurium* strains contain OMPs of 37 and 40 and 41.7 KDa.[78] *S. Enteritidis* OMPs are effective in reducing colonization of *S. Enteritidis* on intestinal mucosa in chickens and could be used as potential vaccines to reduce *S. Enteritidis* colonization in chickens.[6] (Adjuvanted OMP vaccines gave better protection than sonicated extract of *S. Enteritidis* vaccines.[79]

Although *Salmonella* bacterins may not be effective against intestinal colonization, albeit moderately against invasion of chicken visceral organs,[80] better protection can be obtained in laying hens when a combination of both live *S. Enteritidis*, followed by a bacteria is used.[81] In poultry vaccines against *Salmonella* infection are thus incompletely effective, and must be seen as a single component in *Salmonella* control regimens involving a combination of vaccination programs together with hygienic measures.

Conflicts of Interest

The authors declare no conflict of interest.

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