Salmonella Enteritidis' Proteins produce *in Vitro* and *in Vivo* Protection against Colonization

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Abstract:

Salmonella enterica 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 analysed 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 bacterial-precipitated protein harvested from chickens is thought to be more protective than bacteria grown in broth culture, the immunogenicity of protein-precipitated vaccines harvested from chicken intestine and those from broth culture (*in vitro*), were compared using bacterial proteins as an orally inoculated vaccine candidate in chicken. The results demonstrated that 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.

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 *Salmonella*-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). Our team studied newly-hatched chickens infected with *S*. Enteritidis. We 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 60 kDa chaperonin *groEL* and glyceraldehyde-3-phosphate dehydrogenase *gapA*. 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 lipopolysaccharide "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 vaccine 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/colonization. Accordingly, and to investigate the hypothesis, the immunogenicity of protein-precipitated vaccines harvested from chicken intestine and those from broth culture (*in vitro*), were compared using bacterial proteins as an orally inoculated vaccine candidate in chicken.

Material and methods:

Preparation of protein-precipitated vaccines 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. Subsequently the pellet from each tube was re-suspended with 3.33ml NB, then the content of three Falcon tubes were mixed into one Falcon tube. The contents (10ml NB 10^9 bacterium/ml) which is equivalent to 10^8/0.1ml = 3 x 10^8 bacterium/0.3ml = 5 x 10^8/0.05ml which used for chicken injection (i.m) to both breast sides.

Preparation of *in vivoS.* Enteritidis protein vaccine:

A total of 60 newly hatched chickens were inoculated 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 10^7 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, placed on ice to test viable bacterial number on MacConkey agar and nutrient agar. The caecal contents of the remaining chickens were put in 50 ml Falcon tubes, stored at -80^{o}C until needed. Three chicks were left without inoculation, their caecal contents were used to streak on MacConkey agar and nutrient agar plates, 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 20,000 x g 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^{o}C. Subsequently, the supernatant was filtered using 0.45 µm filters and stored in 1 ml aliquots in Eppendorff tubes at -20^{o}C until required.

Preparation of *invitro S.* Enteritidis protein vaccine:

A single colony of the parental S. Enteritidis PT4 sensitive strain was picked and used to inoculate 10ml NB in a universal bottle which was then incubated overnight at 37^{o}C. On the following day 250ml flasks, each containing 100ml nutrient broth, were inoculated with 1ml of the overnight broth culture of S. Enteritidis PT4 and incubated overnight at 37^{o}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 15,000x g for 10 min at 4^{o}C and filtration as mentioned above. The proteins preparations were then stored at -20^{o}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 check for any Salmonella growth.

First vaccination experiment

A lot of 60 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 three groups each of 20 birds, being placed in separate cleaned rooms (Trigene Disinfectant 20L Clear from Scientific Laboratory Supplies Ltd (CLE1320). Followed by chemical fogging with Virkon disinfectant from Sigma (Z692158). Chicks were distributed between the rooms as follows (*in vivo* sonicated proteins group – Room I; *in vitro* sonicated proteins group – Room II; unimmunised group – Room III). All birds in all groups were inoculated with 0.1 ml of neat Avigard gut microflora (Microbial Developments Limited, UK), then at the fifth day of age all chickens were inoculated intramuscularly (i.m), into the breast muscle, with 0.05 ml containing protein preparation. Chickens were also inoculated orally with 0.1 ml of the corresponding vaccine for each group as shown in Table 1. The unimmunized group (control) was inoculated with sterile NB. At three weeks of age the vaccination program was repeated with all birds inoculated 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 Nal^{R} by plating on BG agar supplemented with nalidixic acid (20 µgm/ml^{-1}) and novobiocin (1 µgm/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 Nal^{R} count estimation.

Day/Group	Group I In vivo sonicated proteins	Group II In vitro sonicated proteins	Group III Unvaccinated Control group
1	0.1ml Avigard orally	0.1ml Avigard orally	0.1ml Avigard orally
5	0.05 ml in vivo proteins; i.m	0.05 ml in vitro proteins; i.m	0.05 ml sterile NB
	0.05 ml in vivo proteins; orally	0.05 ml in vitro proteins; orally	0.05 ml sterile NB
21	0.1 ml in vivo proteins;		0.1 ml in vivo proteins;
	0.3 ml in vivo proteins; orally	0.3 ml in vitro proteins;	0.3 ml in vivo proteins; orally
31	Challenged orally with 0.1ml (5 x 108) live SE NalR	Challenged orally with 0.1ml (5 x 108) live SE NalR	Challenged orally with 0.1ml (5 x 108) live SE NalR
Post challenge sample collection	Five randomly selected birds from each group were killed at day 1, 4, 6 and 8 post infections; tissue portion of their spleen, liver and caecal contents were collected for salmonella count		

Table 1: Experiment-I of vaccination and challenge regime (orally challenged). NB = nutrient broth

Second vaccination experiment

signs, then collected in pre-labelled, pre-weighed sterile universal bottles. 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 (10 x weight expressed as volume) of PBS (pH 7.2) were added into each tube. Each tissue portions was

This experiment was different from the first experiment only in the route of challenge and types of sample collected. The birds, groups, vaccination programs were identical to those in the first experiment. Subsequently, all birds were challenged intravenously via the wing vein with 0.1 ml (1 x 10^6 cells) of S. Enteritidis NalR 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, spleen and liver samples were observed for any clinical

counting. This together with a x 9 dilution of the caecal contents were used for bacterial count estimations.

homogenised in a Griffiths tubes in PBS (pH 7.2) to obtain homogenous suspension (2) prior to dilution for

Day/Group	**Group I** ***In vivo*** **sonicated proteins**	**Group II** ***In vitro*** **sonicated proteins**	**Group III** **Unvaccinated** **Control group**
1	0.1ml Avigard orally	0.1ml Avigard orally	0.1ml Avigard orally
5	0.05 ml *in vivo* proteins; i.m	0.05 ml *in vitro* proteins; i.m	0.05 ml sterile NB i.m
	0.1 ml *in vivo* proteins; orally	0.1 ml *in vitro* proteins; orally	0.1 ml sterile NB Orally
21	0.1 ml *in vivo* proteins; i.m	0.1 ml *in vitro* proteins; i.m	0.1 ml sterile NB i.m
	0.3 ml *in vivo* proteins; orally	0.3 ml *in vitro* proteins; Orally	0.3 ml sterile NB Orally
35	Challenged intravenously with 0.1ml (5×10^8) live SE Nal^R	Challenged intravenously with 0.1ml (5×10^8) live SE Nal^R	Challenged intravenously with 0.1ml (5×10^8) live SE Nal^R
Post challenge sample collection	Five randomly selected birds from each group were killed at day 1, 4, 6 and 8 post infections; tissue portion of their spleen, liver and caecal contents were collected for salmonella count		

Table 2: Experiment-II of vaccination and challenge regime (intravenously challenged).

NB = nutrient broth

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Enumeration of bacteria in chicken faeces (Experiment I)

After collection of all swabs 2 ml selenite broth (Oxoid, CM0395) were added to each tube, followed by brief vortexing. Each swab was plated in a standard manner on brilliant green agar plate (BGA) supplemented with nalidixic acid (20 µgm / ml^{-1}) and novobiocin (1 µgm / ml^{-1}) (9). The inoculated 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 inoculated directly were read and observed for *Salmonella* growth using a semi-quantitative estimation of faecal shedding and caecal colonisation 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 (13). Aliquots of each dilution were plated on BGA plates supplemented with nalidixic acid (20 µgm / ml^{-1}) and novobiocin (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 inoculation for culture to guarantee that the chicks are free from Salmonellae. Differences in percentage excretion rates between groups of birds were compared using $\chi 2$, 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 2010). 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 (Barrow *et al.,* 1987; Barrow *et al.,* 1988). Using birds aged from 2-6 weeks is the best model to study *Salmonella* colonisation 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. 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. 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) whole cellular proteins prepared from in vivo-cultured bacteria and (ii) whole cellular proteins prepared from *in vitro*-grown bacteria in NB, all of which would be tested for their ability to protect against Salmonella colonisation in chicken.

Quality control of the vaccine

No growth was detected after culturing the *S*. Enteritidis PT4, with 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 (14). 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.

Percentage of chickens (20 birds per group) excreting *S*. Enteritidis Nal^R (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* proteins			*In vitro* proteins			Unvaccinated		
		Direct		Enriched	Direct		Enriched	Direct		Enriched
		≥50	≥1	Birds (No& %)	≥50	>1	Birds (No& %)	≥ 50	≥ 1	Birds (No& %)
Cloacal swabs	1	0%	10%	10 (50%)	0%	16%	5 (25%)	0%	20%	9 (45 %)
	4	14%	38%	16 (80%)	0%	16%	5 (25%)	0%	25%	10 (50 %)
	7	5%	5%	7 (35%)	0%	0%	6 (30%)	5%	5%	10 (50 %)
	14	0%	0%	3 (15%)	0%	0%	0 (0%)	5%	10%	3 (15 %)
	21	0%	0%	2 (10%)	0%	0%	0 (0%)	5%	15%	4 (20 %)
	28	0%	0%	2 (10%)	0%	0%	0 (0%)	0%	15%	4 (20 %)
Caecal content	28	0%	0%	0 (0%)	0%	0%	0 (0%)	5%	20%	7 (35 %)

Table 1: Effect of vaccinating with whole-cell sonicated protein preparation on faecal excretion of *S.* Enteritidis Nal^R (challenge strain), results obtained from direct plates; plus results of BGA enriched plates shown number of *S*. Enteritidis Nal^R positive birds (positivity %), from cloacal and caecal sample collected at different time points post infections, chicks were orally inoculated

compared with 45% in untreated control (group three). Then there was a noticeable decrease in the percentages of faecal excretion in all groups two weeks post infection as the percentages were 14%, 0% and 15% in groups from one to three respectively. Moreover, three weeks post challenge the percentage of positive birds' faecal

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 unimmunized control (group three), while no growth of any *Salmonella* were observed in the two treated groups (< 1 $x10^2$ cfu/ml) as shown in Table 3 and Figure 1

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 52%for the birds in group one which were treated with *in vivo* sonicated proteins preparation and 26% in group two which were treated with *in vitro* sonicated proteins preparation excretion was 10% for the *in vivo* sonicated proteins (group one), while there was no faecal excretion (0%) in group two which was vaccinated with *in vitro* protein preparation with no difference from 2^{nd} week post infection compared with (20%) of unvaccinated control group as shown in Figure 1 below. In summary, both *in vitro* and *in vivo* protein preparations had a much great immunization effect. The *P* values were ($\chi 2$=16.77. *P*<0.001) and (χ^2=28.3. *P*<0.001) for the *in vivo* and *in vitro*

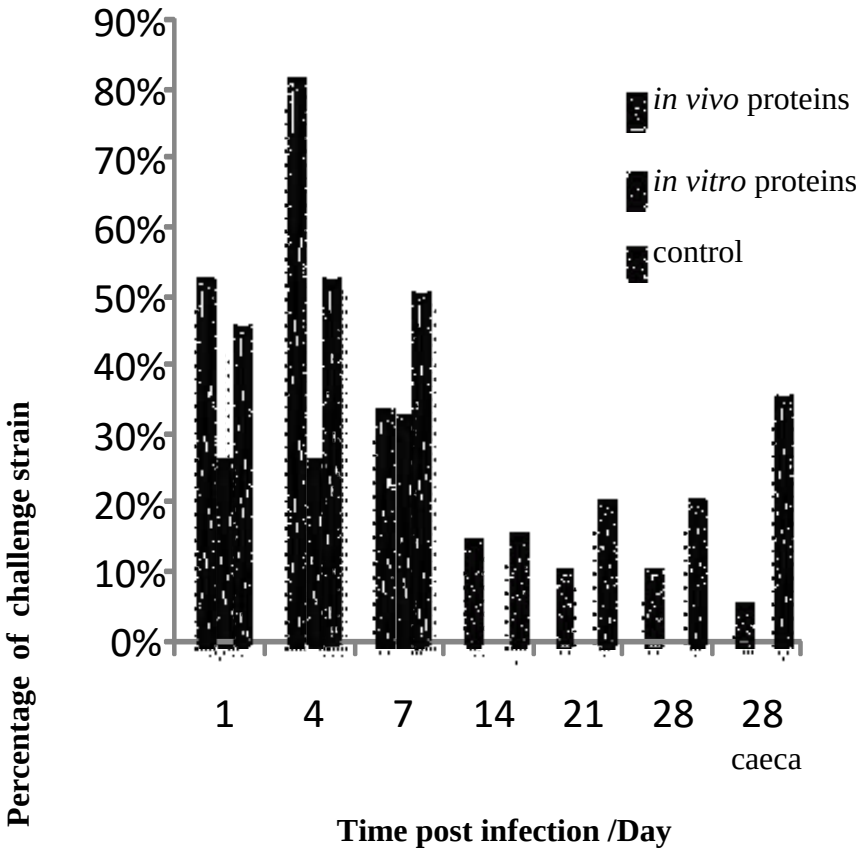

Figure 1: Faecal excretion of challenge S. Enteritidis strain following vaccination with Salmonella proteins produced from bacteria cultured either in chickens (*in vivo*) or in nutrient broth (*in vitro*) compared with unvaccinated control, this figure also shown that no growth of any Salmonella were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

Results for experiment II (Intravenously challenged chicks)

were detected in the control group. Moreover, as shown in Figure 2 below there was no difference of the viable counts (Log_{10}) of *Salmonellae* in the spleen on the 1st day post-infection between all groups. Surprisingly, the count of *Salmonellae* in spleen tissues on the 4th day post-infection in immunized groups (Log5.2 and 5.1 cfu/ml) respectively, were all higher than unimmunized control group which was Log 4.7 cfu/ml

At 1 day post-infection the bacterial count in liver were Log 3.8 and 4.0 cfu/ml in

The results presented in Table 4 shows the averages of Log_{10} *Salmonellae* counts in liver and spleen of five chickens taken at different time points post-infection from the two 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 either immunised with *in vivo* sonicated proteins, or group treated with *in vitro* sonicated proteins; although some other bacterial growth such as *E. coli, Klebsiella* were observed as illustrated in Table 5 and Figure 4. *Salmonellae* challenge organisms

collection (the 8th day post infection), the mean Log_{10}*Salmonella*e count in liver was 2.06 and 1.0 for *in vivo* and *in vitro* protein vaccines respectively and 2.3 for unimmunized 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 fermenter bacteria cultured from different group bird's caeca, no *Salmonella*e were detected (< Log 2 cfu/ml) in caecal contents of any bird.

groups immunized with *in vivo* and *in vitro* proteins preparations respectively. This result was unexpected as the counts in the two vaccinated groups were again higher than that of unimmunized birds (Log 3.4 cfu/ml) as illustrated in Table 4 below. *Salmonella* counts in liver on 1st, 4th, 6th and 8th day post infection steadily decreased in all vaccinated and unimmunized birds as shown in Table 4 and Figure 3 below. Consequently, however, on the last day of sample

Days PI	***In vivo* proteins**						***In vitro* proteins**						**Unvaccinated**					
	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***
1	**3.8**	0.1	**0.07**	**5.0**	0.03	**0.2**	**4.0**	0.1	**0.03**	**4.7**	0.1	**0.7**	**3.4**	0.08	-	**4.8**	0.15	-
4	**3.5**	01	**0.08**	**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**	0.2	**0.08**	**4.8**	0.1	**0.08**	**3.0**	0.1	**0.02**	**4.8**	0.1	**0.1**	**0.9**	0.6	-	**3.1**	0.48	-
8	**2.06**	0.06	0.8	**4.0**	1.0	0.8	**1.0**	0.2	0.12	**4.1**	0.2	0.7	**2.3**	0.7	-	**4.0**	0.13	-

Table 4: The protective effect of 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. Log10 mean viable counts of Salmonella per ml of homogenized liver tissue of 5 birds from each group/time point.

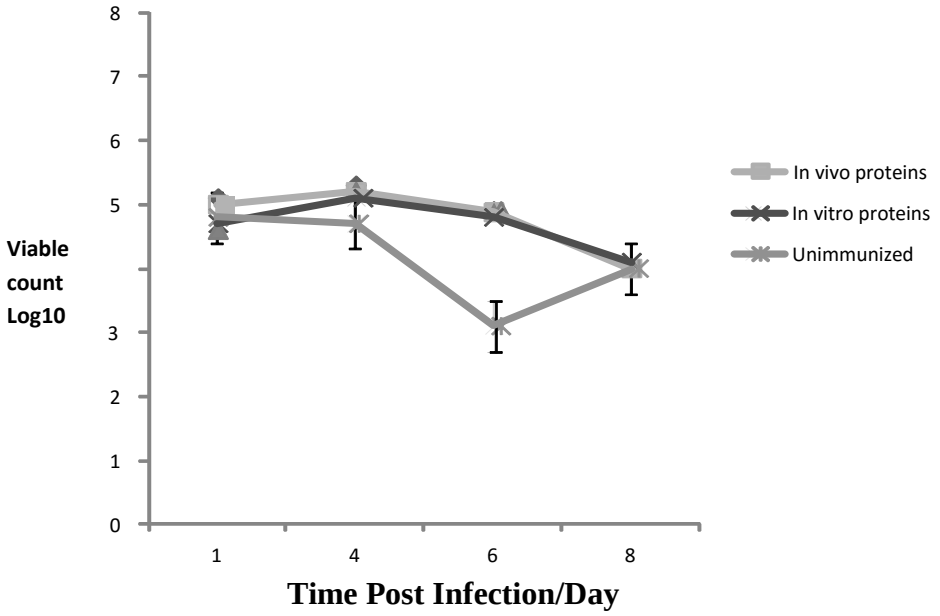

Figure 2: The number of *Salmonella* Log_{10} cfu/ml in chicken's spleen tissue in the groups of birds (each of 20 birds) treated with *S*.Enteritidis whole cellular *in vivo* and *in vitro* sonicated proteins preparations compared with unimmunised control post challenge with parent strain (*S.* Enteritidis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

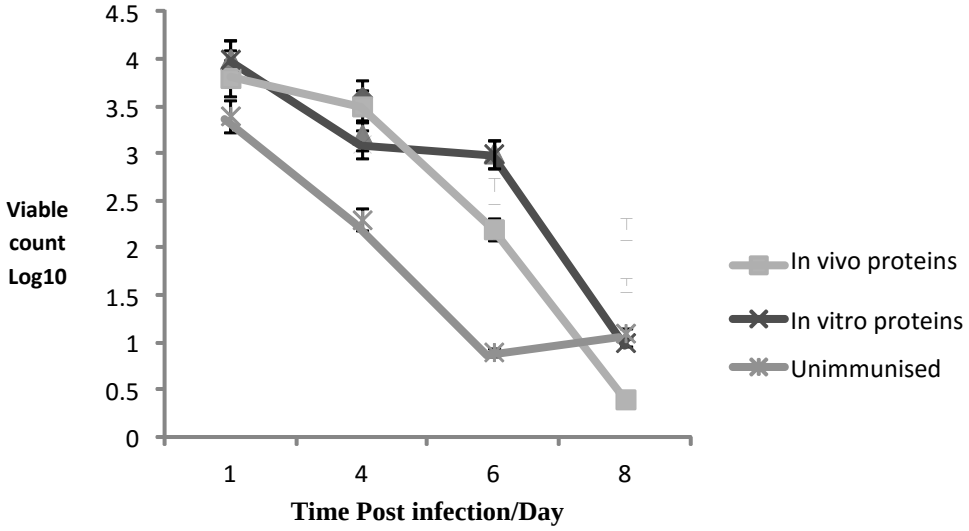

Figure 3: The number of Salmonella Log10 cfu/ml in liver tissue in the groups of birds treated with either S. Enteritidis whole cellular *in vivo* and *in vitro* sonicated proteins preparation compared with unimmunised control post challenge with parent strain (S.Enteritidis Nal^R) intravenously.

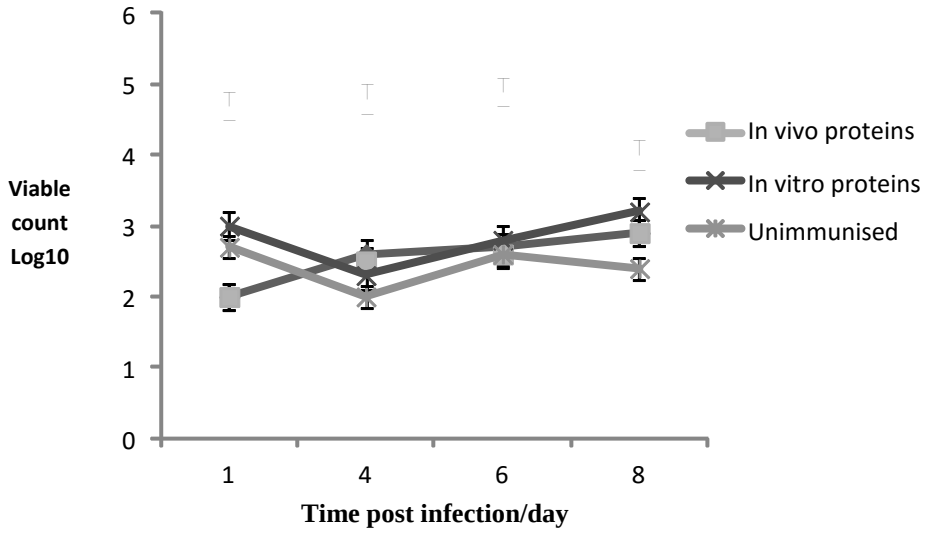

Figure 4: The number of lactose fermentor bacteria Log10 cfu/ml-1 in caecal contents in the two groups of birds treated with *in vivo* or *in vitro* protein preparations of S.Enteritidis compared with unimmunised group at 1st, 4th, 6th and 8th day post intra-venous infection with challenge strain S.Enteritidis Nal^{R}

Table 2: The effect of 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 Nal^{R}. (Average viable counts $\log_{10}$ per 1ml of caecal contents). SE=Standard Error, P=P value

Dayspost Infection	*In vivo* proteins			*In vitro* proteins			Unvaccinated		
	Log 10	SE	*P*	Log 10	SE	*P*	Log 10	SE	*P*
1	**2**	0	0.07	**3**	0.2	0.6	**2.7**	0.7	
4	**2.5**	0.3	0.08	**2.3**	0.2	0.18	**2.0**	0	-
6	**2.6**	0.2	0.08	**2.8**	0.2	0.64	**2.65**	0.4	-
8	**2.9**	0.5	0.3	**3.2**	0.8	0.61	**2.46**	0.3	-

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 poultry and pigs. 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 two 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. The hypothesis was that the type of vaccine prepared from *Salmonellae* harvested directly from the chicken intestine would be more immuno-protective than those cultured *in vitro* in 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 (18). 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 nutrient broth, as they were prepared from the same environment where protection would be required (gut). The protective effect of 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, 15) 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, 15). However, when direct *Salmonella* counts were made from caecal

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 (2003), who found that outer membrane proteins of *Salmonella* when inoculated with adjuvant are effective against *S*. Enteritidis in chickens.

At 4^{th} week post infection no *Salmonella*e were detected from caecal swabs from the vaccinated groups (<1 x 10^2 cfu/ml) compared with unimmunized 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. 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 (16, 19).

The result of this study is in agreement with previous work conducted by Toyota-Hanatani et al. (2009), 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 (20). 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 (21). 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.

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.

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 (17, 22 and 23). 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 Salmonellae is the key in the progress of the systemic infection (24). Salmonella clearance into gastrointestinal tract from the tissues is through gall bladder (17). It has been previously reported that in chickens biliary antibodies are involved in S. Typhimurium clearance from the gut (25). This observation correlated with the results of Woodward et al. (2002) who reported that the Salmonella count in gall bladder is higher in unimmunized group compared with vaccinated birds (26). However, other authors used a similar route of challenge and reported that bacterial shedding in the faces reached

(27), which indirectly should reduce the number of human food-borne salmonellosis cases (28). 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 withhygienic measures

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the highest number 1 – 2 weeks post infection (23), which might explain the absence of Salmonella from the caecal sample at day 8. Poultry immunization against Salmonellae 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

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