Digestive microbiota changes during application of an effective, feed presentation based, mitigation option against *Salmonella* shedding in pigs.

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Abstract
If some studies have attempted to mitigate the *Salmonella* spp. excretion in pigs by feed related interventions, none clearly demonstrated the impact of the presentation (mash or pellet and particular size). Thus this study aimed to determine if the modification of the pigs feed presentation alone can lower the *Salmonella* spp. excretion. To do so, 144 eight weeks aged piglets, previously confirmed as homogeneously in contact with Salmonella during post-weaning, were given diets that varied only by the particle size (500, 750 or 1250 µm) and/or the texture (mash or pellet). During the fattening period, they were individually sampled for blood and feces collection after 0, 21, 46 and 88 days of specific diet. Colons and caecums content were also sampled at the slaughterhouse. There were more pigs from the pellet groups shedding *Salmonella* spp. in their feces after 21 (p=0.012) and 88 days (p=0.002) and in their colon content at slaughter (p=0.026) than from the mash feed groups. In contrast with the literature, no seroconversion significant differences were found between the different groups. Real-time PCR analyses revealed that pigs from the pellet groups had significantly less *Bifidobacterium* spp. in their feces than those from the mash feed groups at day 21. At the same date, a 16S rRNA gene amplicon analysis of the fecal microbiota using Ion Torrent™ sequencing revealed a significantly lower representation of the *Spirochaetes* phylum in the feces of pigs from mash feed groups. It also indicated significantly more bacteria of the *Fibrobacteres* phylum and less Chloroplast in the feces of pigs from pellet feed groups. Correlation between Salmonella mitigation efficiency and changes in microbiota will be tested. Our data are compatible with the fact that a mash feed would promote a healthier gut microbiota while pellet feed would promote better digestion efficiency.

Introduction
Modifying the pigs feed to mitigate the presence of *Salmonella* spp. in herds at the farm level while increasing gut health and reducing the use of antibiotics is gaining in popularity over the years. Many solutions have been explored but none resulted in the elimination of the pathogen. These strategies are believed to be associated with beneficial modifications of the gut microbiota and its production of antibacterial element (Mountzouris, et al., 2006). One promising strategy is the use of mash feed instead of the commonly used pellet feed (Lo Fo Wong, et al. 2004). In their review comparing feed management practices and feed characteristics associated with *Salmonella* prevalence, O’Connor et al. (2008) came to similar conclusions based on serological data. Unfortunately, none demonstrated a *Salmonella* spp. shedding reduction or any modification of the microbiota that would go along with it. It is also important to notice that in most studies many variables were different between the compared diets (composition, particle size, texture, heating process, etc.). The goal of this study was to investigate on modifications of the pigs gut microbiota that would explain or go along with a reduction of *Salmonella* spp. shedding on the farm when only the feed presentation is modified (mash or pellet/particle size).

Material and Methods
On farm
A batch of nine hundred 5 weeks old piglets, known to have been in contact with *Salmonella* spp. at the nursery, were split (10 per pen) into 6 groups. Each group received a different diets varying only by their particle size (500, 750 or 1250 µm) or texture (mash or pellet). Pellet 500 being the reference group from the industry. Individual blood and feces samples were taken as well as colon content at slaughter on 144 of the pigs (24 per diet, 2 per pen) on days: 0, 21, 46 and 86. An aliquot...
of the feces was put into liquid nitrogen and later kept at -80° C for molecular biology analysis and the rest was kept at 4° C until beginning of the analyses.

Blood analysis
Salmonella spp. seroconversion was followed by a commercial ELISA kit (Maxivet, St-Hyacinthe, Quebec, Canada) developed to detect serological response to more than 95% of Salmonella serotypes commonly found in Canada (Letellier, A., 2009). Sera were analysed by the diagnostic service of the Faculté de Médecine Vétérinaire of the University of Montreal.

Salmonella spp. detection
We used a modified version of the ISO 6579 annexe D for the detection of Salmonella spp. in feces and environment (BPW 18-24h at 37° C, MRSV 48h at 42° C, isolation on BGS and XLD 24h at 37° C followed by biochemical and sero-agglutination confirmation).

Real-time PCR
DNA extraction (mechanical lysis followed by a phenol-chloroform extraction) was performed on all samples. Real-time PCRs, using the parameters shown in table 1, were performed on the samples of interest using an Eco® Illumina® real-time PCR with EvaGreen® qPCR mix as recommended by the manufacturer. For lactobacilli and enterobacteria, 15 ng of DNA and a final primer concentration 1 µM was used. For the Bifidobacterium genus, the amplifications reactions consisted of 10 ng of DNA with a final primer concentration of 0.25 µM. The standard curves for each reaction were obtained by diluting the precipitate of PCR product realized in the same conditions with the following reference strains L. acidophilus ATCC314, E. coli 25922 and B. Longum ATCC 15707. Results were expressed as log of copies/10 ng of DNA used.

Table 1. Real-time PCR parameters.

<table>
<thead>
<tr>
<th>Group</th>
<th>Primers</th>
<th>Length</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacilli</td>
<td>LB-F: GCAGCAGTAGGGGATCTTCCA (Tm=57 °C)</td>
<td>≫200 pb</td>
<td>Init. Den : 10m@95°C</td>
</tr>
<tr>
<td>(Castillo, et al. 2006)</td>
<td>LB-R: GCGATYACCGCTCACATG (Tm=57 °C)</td>
<td></td>
<td>40 cycles: 15s@95°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1m@60°C</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>Ent-F: ATGGCTGTTCGTACGTCGT (Tm=60 °C)</td>
<td>364 pb</td>
<td>Init. Den : 10m@95°C</td>
</tr>
<tr>
<td>(Castillo, et al. 2006)</td>
<td>Ent-R: CCTACTTCTTTTGCAACCCACTC (Tm=60 °C)</td>
<td></td>
<td>40 cycles: 15s@95°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1m@60°C</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>Bif-F: CTCCCTGGAAACGGGTGG (Tm=53 °C)</td>
<td>550 pb</td>
<td>Init.: 5m@94°C</td>
</tr>
<tr>
<td>(Matsuki, et al. 2004)</td>
<td>Bif-R: GTGTTTCCTCCCGATATCTCA (Tm=53 °C)</td>
<td></td>
<td>40 cycles: 20s@94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20s@55°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50s@72°C</td>
</tr>
</tbody>
</table>

16S rRNA gene sequencing
PCR of the partial 16S rRNA was performed using the universal primers F343 (5’- TACGGRAGGCAGCAG-3’) and R533 (5’- ATTACCGCGCGGTGG-3’) containing the 10-bp multiplex identifiers (MID) and adaptor sequences for Ion Torrent sequencing on DNA extract from the fecal samples of each pig (Yergeau, et al. 2012). PCR products were purified on 2% agarose gel and pooled in an equimolar ratio and sequenced together. A total of 3.50x10⁷ molecules were used in an emulsion PCR using the Ion OneTouch 200 Template Kit v2 (Life Technologies) and the OneTouch instruments (Life Technologies) according to the manufacturer’s protocol. The sequencing of the pooled library was done using the Ion Torrent Personal Genome Machine (PGM) system and a 316 chip with the Ion Sequencing 200 kit according to the manufacturer’s protocol. Sequence data were analyzed by using the Ribosomal Database Project Pyro-sequencing Pipeline (http://pyro.cme.msu.edu/, RDP release 10, update 26). The sequences, deconvoluted and binned, were trimmed by using the Pipeline Initial Process tool. Datasets containing MID sequences associated with the 16S rRNA gene amplifications were individually classified using the RDP Classifier tool with an 80% bootstrap cutoff (6).
Results

Salmonella spp. detection
As shown in tables 2, no significant differences in Salmonella spp. shedding was found at D0. At D21, D88 and in the colon content at slaughter, more pigs from the pellet feed diets were shedding Salmonella spp. (p=0.012, p=0.002 and p=0.026 respectively) than from pigs on mash diet (Table 2).

Real-time PCR
The lactobacilli and enterobacteria groups and their ratio (lactobacilli/enterobacteria) were similar from group to group. On the other side, Bifidobacterium spp. were more present in feces from the mash feed groups compared to the pellet feed groups (all particle size together) and higher values in the mash 1250 µm compared to the 3 other groups tested on day 21 (p<0.05) were noted (Table 3).

16S rRNA gene sequencing
At day 21, four phyla showed significant differences in between the pigs from the different diets. While Spirochaetes and Fibrobacteres were found in greater numbers in the feces from the pellet groups compared to the mash groups; Firmicutes and Cyanobacteria/Chloroplast phylums were found in lesser numbers in the same samples. The higher representation of Spirochaetes is mainly due to bacteria from the Treponema genus. Similar link can be made for the Fibrobacteres: Fibrobacter being the only representing genus of its phylum in this analysis. Unfortunately, such a link cannot be made for now to conclude on the higher presence of Firmicutes in the samples from the mash groups because of the higher diversity of bacterial from this phylum in the gut microbiota. Finally, the higher proportion of Cyanobacteria/chloroplast in the mash is caused by a greater presence of cells of the Chloroplast family.

Discussion
This study clearly showed that it is possible to modify the Salmonella spp. excretion in pigs by the modification of feed texture and size of particles without modification of the formula. This reduction was present at the beginning and the end of the fattening period (D21 and D88) and maintained in the colon content at slaughter despite the known increasing effect of transportation stress on this pathogen.

These reductions go along with the raise of Bifidobacterium spp. count observed in the mash feed groups. In fact, bacteria of this genus have often been associated with a raise in antibacterial compound such as volatile fatty acids (Mountzouris, et al., 2006). A higher presence of Treponema spp., another potential pathogen, was also revealed in the feces from the pigs fed pellet feed by the metagenomic analyses. These three put together indicate a better gut health for the pigs fed the mash feed diets compared to those feed pellet feed diets (all particle size together).

On the other side, more Fibrobacter spp., an important agent in the degradation of cellulose was found in greater number in the groups fed pellet feed. Which also correspond with the lesser presence of chloroplast, an indicator of the presence of vegetal cells. Therefore, the higher presence of bacteria being able to digest vegetal cells in the pellet groups linked with a lower presence of vegetal cell fits with the observations made in other studies where pigs fed mash feed would get a better gut health but would suffer a lesser digestion efficiency.

Conclusion
Our study is the first one to demonstrate the benefit of the pigs feed presentation (texture or particle size) alone on Salmonella spp. fecal shedding. Other specific changes in the microbiota were measured as an effect of the different diets used. The data from this study are compatible with the fact that mash feed would promote a healthier microbiota compared to a

Table 2. Total of pigs in the mash or pellet groups excreting Salmonella spp. on days 0, 21, 46, 88 and positive colon content at slaughter. Legend: Data with (b) notice are significantly higher than data with (a) notice from the same date.

<table>
<thead>
<tr>
<th>Texture</th>
<th>D0</th>
<th>D21</th>
<th>D46</th>
<th>D88</th>
<th>Colon content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mash (n=72)</td>
<td>31</td>
<td>16 (a)</td>
<td>9</td>
<td>5(a)</td>
<td>10(a)</td>
</tr>
<tr>
<td>Pellet (n=72)</td>
<td>36</td>
<td>30 (b)</td>
<td>14</td>
<td>19 (b)</td>
<td>21 (b)</td>
</tr>
</tbody>
</table>

Table 3. Log of copies of Bifidobacterium spp. 16S RNA gene/10 ng of DNA from pig feces on day 0 and 21. Legend: Data with (a) or (c) noticed are significantly higher than data with respectively (b) or (d) notice.

<table>
<thead>
<tr>
<th>Date</th>
<th>Pellet 500 (n=24)</th>
<th>Pellet 1250 (n=24)</th>
<th>Mash 500 (n=24)</th>
<th>Mash 1250 (n=24)</th>
<th>Total Pellet (n=72)</th>
<th>Total Mash (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0</td>
<td>3.52</td>
<td>3.62</td>
<td>3.40</td>
<td>3.52</td>
<td>3.57</td>
<td>3.46</td>
</tr>
<tr>
<td>D21</td>
<td>3.25 (b)</td>
<td>3.32 (b)</td>
<td>3.39 (b)</td>
<td>4.23 (a)</td>
<td>3.29 (d)</td>
<td>3.81 (c)</td>
</tr>
</tbody>
</table>
better digestion efficiency for pellet feed.

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References


