Host response to different treatments to reduce *Salmonella* infections in swine.

**Letellier A**¹, Messier S¹, Lessard L², and Queisy S²

¹Université de Montréal, Faculté de Médecine Vétérinaire, 3200, Sicotte, St-Hyacinthe, Québec, Canada
²Santé Canada, Laboratoire d’hygiène vétérinaire et alimentaire, 3400 Casavant Ouest, St-Hyacinthe, Québec, Canada

**Abstract**

Host-response was evaluated following the administration of various treatments to reduce *Salmonella* in swine. Response to the treatments were studied by the evaluation of phagocytosis rates by flow cytometry, activation of whole blood phagocytes by bioluminescence and production of immunoglobulins A against *S. Typhimurium*. Results were analyzed by ANOVA and significant differences were observed in the activation of whole blood phagocytes in all groups of treated pigs in comparison to control pigs (p<0.0001). In SC54™ vaccinated pigs, reduction of *Salmonella* in mesenteric lymphatic nodes was observed and production of immunoglobulins A against *S. Typhimurium* was higher in this group in comparison to uninfected (p<0.0007) and other groups. Taken together, these findings suggest that SC54™ vaccine can stimulate local immunity and reduce carriage of *Salmonella* in pigs. Use of SC54™ vaccine should thus be considered in further field experiments in order to increase the host resistance to this infection.

**Introduction**

Salmonellosis is a major disease of swine and is one of the most economically important of the enteric and septicemic diseases affecting growing pigs in many parts of the world (1). *S. typhimurium* is the most frequently reported *Salmonella* serotype from human sources and typically causes acute gastroenteritis (2). Failure to control *Salmonella* infection in meat animals has resulted in salmonellosis becoming one of the most important zoonosis in developed countries. A study of food-borne disease from 1977 to 1984 (3), observed that pork was responsible for 11% of the *Salmonella* outbreaks attributed to meat. At slaughterhouse, carrier pigs are source of contamination and transmission is thought to occur by pig-to-pig contact or from exposure to the contaminated physical environment (4).

In many countries, efforts are now being made to reduce the incidence of infection of live animal at farm-level (5,6,7). For the establishment of efficient on-farm strategies to control *Salmonella* there is a need to increase the animal resistance to this pathogen.

The aim of this study was to evaluate the host response to probiotics Ferlac-2™, fructooligosaccharides and SC54™ vaccination and their capacity to control *Salmonella* infections in pigs.

**Materials and Methods**

*Animals*: Early-weaned 12 day-old *Salmonella* free piglets were randomly assigned to either control or treatment groups. Each group was initially composed of 10 pigs. Clinical signs were monitored daily throughout the experiment.

*Pig Treatments*: FOS (1% in feed; Encore Technologies, USA), probiotics (Ferlac-2™, 2 x 10⁹ CFU/day in feed, composed of *Lactobacillus acidophilus* (4%), *L. rhamnosus* (65%), *Enterococcus faecium* (25%), *Streptococcus thermophilus* (5.9%) and *L. bulgaricus* (0.1%) in feed, Rosell Institute, Montreal, Canada) and vaccination (SC54™, i.n. 2 mL, Boehringer, Iowa, US) were used in this study. One group of twenty-one day-old pigs (Day 0) was vaccinated (SC54™) and others groups were supplemented for 14 days with each treatment describe above. Control group was not supplemented and each group was housed in separate controlled facilities.

*Challenge*: A clinical isolate of *Salmonella* Typhimurium, isolated from a septicemic pig (Faculté de Médecine Vétérinaire, Université de Montréal, Québec, Canada) was used. This strain was inoculated into Nutrient broth (NB, Difco, Detroit, MI) and incubated at 37°C for 18 h. The starting culture was used to inoculate fresh NB tube (1:100). This culture was incubated and log phase bacteria were used for the challenge. A dose of 10⁷ C.F.U. was given orally to each piglet in the different groups, 14 days after the beginning of the treatment (Day 14).

*Necropsy and Bacteriology*: Rectal swabs were collected every two days before and after the challenge with S. Typhimurium and processed as described below. Fourteen days after the challenge, pigs were euthanized and necropsied (Day 28). Tissues collected for bacteriology were tonsils, liver, spleen, middle ileum, colon and mesenteric lymphatic nodes (MLN). One gram of each tissue or feces was homogenized in 9 mL NB and incubated 18h at 37°C. One mL of NB of each specimen submitted to the
primary enrichment was inoculated into 9 mL of Tetrathionate Brilliant Green (BBL, Cockeysville, MD) and incubated for 24-48 h at 37°C, for selective enrichment of Salmonella spp. Then, one loopful (10 μL) of the selective enrichment media was inoculated in Brilliant green sulfa agar (BGS, Difco) containing novobiocin (Sigma Chemical Co., St-Louis, MO) at 20 μg/mL and incubated for 24-48 h at 37°C. Lactose negative colonies were submitted to biochemical testing by urease and Triple sugar iron media (Difco). Colonies typically corresponding to Salmonella spp. were tested by agglutination against polyvalent O antisera (Poly Al-Vi, Difco) and Salmonella isolates were serotyped under the supervision of Dr. C. Poppe, Health Canada in Guelph, Ontario, Canada. Quantitative bacteriology was done on MLN. Dilutions of homogenized tissues (NB) were done in PBS and number of C.F.U. was evaluated by plating dilutions on BGS agar. Colonies typically corresponding to Salmonella were identified as described above.

Collection of blood samples: Peripheral venous blood was collected in tubes containing heparin as anticoagulant. Total and differential leukocyte counts were performed with an Abbott Cell-Dyn 3500 hematologic counter (Abbott Laboratories, Rungis, France) at the hematology laboratory of the College of Veterinary Medicine. If necessary, cells count of a donor was adjusted with phosphate buffer saline (PBS) to approximately 1.0 x 10⁶ cells mL⁻¹. Analyses were performed immediately after collection of blood samples.

Activation of phagocytes by chemiluminescence assay: Whole blood was collected as described above and used immediately to study the myeloperoxidase activity associated with phagocytosis activity as described by Tatsuhiko et al. (8). This process produce electronically excited states which by relaxing the the groundstate emit photons, the phagocytes chemiluminescence (CL). Briefly, 200 μL of whole blood was pre-equilibrated at 37°C in the luminometer chamber for 10 min and 200 μL of luminol solution (10⁻⁴ M) and 400 μL of PBS were added. The background of the samples was monitored for also 10 min before the activation. The stimulation of the phagocytic response was done by the phorbol myristate acetate (PMA, 200 μL, 10 mg μL⁻¹), a soluble initiator of the metabolic burst. The samples were mixed, incubated at 37°C and the light emission was recorded as over a period of 60 min. These results were plotted as different of light emission between activation level after (day 14) and before (day 0) treatment.

Labelling of S. Typhimurium: The strain was grown for 4 h in Nutrient broth at 37°C, and the bacteria were washed and resuspended in PBS to a concentration of 10⁸ colony-forming-units (CFU) mL⁻¹. A volume of 1 mL of bacterial suspension and 25μL of a 20 mg mL⁻¹ fluorescein isothiocyanate (FITC, Sigma, St-Louis, MO) solution were mixed by rotation (100 RPM) for 60 min at 37°C. The FITC-labelled bacteria were washed three times in PBS and finally suspended in PBS to a final concentration of 10⁸ CFU mL⁻¹. Confirmation of the bacterial fluorescence was provided by flow cytometry and fluorescence microscopy. Bacterial suspensions were immediately used for phagocytosis assay.

Phagocytosis assay using flow cytometry: As described by Busque et al. (9), samples of 100 μL of whole blood or whole blood diluted with PBS to adjust the phagocyte:bacteria ratio to 1:10, were incubated with 10 μL of live FITC-labelled S. Typhimurium. Samples were incubated for 30 min at 37°C, and phagocytosis was stopped by the addition of 4 mL of ice-cold PBS. Erythrocytes (RBC) present in samples were lysed using Immuno-Lyse reagent as described by White-Owen et al. (10). Cells were finally fixed by addition of 200 μL of 2% (w/v) paraformaldehyde solution. Phagocytosis rates were obtained by subtracting the percentage of phagocytosis at 4°C (adherence control) from the percentage of phagocytosis obtained at 37°C. Flow cytometry analysis were performed on a FACStar flow cytometer using 488 nm line of an argon ion laser and a 200-mV light output. Data were acquired in mode list for 10 000 events and analyzed by Consort 30 software. The flow system was equipped with a 75 μm nozzle tip, and the analysis was performed at a flow rate of 1 000 events per sec. Green fluorescence (FITC) was collected by using a 530/30 nm filter (FL1). Results were expressed as phagocytosis ratio (J14/J10).

IgA detection by ELISA: A 10 cm portion of ileum was remove of each pig and washed in 5 mL PBS pH 7.2. This preparation was centrifuged and the supernatant was filtered through a 0.22 μm filter. Each preparation was tested undiluted and double diluted in PBS-Tween (100 μL) in the ELISA test. IgA titer was evaluated by an enzyme-linked immunosorbent assay using heat extracted surface membrane antigens of S. Typhimurium, enzymatically treated and supernatant filtrated. Nunc™ Polystreptplates coated with the final dilution of 1/100, in 0.05M carbonate buffer pH 9.6 (Sigma), corresponding to 1.25 μg of total sugar per well (11). Plates were incubated overnight at 4°C and blocked with 0.3% casein for 30 min. Plates were then washed with NaCl-Tween and supernatant was added at different dilutions for 1.5h at 37°C. Plates were washed and anti-swine IgA-HRPO conjugate was added for 1h at 37°C. After washing the plates, revelation was done in citrate buffer with ABTS solution. Plates were read at 414 nm in a Bio-Rad apparatus and sample with OD higher then 0.3 were considered positive.

Results

Phagocytosis from whole blood phagocytes demonstrated no significant difference between groups. A slight increase of latex beads phagocytosis was noted for all groups at J14. On the other hand, at J14, all groups, including control, showed a reduction of S. Typhimurium phagocytosis rate (Figure 1).
PMA-induced chemiluminescence response of whole blood phagocytes from pretreated and control pigs demonstrated that significant differences were observed in the activation in all groups of treated pigs (p=0.0001, Anova analysis). In comparison to control pigs, Ferlac-2, FOS in combination with Ferlac-2 and SC54 vaccinated pigs had a significantly higher cell activation level (p=0.009, p=0.001 and p=0.04 respectively) while FOS alone was not found to activate whole blood phagocytes (Figure 2).

The presence of IgA in small intestine was also evaluated and we observed that vaccinated pigs (SC54™) had a higher titer of IgA in ileum (median O.D. = 0.721) in comparison to uninfected pigs (0.063) and other groups (0.136 to 0.677) (Table 1).

Table 1. Recovery of IgA (median O.D.) from pigs from different groups and percentage of animals colonized by S. Typhimurium in MLN and ileum.

<table>
<thead>
<tr>
<th>Groups</th>
<th>IgA (p valueb)</th>
<th>MLN*</th>
<th>Ileum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>0.063</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Control</td>
<td>0.240</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>SC54</td>
<td>0.721 (0.0007)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>FOS in feed</td>
<td>0.136</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Ferlac-2 in feed</td>
<td>0.677 (0.002)</td>
<td>44</td>
<td>89</td>
</tr>
<tr>
<td>Ferlac-2 + FOS in feed</td>
<td>0.364</td>
<td>100</td>
<td>70</td>
</tr>
</tbody>
</table>


bMann-Whitney p value in comparison to uninfected pigs.

**Discussion**

Host response is crucial to eliminate pathogens. Acquired resistance to infectious disease may be expressed by a predominantly humoral or a cellular mechanism or, more frequently, by a combination of both of them. The host response involves three different cell types: the phagocytes, the thymus-dependent (T) lymphocytes, and the thymus independent (B) lymphocytes. The normal unstimulated phagocytic cell is capable of killing most nonpathogenic bacteria that gain entry to the tissues. However, the presence of opsonic antibodies and activated macrophages is required to eliminate the pathogenic intracellular parasites (12).

The colonization of tissues by Salmonella in pigs following treatments and in control pigs was described elsewhere (13). Briefly, these results showed that colonization in MLN for SC54 vaccinated pigs was reduced in comparison to control group and a significant reduction of Salmonella prevalence in ileum (p<0.05, Fisher test) was noted (Table 1). No significant difference was observed in quantitative evaluation of S. Typhimurium in MLN (data not shown) in the different groups. Bacterial counts under 30 CFU were observed in MLN.

Phagocytosis from whole blood phagocytes was not increased after treatments suggesting that systemic stimulation of phagocytes was not sufficient to increase resistance to S. Typhimurium. However, activation of whole blood phagocytes was higher after SC54™ vaccination of pigs. On the other hand, since supplementation with Ferlac-2™ or FOS in combination with Ferlac-2™ also activated phagocytes without a significant reduction in the MLN colonization, it suggested that local response might be involved in the reduction of Salmonella in MLN and ileum.

Given this, the local response was studied, particularly
production of IgA in the small intestine. Vaccinated pigs with SC54™ were protected from colonization of S. Typhimurium in MLN during experimental infection and those pigs showed a significantly higher IgA titer in small intestine in comparison to other groups. On the other hand, shedding of Salmonella in vaccinated pigs was not reduced, indicating that Salmonella can persist in the gut despite a low prevalence in ileum and colon. These observations suggest that local immunity is involved in reduction of S. Typhimurium in MLN in vaccinated pigs.

Taken together, these findings indicate that the vaccination with SC54™, used intranasal at weaning, can reduce carrier state of S. Typhimurium in swine. Fields study are now under investigation to evaluate the prevalence of Salmonella spp. following vaccination with SC54™. Furthermore, preliminary data revealed histopathological changes at the level of ileum of pigs vaccinated with SC54™, suggesting also that the local response to this treatment may be of significance in the outcome of the infection.

References


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