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16S rRNA microbial community analysis and relationship with *Salmonella* Super shedder status in pigs

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Background

Intestinal microbiota species richness and relative abundance can be linked with the health status of the animals. Recent studies have highlighted the importance of host heterogeneity in infection with zoonotic pathogens, and it has been shown that a minority of the infected individuals are responsible for the majority of the infections (known as 'super-shedders').

A better understanding of the composition of the microbiota of super-shedders may facilitate targeted interventions with, for example, pre and probiotics, to reduce colonisation and shedding. The aim of this study was to investigate whether there was any association between *Salmonella* shedding status in pigs and microbiota heterogeneity.

The study detected small, but statistically significant differences in the bacterial species richness between sample types, between the different groups of pigs and also according to the shedding status (versus the control group) with implications for our understanding and potential mitigation of foodborne zoonoses. Further analysis using machine learning and mathematical modelling tools is under development to further assess the relationships between bacterial communities.

Methods

- 458 samples (faeces and GI content) were collected at different time points. DNA was extracted by using a commercial kit and 16s sequencing was performed.
- Raw sequences obtained were checked for quality and low quality sequences were excluded from the analysis.
- The analysis was performed using the software Qiime2 taking in account different metadata such as husbandry data (i.e. age of the pigs, pen where they grew, sex), clinical parameters (i.e. immunological status, *Salmonella* CFU) and *Salmonella* shedding status.
- To evaluate total *Salmonella* shedding for each pig, the CFU/g in faeces from each sampling point was log-normalized and plotted in order to calculate the area under the log curve (AULC) (Huang *et al.*, 2011; Knetter *et al.*, 2015). Then, a hierarchical clustering (function hclust) was performed on AULC data to cluster pigs in three classes according to their level of *Salmonella* shedding (high, intermediate and low shedders (HS, IS, and LS)). A variance analysis and a Newman & Keuls test were performed to confirm that these three classes were significantly different. All statistical analysis was performed using R software (R version 3.5.2).
- The similarity matrix between the OTUs at Order level (L4) was calculated using Pearson correlation; the matrix was used to generate heat maps using the online tool Morpheus (<https://software.broadinstitute.org/morpheus/>).
- Pearson correlation based network plots were generated using the statistical software PAST4 (v4.04).

Results

16s rRNA analysis (Qiime2)

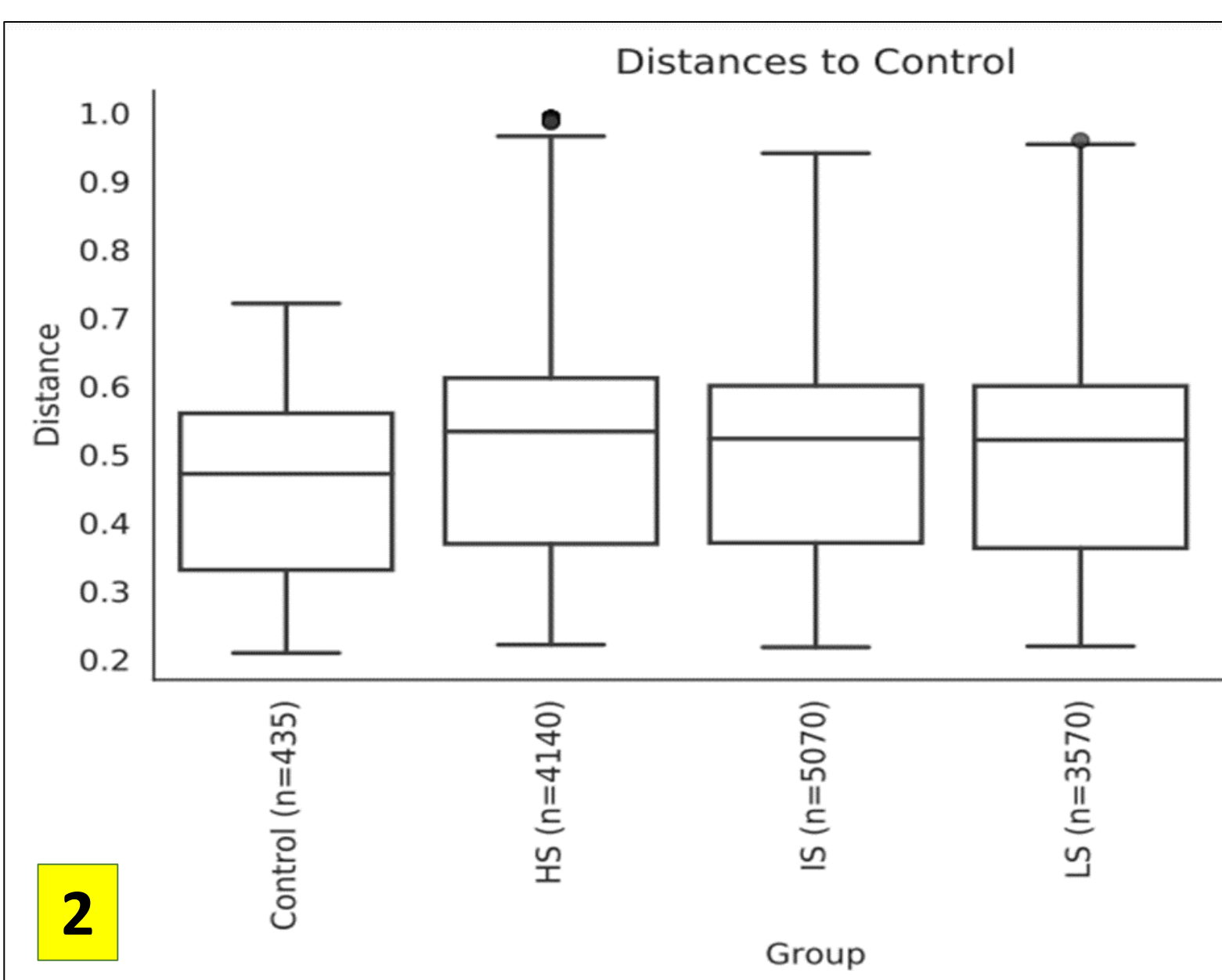
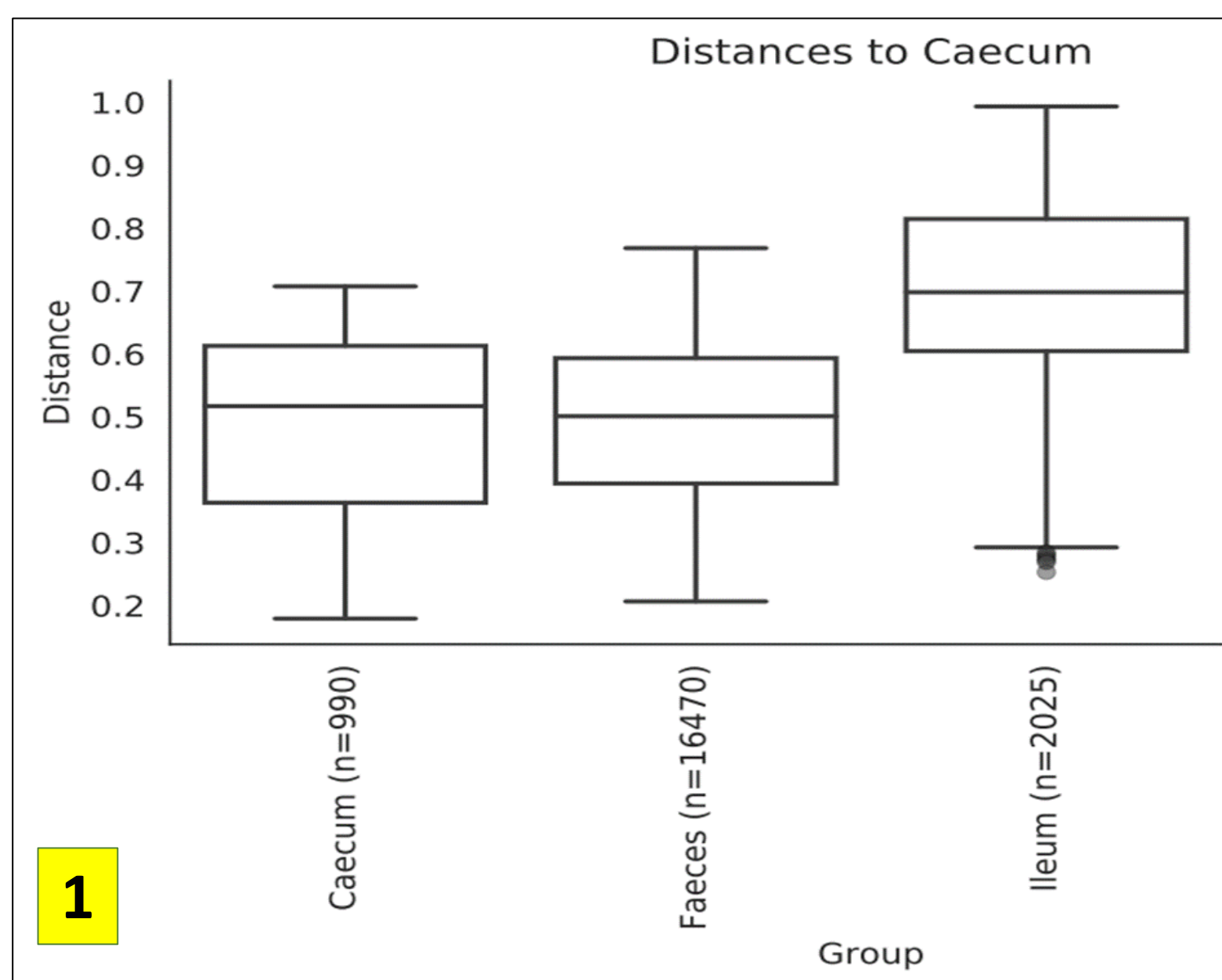


Figure 1: Type of sample (faeces/ileum/caecum). Beta distribution.

Statistically relevant differences can be noted in the OTUs composition of samples collected from ileum while faecal and caecum samples don't show remarkable differences.

Fig 2: Shedding classes (HS/IS/LS). Beta distribution.

It was not possible to discriminate between HS, IS and LS with the 16s analysis alone. However, the three shedding levels show statistical representative differences when compared with the control group.

OTU correlation at Order level (L4)

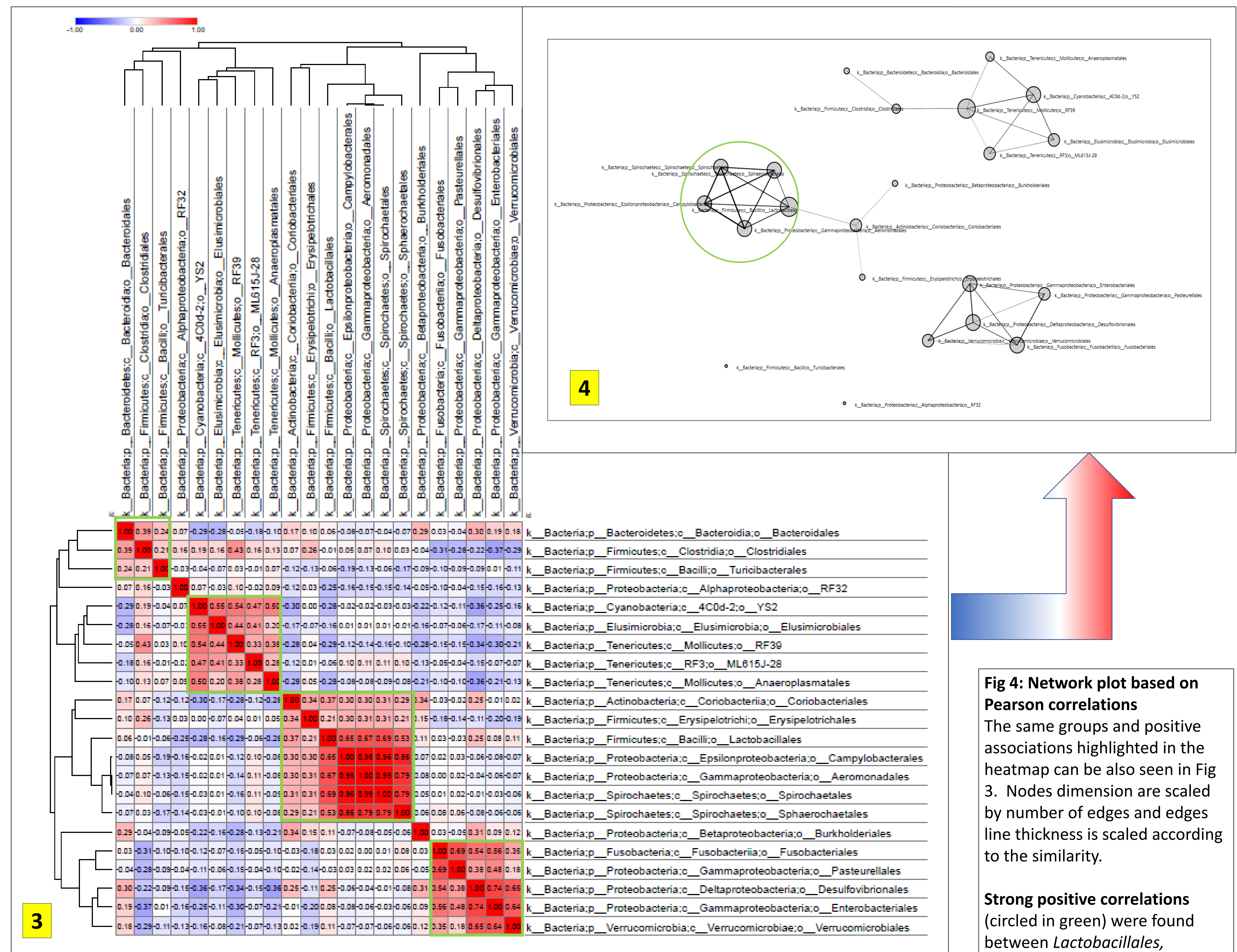


Fig 3: Heat map based on Pearson correlation. Positive correlations are in shades of red, negative correlations in shades of blue (range= -1 to 1) . Four highly correlated OTUs groups (marked in green) have been identified at Order level.

Positive associations between bacteria of interest: From bottom right to upper left green squares: 1) *Enterobacteriales* group, 2) *Lactobacillales* group, 3) *Clostridiales* group.

Negative correlations: The group containing *Tenericutes* Philum it's negatively correlated to the vast majority of the other bacteria. *Lactobacillales* present in our samples didn't show any remarkable negative correlation with *Enterobacteriales*.

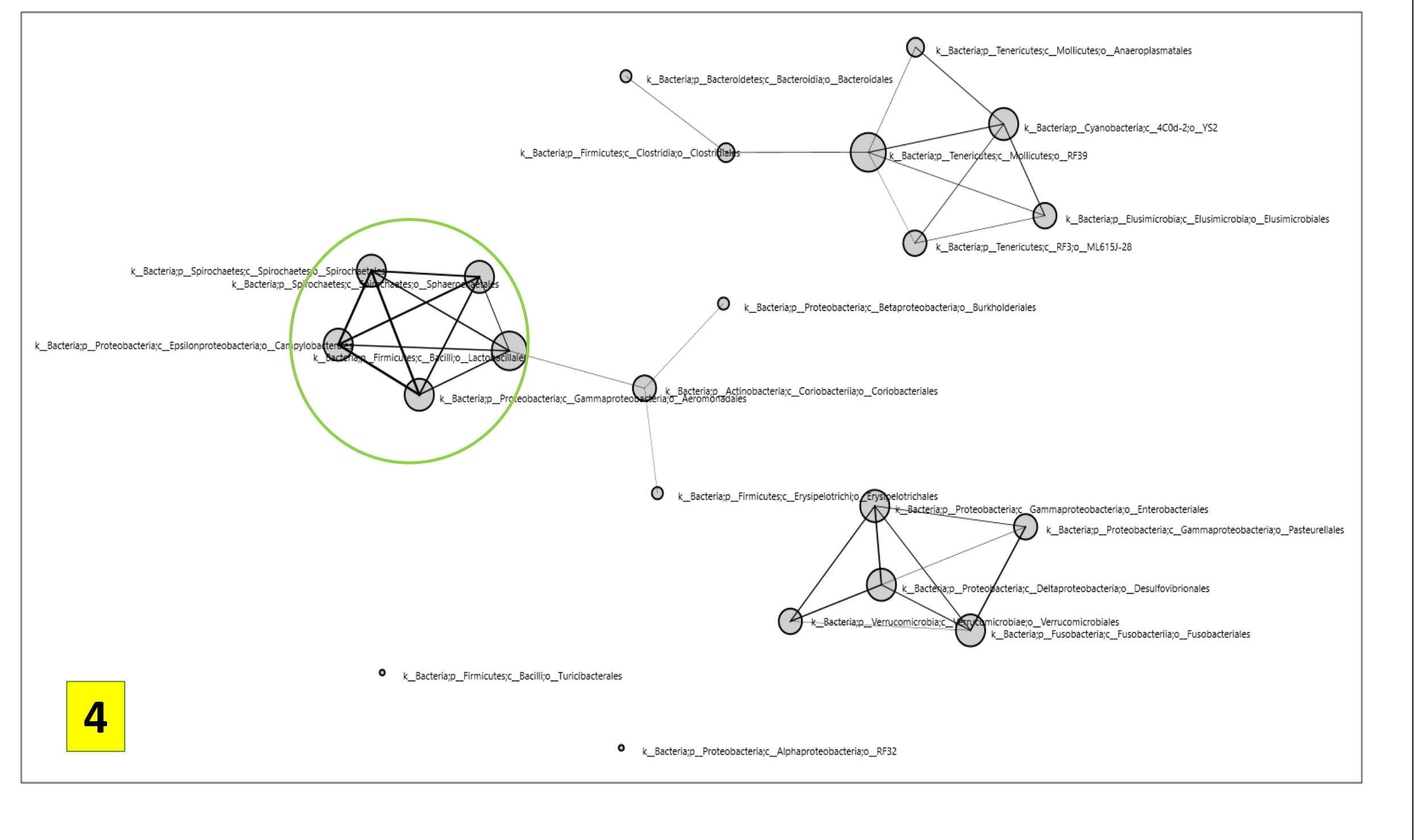


Fig 4: Network plot based on Pearson correlations

The same groups and positive associations highlighted in the heatmap can be also seen in Fig 3. Nodes dimension are scaled by number of edges and edges line thickness is scaled according to the similarity.

Strong positive correlations (circled in green) were found between *Lactobacillales*, *Campylobacteriales*, *Aeromonadales*, *Spirochaetales* and *Sphaerochaetales* (average correlation on heat map = 0.79).

Lactobacillales are mildly correlated (0.37) with the Order of *Coriobacteriales* and with *Burkholderiales* and *Erysipelotrichales* (0.27).

Conclusion and Discussion

- Statistically significant variations were detected in the relative abundance of bacteria in different intestinal locations (ileum, caecum and faeces), between the control and challenged groups.
- This study concurs with previous studies demonstrating that the composition of microbiota varies in different parts of the intestinal tract.
- To date, for all the other metadata it was not possible to find statistically significance in the OTUs composition/abundance.
- The bacterial positive and negative associations observed shed new light on the relationship between bacterial Orders. This data could be used to identify potential new probiotic bacteria able to mitigate known pig pathogens (e.g. *Salmonella*, *Escherichia*, *Brachyspira*).

Future plans

- The biological importance of small variations in different parts of the intestine requires further investigation.
- Shotgun metagenomic data may provide more detailed information on species level differences.
- Further analysis using alternative similarity measures, conducted at Family, Genus and Species level, may provide a more detailed picture of the bacterial associations.
- A combined gene-targeted bacterial and virologic metagenomic analysis may shed further light on the interactions (synergic or divergent).
- Studies are ongoing (following EFSA guidelines) to characterise lactobacilli isolates that exhibit probiotic activity *in vitro* and *in vivo* against *Salmonella* Typhimurium and *Escherichia coli*.