Salmonella associated taxonomic and functional changes in the pig digestive tract during application of feed associated mitigation option in production

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ABSTRACT

Salmonella spp carriage and excretion by pigs during production has to be controlled as it increases the risk of introduction of the pathogen in the food chain. Feed related options developed to mitigate excretion and carriage are numerous. But whatever the effectiveness of the option, excretion still exists for some pigs. The question that raised is: are there some individual characteristics that could explain why some pigs are still or became *Salmonella* shedders in the context of the application of an efficient mitigation option? Coarse feed has been suspected (Mikkelsen et al 2004), and recently demonstrated (Lebel et al, I3S2013), to decrease excretion of *Salmonella* in pigs. In the present study, a group of 46 eight-weeks aged pigs, fed with 1250 μm granulometry during 21 days, was followed. Among the 18 initially shedders, 15 changed their status but 6 out of the 28 non-shedders presented *Salmonella* in their feces. To compare function or composition of flora associated with excretion, short-chain fatty acids (SCFA) concentration in colon were measured: individual *Enterobacteriaceae*, *Lactobacillus* and *Bifidobacterium* were quantified (Q-PCR) and phylogenetic and functional metagenomic analyses were conducted. The group presented high level of SCFA: mean value: 4.6, 3.0 and 2.1g/L in colon content for acetic, propionic and butyric acids respectively. *Salmonella* new shedders showed higher mean value of fecal *Lactobacillus* (0.6 Log gene copy more at D21) and lower *Bifidobacterium* (0.3 Log gene copy less) when *Enterobacteriaceae* (5.2 Log gene copy /10ng of fecal DNA extract) didn't changed. Metagenomic analyses, taxonomic after 16S PCR, were conducted on Illumina platform. Sequences were analyzed using publicly available pipelines (MG-RAST). This permit to describe taxonomic flora characteristics in pigs at D0 and to follow individual evolution, depending on they still shed (n=6) or clear (n=3) *Salmonella* and those, initially non shedders that became shedders (n=3) in this context of favourable feeding strategy.

INTRODUCTION

Salmonella still represent a major concern for public health policy makers and industrials as the zoonotic agent is frequently reported in foodborne grouped infections cases. In Quebec, 44% of these cases are associated to *Salmonella* (1). The surveillance system specifies rarely the food origin of the cases but, according to EFSA (2) the part of pig tends to be high (10 to 20%) particularly in the situation where other implicated production have implemented a control program as the *Salmonella* control program in egg production in Quebec. A *Salmonella* surveillance program in pig production is present since 2004 in Québec province. Control measures at farm were proposed (3) mainly based on good hygiene practices during production. Then when applicable, food derived mitigation options are recommended, including feed additives or modification (pre-biotic probiotic and acidification) (4). These authors underlined that research should document the benefits, and sometimes the absence of effect, using the recently open field of digestive microbiota analyses. Working on a curative method for *Salmonella* colonisation mitigation in pigs we recently demonstrated the previously suggested benefits of using coarse feed to mitigate the *Salmonella* load during the growing phase (5). The object of the present study is to compare fecal contents of pigs that eliminated *Salmonella*, from those which failed or acquired *Salmonella* despite the favorable situation. To do so, compositions of the fecal microbiota and concentrations of acids in the digestive tracts were compared.

MATERIAL AND METHODS

Samples :

10g faeces from individual pigs in a group of 46 naturally *Salmonella* challenged pigs (exposed during post nursery phase) and submitted to a 1250 µm coarse grinded feed from the growing facilities period (D0) to day 21 of the trial (D21). Colon contents for 22 out of 46 pigs were available when slaughter.

***Salmonella spp.* Detection :**

Salmonella presence was assessed using a modified version of the ISO 6579 annexe D (BPW 18-24h, MRSV 48h, isolation on BGS and XLD followed by biochemical and sero-agglutination confirmations).

Real-time PCR :

DNA extraction (glass beads lysis followed by a phenol-chloroform extraction) was evaluated (quality and quantity) by NanoDrop. Real-time PCR using the parameters from (5), have been conducted using a Eco® Illumina® real-time PCR and EvaGreen® qPCR mix. For the *Lactobacillus*, *Bifidobacterium* genus and *Enterobacteriaceae* groups the standard curves were obtained by dilution of the precipitate of a regular PCR product of *L. acidophilus* ATCC314, *B. longum* ATCC 15707 and *E. coli* 25922 respectively.

Short Chain Fatty Acid (SCFA) :

At the end of growing phase, at slaughter, some colon contents were available for acetate, propionate and butyrate quantitation. Contents were sampled in a 15 ml tube and stored at -20ºC. Ten grams of 1/1 diluted in distilled water sample were centrifuged at 41 000*g*, 30 min at 15 °C. One microliter of 0.5 M H_2SO_4 was added to 5 mL of the supernatant and centrifuged at 21,800*g*, 15 min at 15 °C. An internal standard (2-ethylbutyrate at 2 g L^{-1}) was added (0.5 mL) to a tube with 0.5 mL of the acidified–centrifuged supernatant and 0.1 g of DOWEX 50WX8 resin (The Dow Chemical Company, Midland, MI) and vortexed. SCFA were measured with a Perkin Elmer gas chromatograph model 8310 (Perkin Elmer, Waltham, MA), mounted with a DB-FFAP high resolution column. Results were analyzed using TurboChrom version 6.2.1 software (Perkin Elmer).

Metagenomic analyses :

V2 16S rRNA amplicons were obtained after amplifications primed with adapters and MIDS according to match Ion Torrent recommendations. 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 were deconvoluted and binned according to their MID tags, and the MID and forward primers 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).

Statistics Analyses were conducted on SPSS version 17.0 (Lic. UdeMontreal). Non parametric Man&Wtihney test was used for date to date or type to type data comparisons.

RESULTS

Among the 46 pigs, 8 week aged, submitted to a 1250 µm diet, 18 were initially (D0) shedders. After 21 days in the mitigating conditions (D21), only 9 pigs out of the 46 shed *Salmonella* (Fig 1) (Khi2 $p<0.05$), 3 of them were previously *Salmonella* shedders at D0 and 6 could be considered as new shedders. This evolution allows defining 4 groups (types) depending on their *Salmonella* status evolution (Table 1).

No significant difference in fecal content for *Lactobacillus*, *Enterobacteriaceae* and *Bifidobacterium* appear at D0 (Table 1) between groups. At D21 it should be noticed that the mean value in fecal *Enterobacteriaceae* was 0.6Log Unit (copy/10ng DNA extract) lower in the type +/+ compared to other types. A significant increase of *Lactobacillus* occurs during the 3 weeks period. This increase is equivalent whatever the type considered. But it should be underlined that, at D21 the type -/+

presented the highest value in *Lactobacillus* concentration (at least 0.6 Log Unit over compared with the other types) and the lowest concentration in *Bifidobacterium*.

Table 1 Quantitative assessment of Lactobacillus (Lact), Enterobacteriaceae (Ent) and Bifidobacterium (Bif) at D0 and D21, depending on Salmonella *type.*
Type : Salmonella Status at D0/D21, pos. +, neg. – (n number of pig/group). Q-PCR mean value values in Log nb of target copy /10ng DNA extract. SD :standard deviation

Type D0/D21	Lacto D0 (SD)	Ent D0 (SD)	BifidoD0 (SD)
+/+ (n=3)	4.1 (0.8)	4.7 (0.6)	4.1 (0.3)
+/- (n=15)	3.9 (0.7)	5.4 (1.0)	3.8 (0.9)
-/+ (n=6)	4.1 (1.1)	5.6 (1.3)	3.8 (0.2)
-/- (n=22)	4.3 (0.9)	5.1 (1.1)	3.3 (0.5)

Type D0/D21	Lacto D21 (SD)	EntD21 (SD)	Bifido D21 (SD)
+/+ (n=3)	5.3 (0.5)	5.6 (1.0)	4.3 (0.6)
+/- (n=15)	5.1 (1.4)	5.2 (0.8)	3.8 (0.8)
-/+ (n=6)	6.0 (0.6)	5.2 (0.8)	3.5 (0.4)
-/- (n=22)	5.3 (0.9)	5.3 (0.8)	3.7 (0.9)

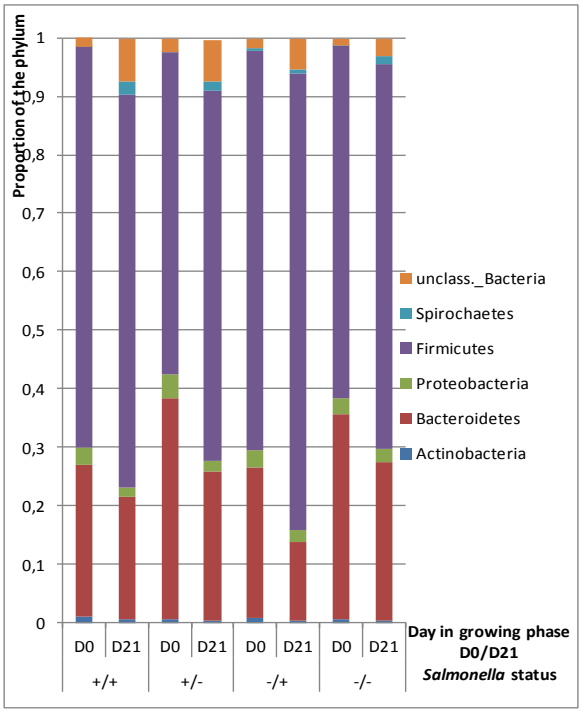
The ability of these two last bacterial populations to contribute to the presence of SCFA in the contents justified the analyses of SCFA in colon at slaughter (Table 2). The concentrations of the 3 SCFA in the colon are in the range documented for high particle size feed (7). No difference could be registered for the concentration of SCFA in the type defined by the excretion status but for pig fed with 1250mm particle size diet.

Table 2 SCFA concentrations in colon contents of pigs depending on their Salmonella *shedding evolution during 3 weeks*
+ shedder, – Salmonella *not present in (n= number) colon samples. SD standard deviation.*

Type D0/D21	Acetic g/L (SD)	Propionic g/L (SD)	Butyric g/L (SD)
+/+(n=2)	3.6 (0.6)	3.3 (0.4)	2.3 (0.1)
+/- (n=8)	4.9 (0.8)	2.9 (1.0)	2.0 (0.7)
-/+ (n=4)	4.7 (1.7)	2.7 (1.6)	2.0 (1.4)
-/- (n=8)	4.6 (0.7)	3.2 (1.1)	2.2 (0.8)

Metagenomic analyses of fecal content were done and results corresponded to a mean value of 37.6 X1000 reads per sample (8.2-78.8). Most important changes in phylum proportion appeared related to the age of the pigs in the groups. Firmicutes and Bacteroidetes represent 90% of the flora, with increased part of Firmicutes with age (Fig 1). The greatest evolution of phylum proportion between 0 and 21 was observed for Spirochetes (Fig 2). Their representation increased less than10 fold for type -/+ and -/- and respectively more than 20 and 50 fold for the groups that shed *Salmonella* at D0 and shed at D21 (+/+) or no more (+/-), respectively.
Fig 1) Metagenomic taxonomic classification of fecal contents of pigs (n=3) raised 21 days in coarse grid feeding.

Salmonella Status at D0/D21, pos. +, neg. –



DISCUSSION

Whatever the mitigation option considered in a pig primary production it should be kept in mind that the *Salmonella* shedding is associated to multiple factors. We analysed a group of pig that is raised in conditions that were demonstrated as favourable to mitigate *Salmonella* excretion. Some pigs were still shedders or some became shedders.

Fig 2 Evolution of the representation of the more frequent phyla in fecal contents of pig from D0 to D21.
Salmonella Status at D0/D21, pos. +, neg. –