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Note

A metagenomic analysis of the pre-enrichment step for the isolation of *Salmonella* spp. from pig feces

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A B S T R A C T

The bacterial short pre-enrichment culture step is important for the proper detection and isolation of *Salmonella* spp. from pig feces. Using metagenomics, we showed that pre-enrichment of *Salmonella* was favored not only by inhibiting the growth of competing bacteria but also by increasing its fitness.

Salmonella spp. is an important food-borne zoonotic bacterial pathogen (Hugas and Beloeil, 2014). Salmonellosis, the symptomatic infection, is characterized by fever, vomiting, abdominal cramps and diarrhea (Chaturvedi et al., 2017). It is a major issue in both food safety and public health (Hugas and Beloeil, 2014; Chaturvedi et al., 2017). Several selective media have been developed empirically for *Salmonella* detection and assessed in National Reference Laboratories (EURL, 2018). The improvement of conventional culture techniques for the detection and isolation of *Salmonella* spp. in faecal samples is still a work in progress (EURL, 2018; Champagne et al., 2005; Love and Rostagno, 2008). The pre-enrichment step, the non-selective step where stressed *Salmonella* and competing microbiota can recover in media, is essential and often critical for the improved sensitivity and efficiency of the selection method that will follow. However, the evolution of competing flora during the pre-enrichment is still considered a “black box” (EURL, 2018; Champagne et al., 2005; Love and Rostagno, 2008). Our objectives here were to study the evolution of bacterial communities in *Salmonella*-free pig feces by metagenomics at time 0 h and following a 5 h pre-enrichment step, and to analyze how the bacterial diversity at 5 h is modulated by the addition of *Salmonella* spp. at 0 h.

Feces composite samples, a combination of feces from six different 16-week old pigs in a 40% storage solution (50:50 glycerol: buffered peptone water), previously shown to be negative for the presence of *Salmonella* (Langlais et al., 2019) were stored at −80 °C in 5 mL aliquots. Aliquots were thawed, diluted 1:10 in buffered peptone water and added to a tube containing *Brucella* broth to a final concentration of 0.0167 g/mL.

Three different feces cultures were prepared: one at time 0 h, *Salmonella*-free, and two after 5 h of incubation: the *Salmonella*-free culture, and a culture in which *Salmonella* spp. had been added to a final concentration of 6×10^6 CFU/mL at time 0 h. All experiments were done in triplicate.

Cultures were incubated at 37 °C on a rotary platform at 250 rpm for 0 or 5 h under aerobic conditions. DNA was extracted from 1.5 mL of each *Brucella* broth using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) and stored at −80 °C. 16S rRNA gene amplicon libraries were prepared using the universal primer pair 515FP1-CS1F and 806RP1-CS2R (Life Technologies, Pleasanton, USA) which amplifies a 292 bp segment of the v4 region (Gilbert et al., 2012). For each sample, 12.5 ng of DNA was amplified in a final volume of 30 µL using Invitrogen Platinum SuperFi DNA Polymerase according to the manufacturer's instructions (Invitrogen – Thermo Fisher Scientific, Waltham, MA, USA). The amplification was done with an initial step of 5 min at 95 °C followed by 20 cycles at a denaturing temperature of 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 180 s, and a final extension at 72 °C for 10 min. Here, a sample containing the ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA) was used as a positive control; three different samples were used as negative controls: feces-free *Brucella* broth following a 5 h incubation and water, both submitted to DNA extraction as described above, and a v4 PCR negative control. Barcoding and DNA sequencing were done on an Illumina Miseq PE250 at The McGill University and Génome Québec Innovation Centre (Montreal, QC, Canada).

Reads were cleaned and analyzed using Mothur version v.1.39.5 (Schloss et al., 2009) with some modifications according to Larivière-Gauthier et al., (Larivière-Gauthier et al., 2017). For the bacterial diversity analyses, sequences were subsampled in Mothur with the lowest number of reads found in a single sample. Alpha-diversity indices, the species diversity within sample, operational taxonomic units (OTUs) Shannon even, Shannon and Inverse Simpson's, were calculated in Mothur and compared with GraphPad Prism 7.04 (GraphPad Software, La Jolla, CA, USA) using one-way ANOVA with multiple comparisons

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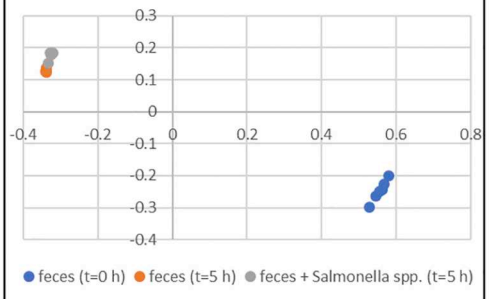
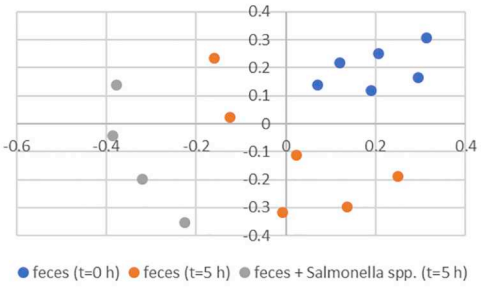
Table 1
Comparison of alpha-diversity indices of pig feces pre-enrichment.

	Feces (t = 0 h) *Salmonella*-free	Feces (t = 5 h) *Salmonella*-free	Feces + *Salmonella* spp. (t = 5 h) in which *Salmonella* spp. had been added at t = 0 h
OTUs	1236 *a	782 *b	613 *c
Shannon even	0.74 *a	0.31 *b	0.28 *c
Shannon	5.28 *a	2.06 *b	1.80 *c
Inverse Simpson's	71.23 *a	2.98 *b	2.41 *b

The values were based on 1000 subsampling of 69,965 sequences. On the same row *a, *b and c* are significantly different, with significant p-values < 0.05.

Table 2
NMDS plot illustrating beta-diversity of pig feces before or after pre-enrichment, and in presence of *Salmonella* spp. with their p-value.

Compared group		Amova (p-value)	
		Yue & Clayton	Jaccard
			
feces (t=0 h)	feces (t=5 h)	< 0.001*	< 0.001*
	feces + *Salmonella* spp. (t=5 h)	0.008*	0.01*
feces (t=5 h)	feces + *Salmonella* spp. (t=5 h)	0.004*	0.002*

*Distance matrix determined with the Yue & Clayton and Jaccard indices have a stress value of 0.007 and 0.289 respectively.

(p-value < 0.05). The beta-diversity analysis, the diversity between samples, was performed using Jaccard similarity coefficient based on the observed richness and the Yue & Clayton theta similarity coefficient. The diversity between the three samples was analyzed using AMOVA (Analysis of MOlecular VAriance; (Excoffier et al., 1992)) and visualized with 2D nonmetric multidimensional scaling (NMDS) graphs (Larivière-Gauthier et al., 2017). OTUs associated with each compared sample were determined with a linear discriminant analysis effect size (LEfSe; (Segata et al., 2011)) using the Galaxy/Hutlab web application (http://huttenhower.sph.harvard.edu/galaxy/). The results were restricted at a linear discriminant analysis (LDA) score over 2.5 with significant OTUs (p-value < 0.05).

Specific populations of lactobacilli (Castillo et al., 2006), *Escherichia coli* (Clifford et al., n.d.) and *Lachnospiraceae* (Wilson et al., 2014) were quantified on each sample by qPCR in a LightCycler 96 Real-Time PCR System (Roche Diagnostics, Laval, QC, Canada), with EvaGreen pPCR mastermix (Montreal Biotech, Montreal, QC, Canada) according to the manufacturer's instructions with some modifications as described by Larivière-Gauthier et al. (Larivière-Gauthier et al., 2017). The comparisons between each of the three samples were done using ANOVA and Student's t-test (p-value < 0.05).

Most of the alpha-diversity indices were significantly different between all three samples (Table 1). The feces (t = 0 h, *Salmonella*-free) sample's richness and evenness are significantly higher compared to the other two samples (feces (t = 5 h)), *Salmonella*-free; and feces + *Salmonella* spp. (t = 5 h). These indicate a wider diversity of evenly distributed OTUs in the initial microbiota. The incubation period (t = 5 h, *Salmonella*-free) and the initial addition of *Salmonella spp.* (t = 5 h, *Salmonella* added at t = 0 h) are two factors that decreased the richness and evenness indicating the emergence of dominant OTUs following incubation and a concomitant loss of diversity (Table 1).

The bacterial population reorganisations that occurred during pre-enrichment and the significant effect of the presence of *Salmonella* in the sample can be visualized on the NMDS graphs (Table 2) (Larivière-Gauthier et al., 2017). It shows that the beta-diversity of pig feces before and after pre-enrichment and in the presence of *Salmonella* for both indices for all three compared groups are significatively different.

Mostly strict anaerobic bacterial species were associated with the feces sample (t = 0 h, *Salmonella*-free) compared to the incubated feces sample (t = 5 h, *Salmonella*-free) where aerobic bacteria were clearly positively selected (Fig. 1a). Interestingly, feces incubated for 5 h without *Salmonella* spp. contained bacteria associated to a healthy pig intestinal tract, notably *Lactobacillus* (Holman et al., 2017), *Ruminococcus* (Suchodolski, 2013) and *Prevotella* (Leser et al., 2002). These were absent in the feces incubated for 5 h that had been inoculated with *Salmonella* spp. (Fig. 1b).

The pre-enrichment step was positively selective for *Salmonella*. The bacterium outgrew lactobacilli members, bacteria known to show anti-*Salmonella* activity (Micciche et al., 2018). Some bacterial populations such as *E. coli* (aerobic), *Lachnospiraceae* (anaerobic) and lactobacilli (facultative anaerobic) evolved differently during the aerobic pre-enrichment (Fig. 1 in Langlais et al., (2018)). This was to be expected. The absence or addition of *Salmonella* spp. had no significant impact on the quantification of these three bacterial indicators.

Our work revealed the bacterial population dynamics during pre-enrichment under aerobic conditions. Anaerobic bacteria were outcompeted by aerobic species and the addition of *Salmonella* spp. at t = 0 h clearly modulated the composition of the bacterial flora at t = 5 h. The pre-enrichment step favored the growth of *Salmonella* not only by inhibiting the growth of competing bacteria but also by increasing its fitness.

Here, metagenomics has shed new lights in the pre-enrichment step

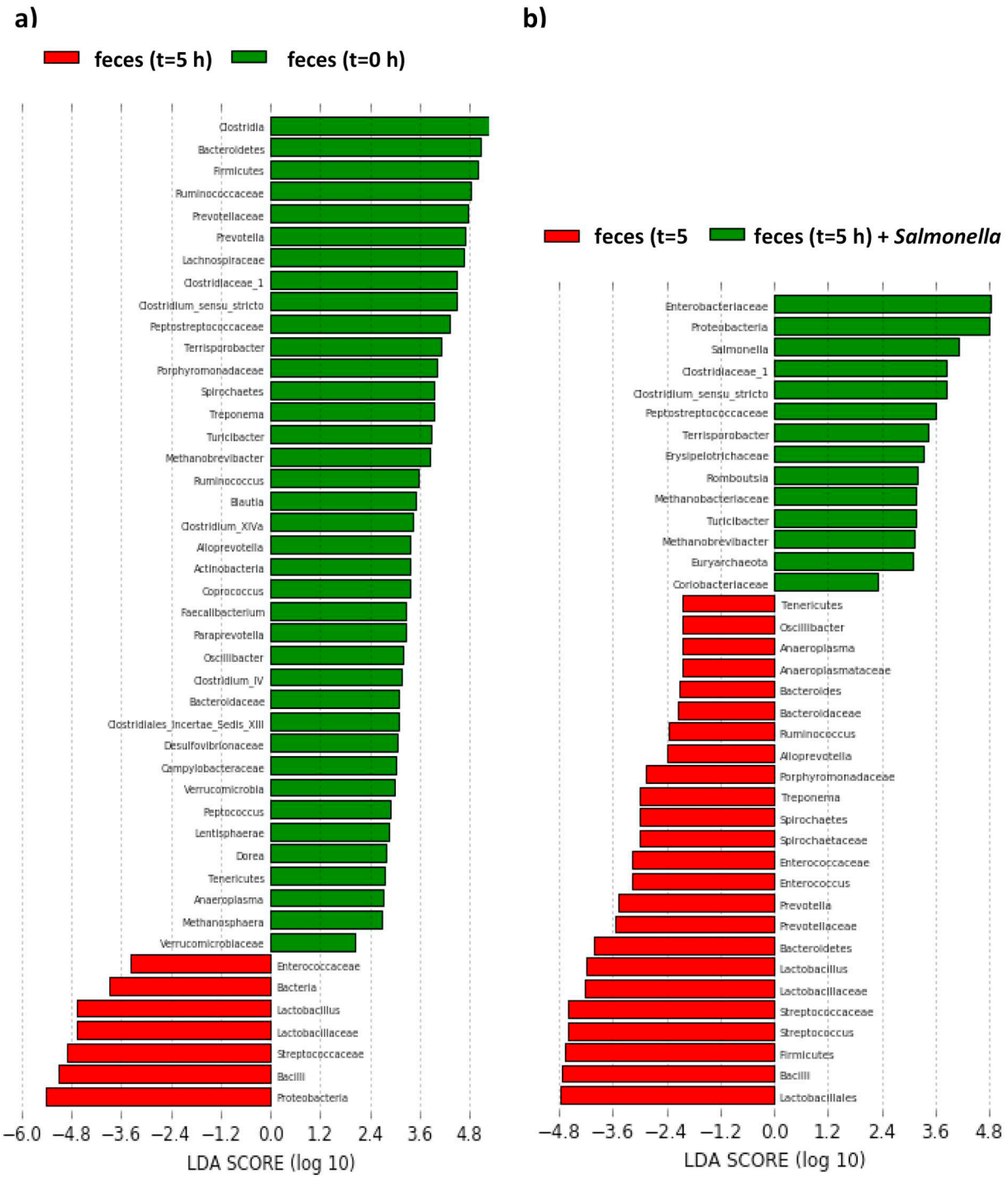


Fig. 1. Histograms of linear discriminant analysis (LDA) effect size (LEfSe) computed a) between *Salmonella*-free pig feces before (t = 0 h) and after pre-enrichment (t = 5 h); and b) between pig feces after the pre-enrichment (t = 5 h) without and with the addition of *Salmonella* spp. at t = 0 h. The x axis is the scale of the logarithmic LDA score.

for the isolation of *Salmonella* spp. from pig feces. Our findings must be taken into consideration in other detections and isolations of *Salmonella* spp. where sensitivity is influenced by the pre-enrichment step.

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