

*Unlocking the potential of  
macroalgae for a thriving  
European blue  
bioeconomy*

Ref. Ares(2025)5211159 - 30/06/2025

2025



## Gut microbiome characterization by 16S rRNA gene amplicon sequencing completed

SEAMARK DELIVERABLE 4.4

University of Copenhagen



Co-funded by  
the European Union

This project has received funding from the European Union's Horizon Europe research and innovation programme under Grant Agreement No 101060379. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union nor the European Research Executive Agency (REA). Neither the European Union nor REA can be held responsible for them.

## SEAMARK DELIVERABLE 4.4: GUT MICROBIOME CHARACTERIZATION BY 16S RRNA GENE AMPLICON SEQUENCING COMPLETED

**Nilay Büdeyri Gökgöz<sup>1</sup>, Marianne Kaiser<sup>2</sup>, Dennis Sandris Nielsen<sup>1</sup>**

### OPEN ACCESS

#### **Edited by:**

Maya Miltell, SUBMARINER Network  
for Blue Growth EEIG, Berlin  
Germany

#### **Reviewed by:**

Urd Grandorf Bak and Ólavur  
Gregersen, Kaldbak, Ocean  
Rainforest Sp/F

**Document Status:** Finished

**Dissemination Level:** PU – Public

**Due Date:** 30.12.2024

**Submission Date:** 30.06.2025

**Accepted Date:** 27.06.2025

**Deliverable Reference:** D4.4

#### **Work Package / Task:**

4 / T4.3 Fermented seaweed's effect  
on the microbiome of sows and  
integration of data

**Total number of pages (incl.  
annexes):**

18

#### **Keywords:**

Fermented feed additive, EP119,  
seaweed, pig nutrition, commercial  
farm, gut microbiome, 16S rRNA gene  
amplicon sequencing

**Front page picture:** Marianne Kaiser

<sup>1</sup>University of Copenhagen, Department of Food Science, Frederiksberg C, Denmark,

<sup>2</sup>Aarhus University, Department of Animal and Veterinary Sciences – ANIVET, Tjele Denmark

### **Abstract**

**Background:** Supplementing sow diets with fermented feed additives has become a promising nutritional approach to influence the gut microbiome, potentially enhancing both sow performance and gut health of their offspring. This work investigated the impact of inclusion of EP119 (commercially available fermented rapeseed meal and the seaweed species *Ascophyllum nodosum* and *Saccharina latissima*) into sow diet on the gut microbiome composition under two different studies named Focal study 1 (FS1) and Focal study 2 (FS2). FS1 investigates two aspects: i) the maternal gut microbiome at baseline, late gestation in gilts, and post-farrowing in first-parity sows, along with the gut microbiome of their first-parity offspring; ii) an extended observation period where the gut microbiome is evaluated in a subset of the gilts followed to third-parity sows. FS2 focuses on evaluating and comparing the gut microbiome of multi-parity sows on the days of pregnancy achieved and farrowing completed.

**Methods:** Sows were randomly allocated to a control and an experimental group. The control group received a standard diet containing rapeseed meal as the protein source, while the experimental group was provided with the same diet but with a fraction of the rapeseed meal replaced by EP119 (6% of the feed in the gestation section and 9% of the feed in the farrowing section). Faeces samples were collected, and the gut microbiome composition was analysed using 16S rRNA gene amplicon sequencing.

**Results:** In FS1, the EP119-supplemented diet led to alterations in the gut microbiome of late gestation gilts and first parity sows, regardless of inter-individual variation. The abundance of genus *Clostridium* was significantly higher in the late gestation gilts and first parity sows fed an EP119-supplemented diet compared to those fed the control diet. Since this genus includes both beneficial and harmful bacteria, species-level analysis may help clarify the significance of this change. The between-group analysis showed lower levels of *Lactobacillus* (a tendency in FS1 sub-study ii) and *Streptococcus* in the late gestation gilts fed an EP119-supplemented diet. Within-group analysis revealed that the relative abundance of *Lactobacillus* and *Streptococcus* in first-parity sows was lower in both groups compared to baseline. This may suggest that the change in relative abundance of these two genera may not be solely due to dietary intervention but also reflect other environmental factors on the farm. Despite maternal feeding with EP119-supplemented diet rendered a selective shift in maternal gut microbiome in the late gestation gilts and first parity sows, the intervention had no notable impact on the gut microbiome diversity and composition of the piglets of the first-parity sows. In FS2, no significant differences between CON and EXP groups were observed in the gut microbiome of multi-parity pregnant and farrowed sows.

**Conclusion:** The EP119-supplemented diet selectively influenced maternal gut microbiome in late gestation gilts and first-parity sows, with increased relative abundance of genus *Clostridium*.

# CONTENT

|                                                               |           |
|---------------------------------------------------------------|-----------|
| <b>1. INTRODUCTION.....</b>                                   | <b>5</b>  |
| 1.1 HYPOTHESES.....                                           | 5         |
| <b>2. METHODOLOGY .....</b>                                   | <b>5</b>  |
| 2.1 ETHICS STATEMENT .....                                    | 5         |
| 2.2 EP119, THE FERMENTED FEED ADDITIVE .....                  | 5         |
| 2.3 ANIMALS AND FEEDING PROTOCOL .....                        | 5         |
| 2.4 STUDY DESIGN FOR GUT MICROBIOME ANALYSIS.....             | 5         |
| 2.3.1 <i>Focal Study 1 (FS1)</i> .....                        | 6         |
| 2.3.2 <i>Focal Study 2 (FS2)</i> .....                        | 6         |
| 2.5 DNA EXTRACTION AND 16S rRNA GENE AMPLICON SEQUENCING..... | 6         |
| 2.6 GUT MICROBIOME DATA ANALYSIS AND STATISTICS .....         | 7         |
| <b>3. RESULTS .....</b>                                       | <b>7</b>  |
| 3.1 FOCAL STUDY 1.....                                        | 7         |
| 3.1.1 <i>Sub-study 1</i> .....                                | 7         |
| 3.1.2 <i>The maternal gut microbiome</i> .....                | 7         |
| 3.1.3 <i>The piglet gut microbiome</i> .....                  | 10        |
| 3.1.4 <i>Sub-study 2</i> .....                                | 10        |
| 3.2 FOCAL STUDY 2.....                                        | 13        |
| <b>4. DISCUSSION .....</b>                                    | <b>15</b> |
| 4.1 FOCAL STUDY 1.....                                        | 15        |
| 4.1.1 <i>Sub-study 1</i> .....                                | 15        |
| 4.1.2 <i>Sub-study 2</i> .....                                | 15        |
| 4.2 FOCAL STUDY 2.....                                        | 16        |
| <b>5. CONCLUSION .....</b>                                    | <b>16</b> |
| <b>6. FUTURE CONSIDERATIONS.....</b>                          | <b>16</b> |
| <b>7. ACKNOWLEDGMENTS .....</b>                               | <b>16</b> |
| <b>REFERENCES.....</b>                                        | <b>16</b> |
| <b>DATA SETS .....</b>                                        | <b>18</b> |

## LIST OF FIGURES

|                                                                                                                                                                              |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1: The maternal gut microbiome profile in FS1 sub-study 1. ....                                                                                                       | 8  |
| Figure 2: FS1 sub-study1: Genus-level bacterial relative abundance profile showing taxa.....                                                                                 | 9  |
| Figure 3: Boxplots show the group-wise differences in the relative abundance of Clostridium, Lactobacillus, and Streptococcus at each sampling days of FS1 sub-study 1. .... | 10 |
| Figure 4: FS1 sub-study 1- The relative abundance of Clostridium perfringens in CON and EXP piglets. ....                                                                    | 10 |
| Figure 5: FS1 sub-study 1-The piglet microbiome. ....                                                                                                                        | 11 |
| Figure 6: FS1 sub-study 2: Monitoring gut microbiome from gilts to first-parity sows to third-parity sows.....                                                               | 11 |
| Figure 7: FS1 sub-study 2: Genus-level bacterial relative abundance profile from gilts to first-parity sows to third-parity sows.....                                        | 12 |
| Figure 8: Boxplots show the group-wise differences in relative abundance of Clostridium, Lactobacillus, and Streptococcus at each sampling days of FS1 sub-study 2. ....     | 12 |
| Figure 10: The gut microbiome of multi-parity sows on Days P and F.....                                                                                                      | 14 |
| Figure 9: FS2: Genus-level bacterial relative abundance profile of multi-parity sows .....                                                                                   | 14 |

## LIST OF TABLES

|                                                                                                                                                                 |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 1: The reproductive stages and the diets given to sow groups at the time of faeces sampling. ....                                                         | 6  |
| Table 2: The FS1 sub-study 1: Type III Wald Chi-square test results for the effects of Group, Day, and their interaction on PCoA1.. ....                        | 8  |
| Table 3: The FS1 sub-study 1: Group-wise differences in gut microbiome community composition across sampling da.....                                            | 9  |
| Table 4: The FS1 sub-study 1: The significance assessment for the changes in abundance of Clostridium, Lactobacillus, and Streptococcus within CON and EXP..... | 9  |
| Table 5: The FS1 sub-study 2: The significance assessment for the changes in abundance of Clostridium, Lactobacillus, and Streptococcus within CON and EXP..... | 13 |

## LIST OF ABBREVIATIONS

| Abbreviation        | Description                                                                                                                                  |
|---------------------|----------------------------------------------------------------------------------------------------------------------------------------------|
| <i>A. nodosum</i>   | <i>Ascophyllum nodosum</i>                                                                                                                   |
| ASV                 | Amplicon Sequence Variant                                                                                                                    |
| AU                  | Aarhus University                                                                                                                            |
| CON                 | Control sows fed a standard feed including rapeseed meal as the protein source                                                               |
| Day ÷ X             | First observation day of gilts in the Focal study 1                                                                                          |
| Day ÷ Y             | Approximately 14 days before the pregnant gilts in Focal study 1 were moved from the gestation barn to the farrowing unit for the first time |
| Day ÷ Z             | The day after the gilts in the Focal study 1 farrowed for the first time (hereafter gilts are often called first parity sows)                |
| Day P               | Day Pregnancy. Last pregnancy before the end of the study                                                                                    |
| Day F               | Day Farrowing. The day when the barn staff for the first time noticed that a given sow had finished farrowing                                |
| EXP                 | Experimental sows fed a standard feed in which rapeseed meal was replaced by EP119                                                           |
| FEXP                | Fermentationexperts A/S                                                                                                                      |
| FS1                 | Focal study 1                                                                                                                                |
| FS2                 | Focal study 2                                                                                                                                |
| Gilt                | A sexually mature female pig that has not never been pregnant (gilts in Day ÷ X) or is in her first gestation (gilts in Day ÷ Y)             |
| GM                  | Gut microbiome                                                                                                                               |
| Parity              | The number of times a sow has farrowed                                                                                                       |
| PCoA                | Principal Coordinates Analysis                                                                                                               |
| <i>S. latissima</i> | <i>Saccharina latissimi</i>                                                                                                                  |
| SCFA                | Short Chain Fatty Acids                                                                                                                      |
| Sow                 | A sexually mature female pig that has farrowed at least once                                                                                 |
| UCPH                | University of Copenhagen                                                                                                                     |

# 1. INTRODUCTION

The growing demand for sustainable and health-promoting feed strategies in pig nutrition has led to a rising interest in functional feed additives such as fermented rapeseed meal (FRSM) [1] and seaweed [2] due to their potential beneficial effects on gut health and pig performance.

Studies in piglets have shown that FRSM can positively influence gut microbiome by increasing the abundance of beneficial bacteria such as *Lactobacillus* [1]. Another study also reported that its inclusion in the diet enhances lactic acid bacteria levels and reduces the number of pathogenic *Clostridium perfringens* [3].

Diets supplemented with a combination of FRSM and brown seaweeds (*A. nodosum* and/or *S. latissima*) have produced variable results: For instance, a 2020 study showed that supplementation with FRSM fermented together with *A. nodosum* produced effects on growth comparable to FRSM alone. However, the inclusion of FRSM fermented together with *A. nodosum* and *S. latissima* appeared to diminish some beneficial intestinal effects observed with FRSM [4]. Another study highlighted that the inclusion rate of the FRSM-seaweed blend in the weaner diet plays a key role, as moderate levels enhanced growth of the relative abundance of putatively beneficial *Prevotella stercorea* and *Mitsuokella* spp. [5].

While the combination of FRSM and seaweed has been studied in piglet nutrition, its effects on the gut microbiome of sows and/or their offspring remain largely underexplored. EP119 is a commercially available fermented feed additive consisting of rapeseed meal and seaweeds *A. nodosum* and *S. latissima* [European Protein, Bække, Denmark].

In this context, this report examines the impact of an EP119-supplemented diet on the gut microbiome of first-parity sows and their piglets, as well as multi-parity sows in a commercial sow farm setting.

## 1.1 Hypotheses

The hypothesis related to deliverable 4.4 is that sow gut microbiome will adapt and preferentially utilize EP119 over time, leading to a shift in the microbiome composition towards a gut microbiome with increased relative abundance potentially beneficial bacteria, which in turn may promote gut health.

# 2. METHODOLOGY

## 2.1 Ethics statement

Approval for the experimental protocol was granted by Danish Animal Experiments Inspectorate, The Danish Veterinary and Food Administration (DVFA) under permit number 2023-15-0201-01427.

## 2.2 EP119, the fermented feed additive

EP119 is a commercial product of European Protein (Bække, Denmark) and supplied by Fermentationexperts A/S (FEXP). It is produced by fermenting rapeseed meals and seaweed with lactic acid bacteria and serves as a complementary feed additive in pig and poultry nutrition [Deliverable 4.6, Appendix 1].

## 2.3 Animals and feeding protocol

The animal and feeding protocol are detailed in Deliverable 4.6, Methodology Section. Here, we provide a summary.

The sow study was carried out on a Danish sow farm housing approximately 2000 sows of breeds Topigs Norsvin (Topigs Norsvin, Herning, Denmark) and DanBred (DanBred P/S, Vejle, Denmark).

The sows were fed a commercial dry feed using an electronic sow feeding system (Bopil, Sønderborg, Denmark) in the gestation section, while the SpotMix feed plant (Bopil, Sønderborg, Denmark) in the farrowing section enabled individual monitoring of the sows' feed intake.

The feed plant was first checked for microbial contamination and none were detected. The farm's feed curves follow guidelines provided by Topigs Norsvin, with individual adjustments made in the farrowing section based on the sows' appetite. These adjustments were carried out by the farm staff, using their assessments as part of standard farm practice. Before farrowing, the sows were fed three daily feedings. Starting from the day of farrowing, sows were fed restrictively with three feedings per day, which were stepped up to four times feeding per day approximately one week after farrowing.

## 2.4 Study design for gut microbiome analysis

The sow trial follows a three longitudinal case-cohort design: focal study 1 (FS1), a multi-parity study, and focal study 2 (FS2). Each study is described in detail in Deliverable 4.6, Methodology Section.

The gut microbiome analysis was only conducted for FS1 and FS2, both of which aimed at evaluating the impact of the EP119-supplemented feed on the gut microbiome of sows — and in the case of FS1 sub-study 1, also a random selection of their offspring.

To investigate the changes in the gut microbiome of sows related to including EP119 in the diet, the pigs were randomly assigned to one of two groups: control (CON) and experimental (EXP). Both groups received the same standard feed, but in the EXP group, the feed ingredient rapeseed meal (protein source) was replaced with EP119 fermented feed additive. The percentage of EP119 differed between the gestation and farrowing sections, with 6% and 9%, respectively. Since EP119 is only expected to work after an initial feeding period, the

study ran over several months (see details for each study below).

### 2.3.1 Focal Study 1 (FS1)

The FS1 covers the period from April'23 to November'24.

As explained in D4.6, from January'24 to March'24, the feed was mistakenly added with benzoic acid [Deliverable 4.6, Table 1], which may modulate the pig gut microbiome [6]. Therefore, the study was extended to also cover the third parity for gut microbiome analysis (originally only planned to cover the second parity). Following adjustments in the study design, a minor and similar change in the mineral composition of the feed mixture was made for both CON- and EXP-feed compositions [Deliverable 4.6, Appendix 3]. In addition, for a period of 11 days in July'24, the EXP-feed was inadvertently provided without EP119, causing a minor deviation in the feeding protocol [Deliverable 4.6, Table 1].

To capture the changes in GM, faecal samples were collected across the reproductive stages:

**Day ÷X:** Faeces samples were collected from gilts when they arrived at the farm.

**Day ÷Y:** Faeces samples were collected approximately 14 days before the pregnant gilts were moved to the farrowing section.

**Day ÷Z:** Faeces samples were collected from first-parity sows the day after farrowing.  
Faeces samples were collected from randomly selected piglets from first-parity sows.

**Day P:** Faeces samples were collected on the day pregnant of third-parity sows.

**Day F:** Faeces samples were collected on the day farrowing completed of third-parity sows.

In FS1, the Day ÷X represents the gut microbiome of non-pregnant gilts before starting the sow trial (baseline GM). The reproductive stages and the diets given to sow groups at the time of faeces sampling are shown in [Table 1].

*Table 1: The reproductive stages and the diets given to sow groups at the time of faeces sampling.*

| Faeces Sampling Time | Reproductive Stage                             | Farm Section | CON Group     | EXP Group                           |
|----------------------|------------------------------------------------|--------------|---------------|-------------------------------------|
| Day ÷Y               | Late gestation gilts                           | Gestation    | Standard Diet | 6% EP119-supplemented Standard Diet |
| Day ÷Z               | First-parity sows the day after farrowing      | Farrowing    | Standard Diet | 9% EP119-supplemented Standard Diet |
| Day P                | Third-parity sows -the day pregnant            | Gestation    | Standard Diet | 6% EP119-supplemented Standard Diet |
| Day F                | Third-parity sows -the day farrowing completed | Farrowing    | Standard Diet | 9% EP119-supplemented Standard Diet |

GM analysis related to FS1 was performed under two sub-studies:

In the first sub-study (from April'23 through December'23), we focused on how the EP119-supplemented diet influenced maternal and offspring gut microbiome. Briefly, maternal gut microbiome was assessed at three reproductive stages: Day ÷X, Day ÷Y, and Day ÷Z. On Day ÷Z, one randomly selected piglet from each sow was anal microbiomes were the maternal feeding effect on the offspring gut microbiome.

In the second sub-study, we assessed a subset of sows from FS1 that were also sampled in third parity to explore how gut microbiome changes during this period. Of note, due to the short-term benzoic acid usage and its potential effect on GM, no faeces samples were collected from second-parity sows. As a result, the gut microbiome was evaluated at these five reproductive stages: Day ÷X, Day ÷Y, Day ÷Z, Day P, and Day F, where P represents the third pregnancy and F the third farrowing.

### 2.3.2 Focal Study 2 (FS2)

This study was introduced as a consequence of the addition of benzoic acid. In this study, the gut microbiome of a subset of multiparity sows from the multi-parity study [Deliverable 4.6, Methodology Section] was assessed to explore the impact of EP119 inclusion in feed. Faeces samples were collected on the day of pregnancy (Day P) before feeding EP119 and the day farrowing was completed (Day F). Again, in the EXP diets, the percentage of EP119 was 6% and 9% in the gestation and farrowing sections, respectively.

All faeces samples and faeces swabs were immediately stored on ice and transported to University of Aarhus (AU) for an initial and short-term freezing at -18 degrees. AU subsequently delivered faeces samples and faecal swabs to UCPH (transported in frozen state), where the samples were stored at -80°C until further analysis.

## 2.5 DNA Extraction and 16S rRNA gene amplicon sequencing

The DNA from sow faeces samples were extracted by following the protocol of QIAamp PowerFecal Pro DNA Kit (Qiagen, Germany), while the Bead-Beat Micro AX Gravity Kit, supplemented with mutanolysin and lysozyme (Cat. 106-100-M1, A&A Biotechnology, Poland) was used to extract DNA from piglet faeces swabs. Since piglet faeces swabs were stored in Cary-Blair medium of FecalSwab® collection kit (Copan, Italy), a pre-washing step with sterile 0.9% NaCl was applied prior to DNA extraction.

The DNA concentration was measured using the Qubit® dsDNA HS Assay Kit (Life Technologies, CA, USA) on Varioskan Flash Multimode Reader (Thermo Fisher Scientific, MA, USA). A two-step PCR approach explained in [7] was applied for library preparation and the size of second-PCR products was verified on a 1.5% agarose gel.

A dual-level barcoding system was employed to construct a sequencing library, which was sequenced on PromethION 2 Solo device (Oxford Nanopore Technologies, Oxford, UK; <https://nanoporetech.com>) connected to a custom-built computing setup (MinkNOW software 24.02.16, Oxford Nanopore Technologies).

In brief, the pooled second-PCR products were barcoded using the Native Barcoding kit 24 V14 (SQK-NBD114.24; Oxford Nanopore Technologies, Oxford, UK) and subsequently loaded onto a PromethION R10.4.1 flow cell. Sequencing, initial demultiplexing (based on native barcodes), and basecalling were carried out using the MinkNOW software 24.02.16 (Oxford Nanopore Technologies). Raw sequencing data were processed via RubyRed, a custom bioinformatics pipeline (<https://github.com/reillyeo/RubyRed/>).

### 2.6 Gut Microbiome Data Analysis and Statistics

Data analysis and statistics were performed in R studio [8] using R version 4.2.1 [9].

Amplicon Sequence Variants (ASVs) only assigned taxonomy to phylum level were included in analysis, while unassigned taxonomy at any level below the phylum level were modified to be labeled as “Unclassified *lowest taxonomy name*”.

Alpha and beta diversity analyses were performed after rarefying the data to 10000 reads per sample using the R phyloseq package [10]. This resulted in the removal of one sow sample (control group) and four piglet samples from the analysis.

Alpha diversity was assessed through the number of observed ASVs and Shannon diversity index. To assess statistical significance, one-way ANOVA was used for normally distributed data (Shapiro-Wilk test); otherwise, Wilcoxon rank-sum test was applied.

Beta diversity was assessed through principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metrics using the R phyloseq package. Overall significance was tested by PERMANOVA using the R vegan package [11]. Significant results were followed by pairwise comparisons with pairwise PERMANOVA using the R pairwiseAdonis package [12], applying Benjamini-Hochberg correction.

In FS1 sub-study 1, to assess the effect of diet (Group) and sampling days (Day) on microbial community composition, while accounting for the inter-individual variation, a linear mixed-effects model was fitted with first PCoA1 scores as the response variable, and group, day, and their interaction as fixed effects. Individual sow ID was included as a random effect. The R lme4 package [13] was used to apply this model. Model diagnostics was checked using the R DHARMA package [14]. The significance of fixed variables was assessed with Type-III Wald Chi-square test from the R car package [15].

Pairwise comparisons with Benjamini-Hochberg correction were conducted using by the R emmeans package [16].

Bacterial relative abundance was determined at genus-level.

The significance assessment for the relative abundance of the genera *Clostridium*, *Lactobacillus*, and *Streptococcus* were performed using pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction from the R stats package [9].

In FS1 and FS2 studies, while the aim was to track the same sows at determined time points, some faeces sample were missing due to issues encountered during sample collection and handling.

## 3. RESULTS

### 3.1 Focal Study 1

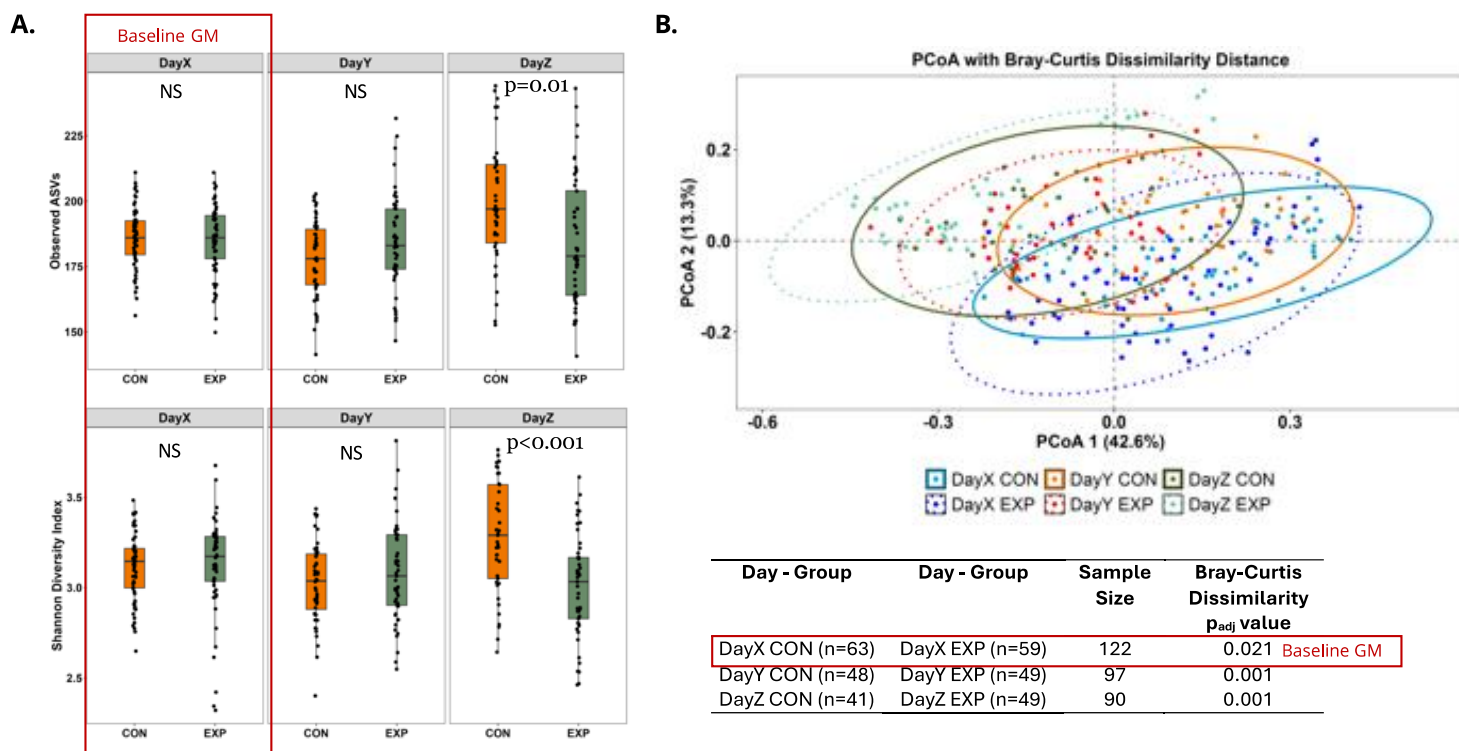
#### 3.1.1 Sub-study 1

In this sub-study, we investigated how EP119 inclusion into sow diet affected the maternal and piglet gut microbiome (Day  $\pm$ X to Day  $\pm$ Z).

#### 3.1.2 The maternal gut microbiome

At baseline, maternal gut microbiome diversity between the CON and EXP was similar as measured by bacterial richness (observed ASVs) and evenness (Shannon diversity index) ( $p>0.05$ ). This pattern of similarity persisted at the late gestation ( $p>0.05$ ); however, a significant reduction in bacterial richness and evenness was observed in the EXP the day after farrowing ( $p<0.05$ ) [Figure 1A].

Subsequent analysis of gut microbiome compositional patterns (beta-diversity analysis) revealed a significant overall difference in gut microbiome community composition across day and group combinations (PERMANOVA,  $p<0.05$ ). Pairwise comparisons showed significant differences between the CON and EXP at both baseline and subsequent sampling days ( $p<0.05$ ), indicating that gut microbiome community composition differed from the beginning and that this difference persisted in late gestation and the day after farrowing [Figure 1B].



**CON:** Sows fed a standard feed containing rapeseed meal  
**EXP:** Sows fed a standard feed in which rapeseed meal replaced by EP119  
**DayX:** The first day of observation  
**DayY:** Approximately 14 days before the pregnant gilts were moved to the farrowing section  
**DayZ:** The day after farrowing of first-parity sows

Figure 1: The maternal gut microbiome profile in FS1 sub-study 1. A. Box plots show alpha diversity observed ASVs (top) and Shannon diversity index (bottom). B. Principal coordinates analysis (PCoA) with Bray-Curtis dissimilarity distance show the gut microbiome community composition (top). The table shows pairwise comparisons performed using pairwise PERMANOVA with Benjamini-Hochberg correction (bottom). Adjusted  $p < 0.05$  considered as significant. A non-significant difference is indicated as “NS” on the plot.

Since the significant baseline difference in gut microbiome community composition between CON and EXP may reflect inter-individual variation, we further explored whether group (CON and EXP) and sampling day effects on gut microbiome community composition were independent of individual variation. To this end, we modeled the first PCoA (PCoA1, explains the largest variation) using a linear mixed-effects model with sow ID as a random effect.

The model showed significant overall effects of group (CON and EXP), day (Day ÷ X, Day ÷ Y, Day ÷ Z), and their interaction on gut microbiome community composition (Type III Wald Chi-square test,  $p < 0.05$ ), indicating that the changes observed were not only due to baseline differences, but also influenced by the dietary intervention [Table 2].

Table 2: The FS1 sub-study 1: Type III Wald Chi-square test results for the effects of Group, Day, and their interaction on PCoA1.  $p < 0.05$  considered as significant.

| Term                            | Chi-square | Degrees of Freedom (Df) | p Value   | Notes                                    |
|---------------------------------|------------|-------------------------|-----------|------------------------------------------|
| <b>PCoA1 Variance Explained</b> | -          | -                       | -         | 42.6% total variation explained by PCoA1 |
| <b>Group</b>                    | 5.5126     | 1                       | 0.019     | Significant effect of diet group         |
| <b>Day</b>                      | 67.7145    | 2                       | 1.977e-15 | Significant effect of sampling day       |
| <b>Group:Day</b>                | 6.6373     | 2                       | 0.036     | Significant interaction effect           |

Pairwise comparisons further supported this, showing that significant group differences persisted at the late gestation and the day after farrowing [Table 3], likely reflecting the impact of the differences between CON and EXP diet on gut microbiome community composition.

**Table 3: The FS1 sub-study 1: Group-wise differences in gut microbiome community composition across sampling days.** Estimates are based on linear mixed-effects model using PCoA1 scores. Group and Day were included as fixed effects, with individual sow ID as a random effect. Degrees of freedom were calculated using the Kenward-Roger method. 95% confidence intervals were used. *p* values adjusted using the Benjamini-Hochberg method for multiple comparisons. *p*<0.05 considered as significant.

| Day     | Group Comparison | Estimate (±SE) | t Ratio | p Value |
|---------|------------------|----------------|---------|---------|
| Day ÷ X | CON vs EXP       | 0.0651±0.0277  | 2.348   | 0.0196  |
| Day ÷ Y | CON vs EXP       | 0.1578±0.0309  | 5.112   | <0.0001 |
| Day ÷ Z | CON vs EXP       | 0.1203±0.0321  | 3.750   | 0.0002  |

Genus-level bacterial composition with relative abundance above 1% revealed that EXP had consistently higher relative abundance of genus *Clostridium* and comparatively lower levels of *Lactobacillus* and *Streptococcus* when compared to CON [Figure 2].

We next assessed whether this pattern was statistically significant for these three genera between groups and found that the abundance of *Clostridium* was significantly higher in the EXP late-gestation gilts and the first-parity sows compared to CON. The genus *Lactobacillus* was lower in baseline and late gestation gilts in the EXP compared to CON. For *Streptococcus*, its relative abundance was significantly lower only in the EXP late gestation gilts compared to CON [Figure 3].

The changes in relative abundance of these three genera within each group [Figure 3 and Table 4] are:

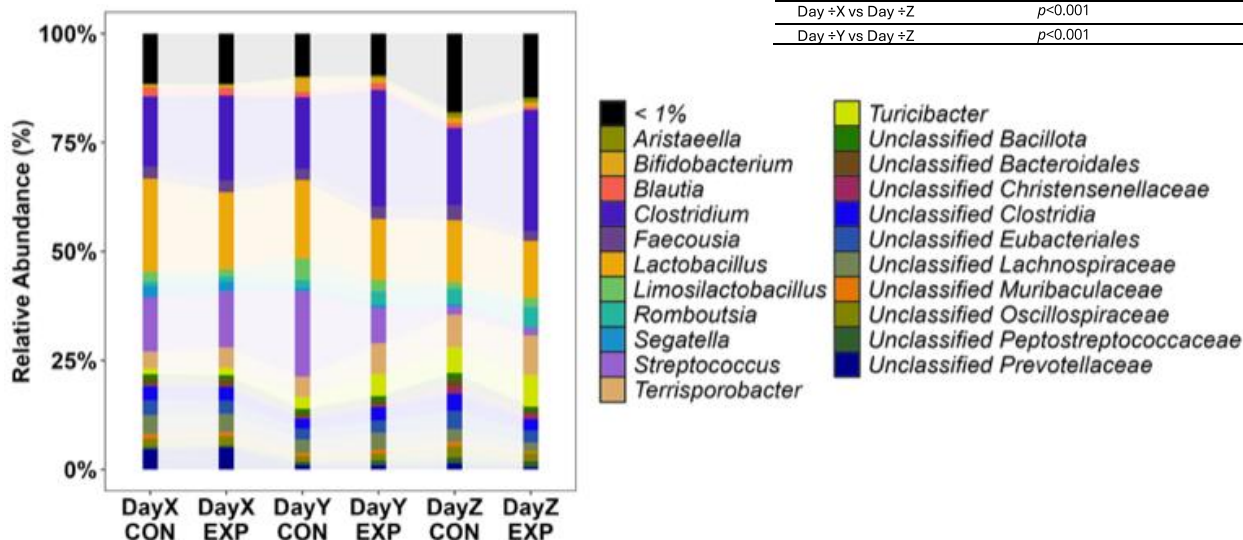


**Figure 2: FS1 sub-study1: Genus-level bacterial relative abundance profile showing taxa with relative abundance greater than 1%.**

In CON, the relative abundance of *Clostridium* remained stable at the late gestation and farrowing stages when compared to baseline (*p*>0.05). In contrast, EXP showed a significant increase in *Clostridium* abundance at these stages compared to baseline (*p*<0.05).

For *Lactobacillus*, in both groups, comparison with the baseline revealed a similar pattern in its relative abundance: levels at the late gestation were comparable to baseline (*p*>0.05), but a significant decrease was observed the day after farrowing (*p*<0.05).

The opposite trends were noted for the abundance of *Streptococcus* at the late gestation: while its abundance increased relative to baseline in the CON, it decreased in the EXP (*p*<0.05). However, the day after farrowing, both groups exhibited a marked reduction in *Streptococcus* compared to baseline (*p*<0.05).

**Table 4: The FS1 sub-study 1: The significance assessment for the changes in abundance of *Clostridium*, *Lactobacillus*, and *Streptococcus* within CON and EXP.** Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction was applied and adjusted *p*<0.05 considered as significant.

| <i>Clostridium</i>   |                                                                    |                                                                    |
|----------------------|--------------------------------------------------------------------|--------------------------------------------------------------------|
| Comparison           | CON                                                                | EXP                                                                |
|                      | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction |
| Day ÷ X vs Day ÷ Y   | <i>p</i> =0.54                                                     | <i>p</i> <0.001                                                    |
| Day ÷ X vs Day ÷ Z   | <i>p</i> =0.32                                                     | <i>p</i> <0.001                                                    |
| Day ÷ Y vs Day ÷ Z   | <i>p</i> =0.52                                                     | <i>p</i> =0.61                                                     |
| <i>Lactobacillus</i> |                                                                    |                                                                    |
| Comparison           | CON                                                                | EXP                                                                |
|                      | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction |
| Day ÷ X vs Day ÷ Y   | <i>p</i> =0.14                                                     | <i>p</i> =0.25                                                     |
| Day ÷ X vs Day ÷ Z   | <i>p</i> =0.004                                                    | <i>p</i> =0.03                                                     |
| Day ÷ Y vs Day ÷ Z   | <i>p</i> =0.10                                                     | <i>p</i> =0.25                                                     |
| <i>Streptococcus</i> |                                                                    |                                                                    |
| Comparison           | CON                                                                | EXP                                                                |
|                      | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction |
| Day ÷ X vs Day ÷ Y   | <i>p</i> <0.001                                                    | <i>p</i> <0.001                                                    |
| Day ÷ X vs Day ÷ Z   | <i>p</i> <0.001                                                    | <i>p</i> <0.001                                                    |
| Day ÷ Y vs Day ÷ Z   | <i>p</i> <0.001                                                    | <i>p</i> <0.001                                                    |

### 3.1.3 The piglet gut microbiome

To evaluate the effect of maternal diet, we compared the gut microbiome of piglets from sows fed a standard diet (CON) with those from fed an EP119-supplemented diet (EXP).

We observed that inclusion of EP119 into maternal diet unaltered piglet gut microbiome diversity (bacterial richness and evenness) [Figure 5A] and community composition [Figure 5B]. The bacterial genus profile, considering taxa greater than 1%, remained consistent across piglet groups [Figure 5C].

Next, we assessed the relative abundance of *Clostridium perfringens*, a bacterium associated with diarrhea. It was observed in both CON and EXP piglets. Despite expectations for a potential reduction in the EXP piglets, its abundance remained similar in both groups [Figure 4].

### 3.1.4 Sub-study 2

This sub-study assessed a subset of sows from FS1 and monitored the differences in gut microbiome from gilts to first-parity sows and then to third-parity sows (Day ÷ X to Day F).

In contrast to sub-study 1, we did not observe significant difference in baseline gut microbiome diversity (bacterial richness and evenness) [Figure 6A] and composition between CON and EXP [Figure 6B].

No significant changes in gut microbiome diversity were observed between the groups from late gestation gilts to first-parity sows to third-parity sows. However, there was a trend toward lower bacterial evenness in the EXP first-parity sows on the day after farrowing compared to CON [Figure 6A].

Differences in gut microbiome community composition between CON and EXP were observed only for the late-gestation gilts and the first-parity sows [Figure 6B].

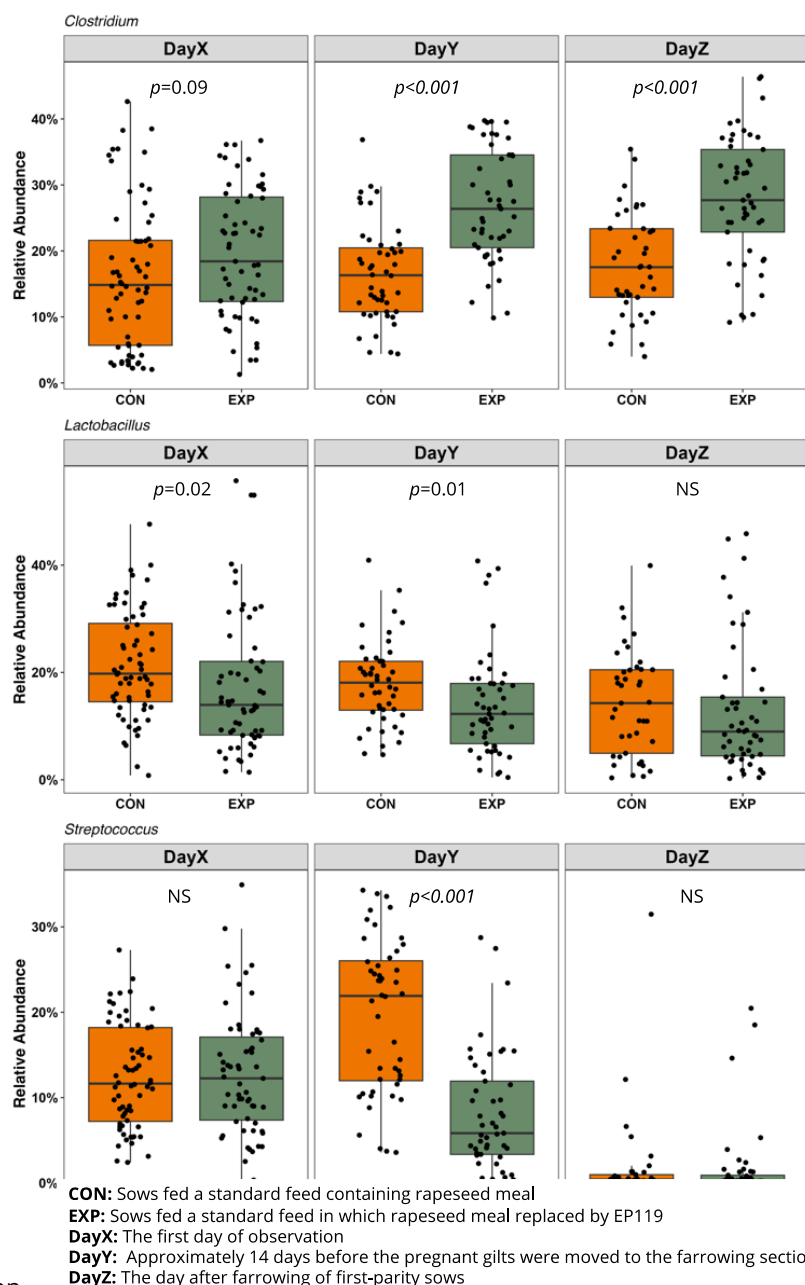


Figure 3: Boxplots show the group-wise differences in the relative abundance of *Clostridium*, *Lactobacillus*, and *Streptococcus* at each sampling days of FS1 sub-study 1. Adjusted  $p < 0.05$  considered as significant. A non-significant difference is indicated as "NS" on the plot.

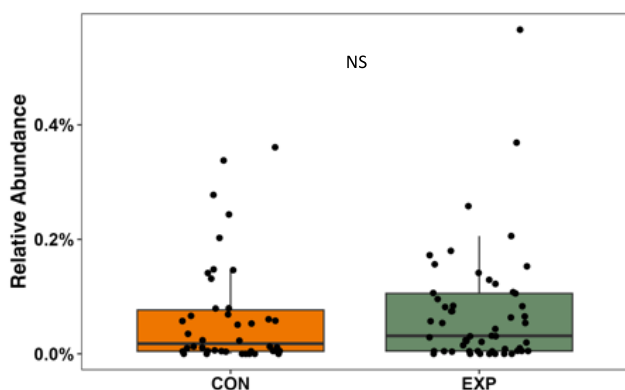
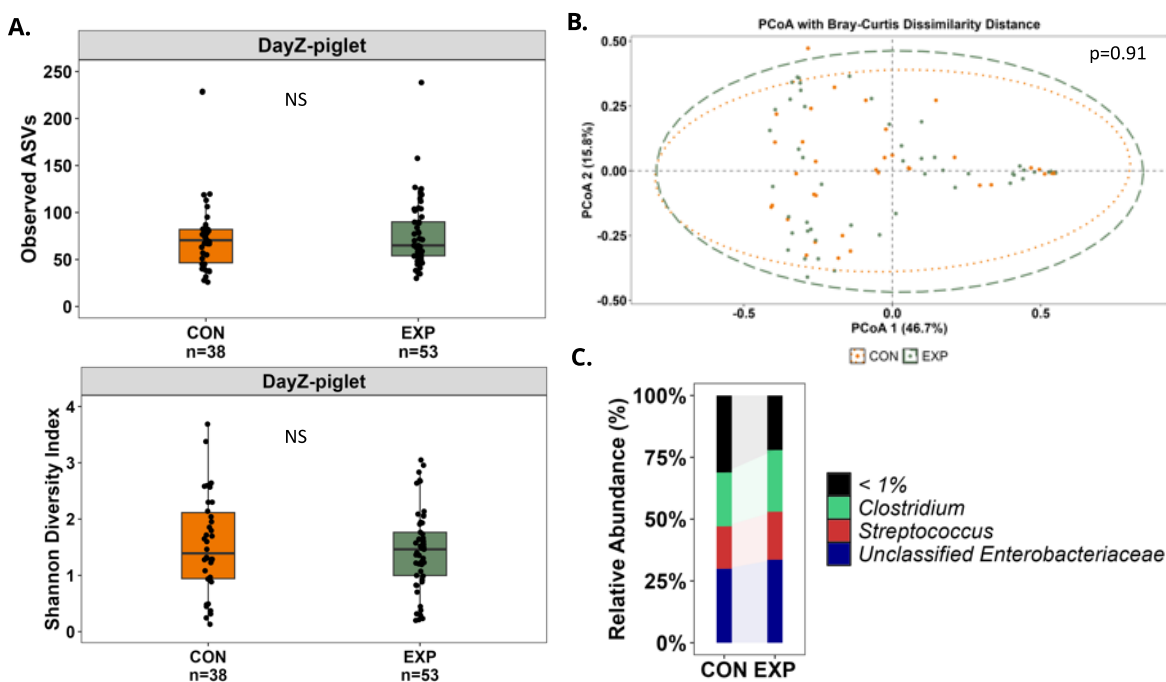


Figure 4: FS1 sub-study 1- The relative abundance of *Clostridium perfringens* in CON and EXP piglets. A non-significant difference ( $p > 0.05$ ) is indicated as "NS" on the plot.

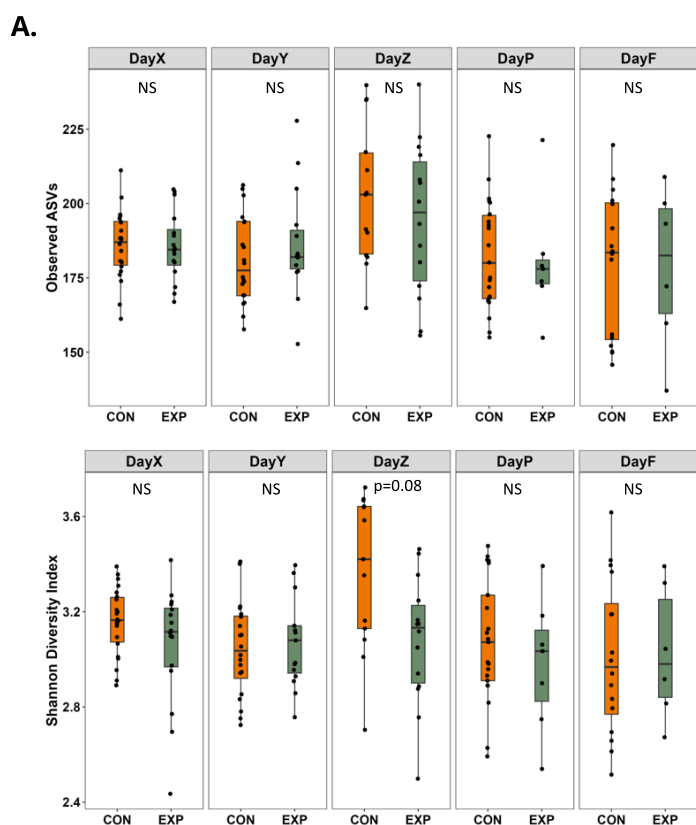
**CON:** Piglets of sows fed a standard feed containing rapeseed meal  
**EXP:** Piglets of sows fed a standard feed in which rapeseed meal replaced by EP119

Figure 5: FS1 sub-study 1-The piglet microbiome.

A. Box plots show the gut microbiome diversity as measured by observed ASVs (top) and Shannon diversity index (bottom). B. Principal coordinates analysis (PCoA) with Bray-Curtis dissimilarity distance shows the gut microbiome community composition. C. Bar plots show the genus-level bacterial relative abundance greater than 1%. A non-significant difference is indicated as "NS" on the plot.



CON: Piglets of sows fed a standard feed containing rapeseed meal  
EXP: Piglets of sows fed a standard feed in which rapeseed meal replaced by EP119



CON: Sows fed a standard feed containing rapeseed meal  
EXP: Sows fed a standard feed in which rapeseed meal replaced by EP119  
DayX: The first day of observation  
DayY: Approximately 14 days before the pregnant gilts were moved to the farrowing section  
DayZ: The day after farrowing of first-parity sows  
DayP: Day pregnant of third parity sows.  
DayF: The day farrowing completed (third-parity sows)

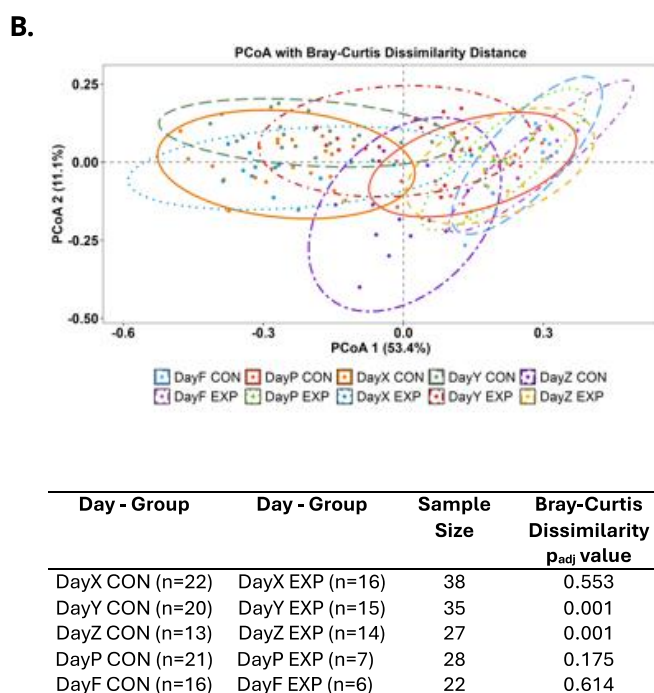


Figure 6: FS1 sub-study 2: Monitoring gut microbiome from gilts to first-parity sows to third-parity sows. A. Box plots show alpha diversity as measured by observed ASVs (top) and Shannon diversity index (bottom). B. Principal coordinates analysis (PCoA) with Bray-Curtis dissimilarity distance shows the gut microbiome community composition (top). The table shows pairwise comparisons performed using pairwise PERMANOVA with Benjamini-Hochberg correction (bottom). Adjusted p<0.05 is considered as significant. A non-significant difference is indicated as "NS" on the plot.

The genus-level bacterial profile showed the following patterns between two groups:

The relative abundance of *Clostridium* was higher in the EXP compared to CON, particularly transition from late gestation gilts to the day of farrowing completed in third-parity sows. The abundance of *Lactobacillus* was lower in the EXP group compared to CON during the same periods. In contrast, when compared to CON, the decrease in *Streptococcus* was only noticeable in the EXP during late gestation gilts [Figure 7].

We examined whether the abundance of these three genera significantly different between CON and EXP groups. At baseline, there were no significant differences between the groups for any of the three bacterial genera. A significant increase in relative abundance of *Clostridium* was observed only in the EXP late-gestation gilts and on the day after farrowing in first-parity sows. At the same period, there was a trend toward a decrease in *Lactobacillus* in the EXP group when compared to CON. Bacterium *Streptococcus* showed a significant decrease only in the EXP late gestation gilts [Figure 8].

Figure 6: Boxplots show the group-wise differences in relative abundance of *Clostridium*, *Lactobacillus*, and *Streptococcus* at each sampling days of FS1 sub-study 2. Adjusted  $p < 0.05$  considered as significant. A non-significant difference is indicated as "NS" on the plot.

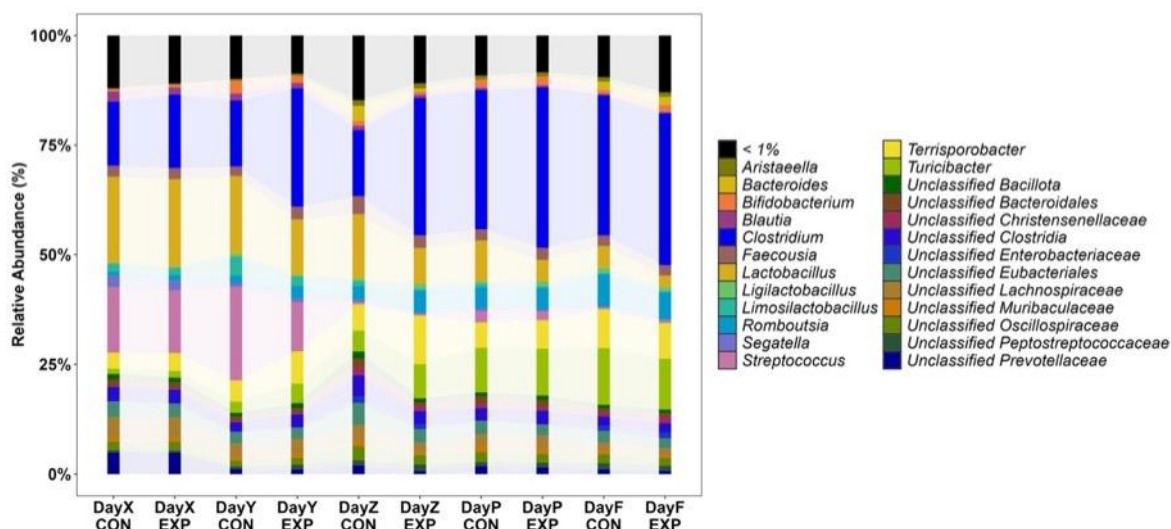
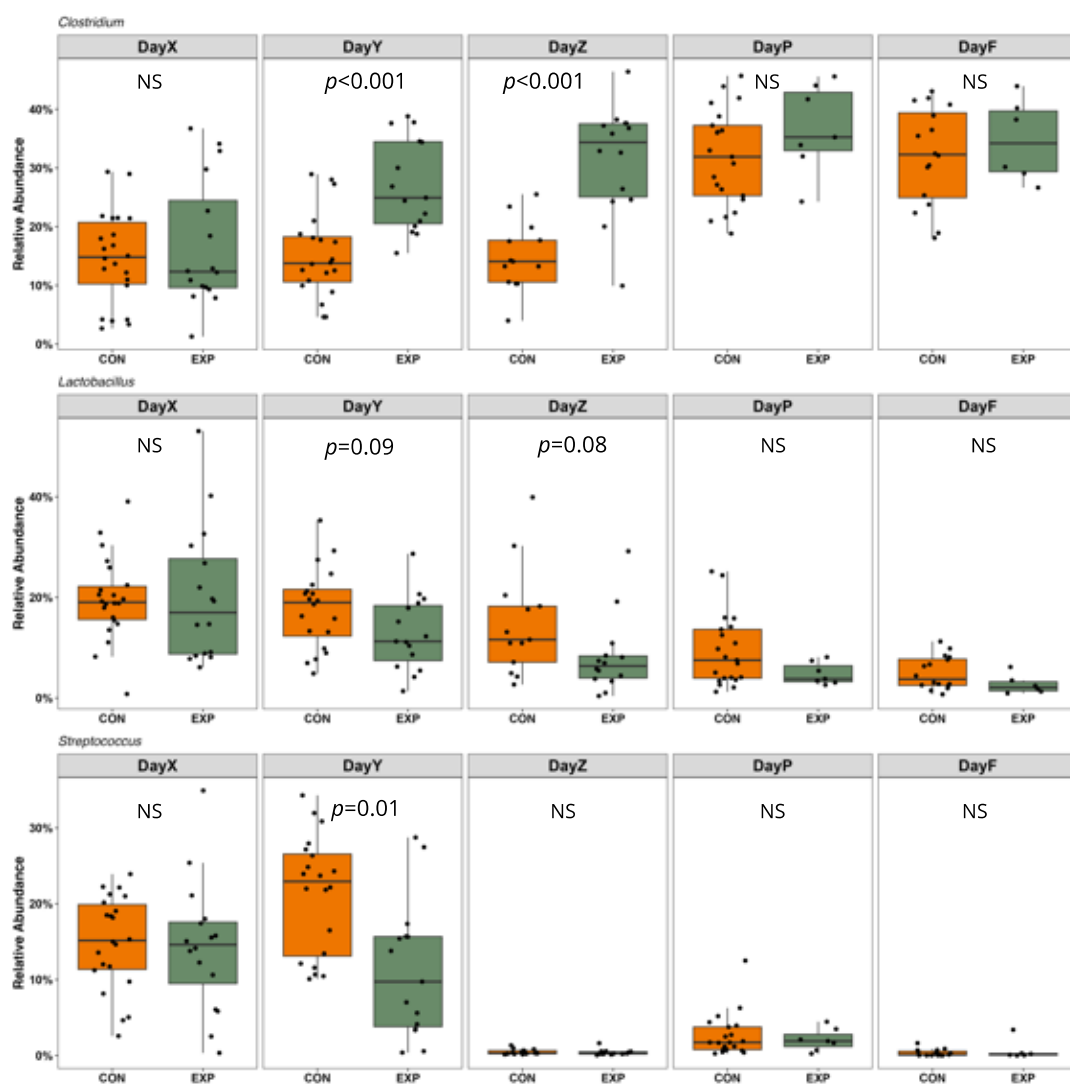


Figure 5: FS1 sub-study 2: Genus-level bacterial relative abundance profile from gilts to first-parity sows to third-parity sows, showing taxa with relative abundance greater than 1%.



CON: Sows fed a standard feed containing rapeseed meal  
EXP: Sows fed a standard feed in which rapeseed meal replaced by EP119  
DayX: The first day of observation  
DayY: Approximately 14 days before the pregnant gilts were moved to the farrowing section  
DayZ: The day after farrowing of first-parity sows  
DayP: Day pregnant of third parity sows.  
DayF: The day farrowing completed (third-parity sows)

Next, we assessed the changes in relative abundance of these three genera within each group [Figure 8; Table 5]:

For *Clostridium*, in the CON group, relative abundance of this genus remained stable in late gestation gilts and first-parity sows but were higher than baseline in pregnant sows and third-parity sows. Third-parity sows had significantly higher *Clostridium* abundance than first-parity sows. In the EXP group, abundances were elevated at all sampling days compared to baseline, with no significant difference between first- and third-parity sows.

The changes in abundance of *Lactobacillus* followed a comparable pattern within both CON and EXP, except for the comparison between late gestation gilts and first-parity sows. In both groups, its abundance was lower than baseline in first-parity sows, pregnant sows and third-parity sows. However, first-parity sows showed significantly higher levels than third-parity sows in the CON.

For *Streptococcus*, we observed that in the CON group, the abundance was higher than baseline only in late gestation gilts. In contrast, its abundance significantly decreased compared to baseline in first-parity sows, pregnant sows, and third-parity sows. The abundance of this bacterium was similar between first-parity and third-parity sows. In the EXP group, no change was observed in its abundance in the late gestation gilts compared to baseline. However, as in the control group, a decrease in its abundance was noted in first-parity sows, pregnant sows, and third-parity sows. Again, its abundance was similar between first-parity and third-parity sows.

### 3.2 Focal Study 2

This study compared the gut microbiome of multi-parity sows on the day pregnant (Day P) and the day farrowing completed (Day F) to evaluate the effect of EP119-supplemented diet.

No significant differences were observed in gut microbiome diversity (bacterial richness and evenness) [Figure 9A], and gut microbiome community composition [Figure 9B] between CON and EXP at either Days P or F ( $p > 0.05$ ).

Bacterial genera with relative abundance above 1% showed no marked variation between EXP and CON [Figure 10].

*Table 3: The FS1 sub-study 2: The significance assessment for the changes in abundance of Clostridium, Lactobacillus, and Streptococcus within CON and EXP. Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction was applied and adjusted  $p < 0.05$  considered as significant.*

| <i>Clostridium</i> | CON                                                                | EXP                                                                |
|--------------------|--------------------------------------------------------------------|--------------------------------------------------------------------|
| Comparison         | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction |
| Day +X vs Day +Y   | $p=1$                                                              | $p=0.007$                                                          |
| Day +X vs Day +Z   | $p=1$                                                              | $p=0.002$                                                          |
| Day +X vs Day P    | $p<0.001$                                                          | $p=0.002$                                                          |
| Day +X vs Day F    | $p<0.001$                                                          | $p=0.008$                                                          |
| Day +Y vs Day +Z   | $p=1$                                                              | $p=0.331$                                                          |
| Day +Y vs Day P    | $p<0.001$                                                          | $p=0.057$                                                          |
| Day +Y vs Day F    | $p<0.001$                                                          | $p=0.078$                                                          |
| Day +Z vs Day P    | $p<0.001$                                                          | $p=0.586$                                                          |
| Day +Z vs Day F    | $p<0.001$                                                          | $p=0.496$                                                          |
| Day P vs Day F     | $p=1$                                                              | $p=0.686$                                                          |

| <i>Lactobacillus</i> | CON                                                                | EXP                                                                |
|----------------------|--------------------------------------------------------------------|--------------------------------------------------------------------|
| Comparison           | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction |
| Day +X vs Day +Y     | $p=0.640$                                                          | $p=0.225$                                                          |
| Day +X vs Day +Z     | $p=0.071$                                                          | $p=0.002$                                                          |
| Day +X vs Day P      | $p<0.001$                                                          | $p<0.001$                                                          |
| Day +X vs Day F      | $p<0.001$                                                          | $p<0.001$                                                          |
| Day +Y vs Day +Z     | $p=0.227$                                                          | $p=0.071$                                                          |
| Day +Y vs Day P      | $p=0.002$                                                          | $p=0.011$                                                          |
| Day +Y vs Day F      | $p<0.001$                                                          | $p=0.003$                                                          |
| Day +Z vs Day P      | $p=0.129$                                                          | $p=0.372$                                                          |
| Day +Z vs Day F      | $p=0.003$                                                          | $p=0.071$                                                          |
| Day P vs Day F       | $p=0.072$                                                          | $p=0.110$                                                          |

| <i>Streptococcus</i> | CON                                                                | EXP                                                                |
|----------------------|--------------------------------------------------------------------|--------------------------------------------------------------------|
| Comparison           | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction | Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction |
| Day +X vs Day +Y     | $p=0.01$                                                           | $p=0.370$                                                          |
| Day +X vs Day +Z     | $p<0.001$                                                          | $p<0.001$                                                          |
| Day +X vs Day +P     | $p<0.001$                                                          | $p<0.001$                                                          |
| Day +X vs Day +F     | $p<0.001$                                                          | $p<0.001$                                                          |
| Day +Y vs Day +Z     | $p<0.001$                                                          | $p<0.001$                                                          |
| Day +Y vs Day +P     | $p<0.001$                                                          | $p=0.011$                                                          |
| Day +Y vs Day +F     | $p<0.001$                                                          | $p<0.001$                                                          |
| Day +Z vs Day +P     | $p<0.001$                                                          | $p=0.003$                                                          |
| Day +Z vs Day +F     | $p=0.104$                                                          | $p=0.308$                                                          |
| Day +P vs Day +F     | $p<0.001$                                                          | $p=0.045$                                                          |

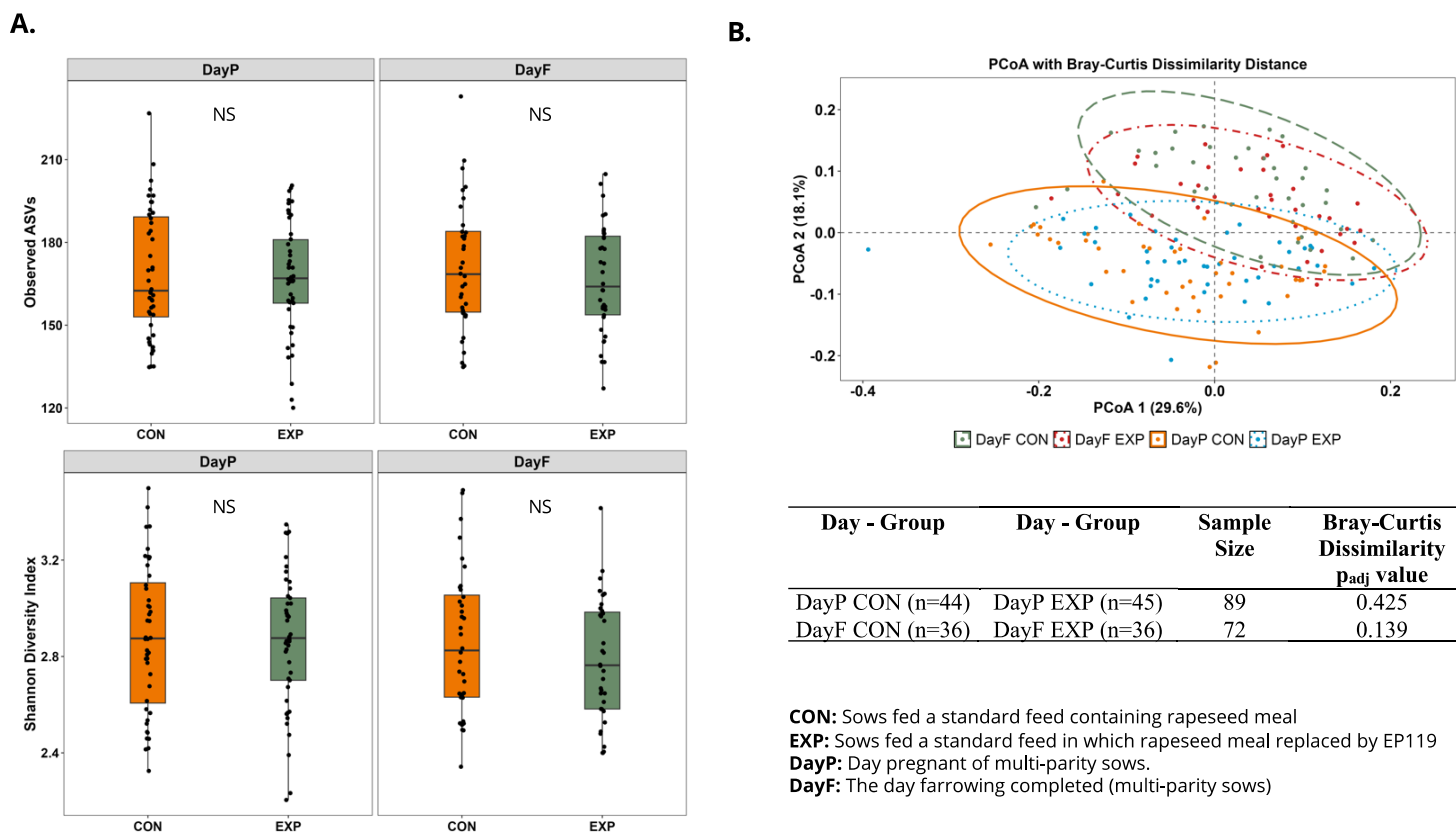


Figure 7: The gut microbiome of multi-parity sows on Days P and F. A. Box plots show alpha diversity as measured by observed ASVs (top) and Shannon diversity index (bottom). B. Principal coordinates analysis (PCoA) with Bray-Curtis dissimilarity distance shows the gut microbiome community composition (top). The table shows pairwise comparisons performed using pairwise PERMANOVA with Benjamini-Hochberg correction (bottom). Adjusted  $p < 0.05$  considered as significant. A non-significant difference is indicated as "NS" on the plot.

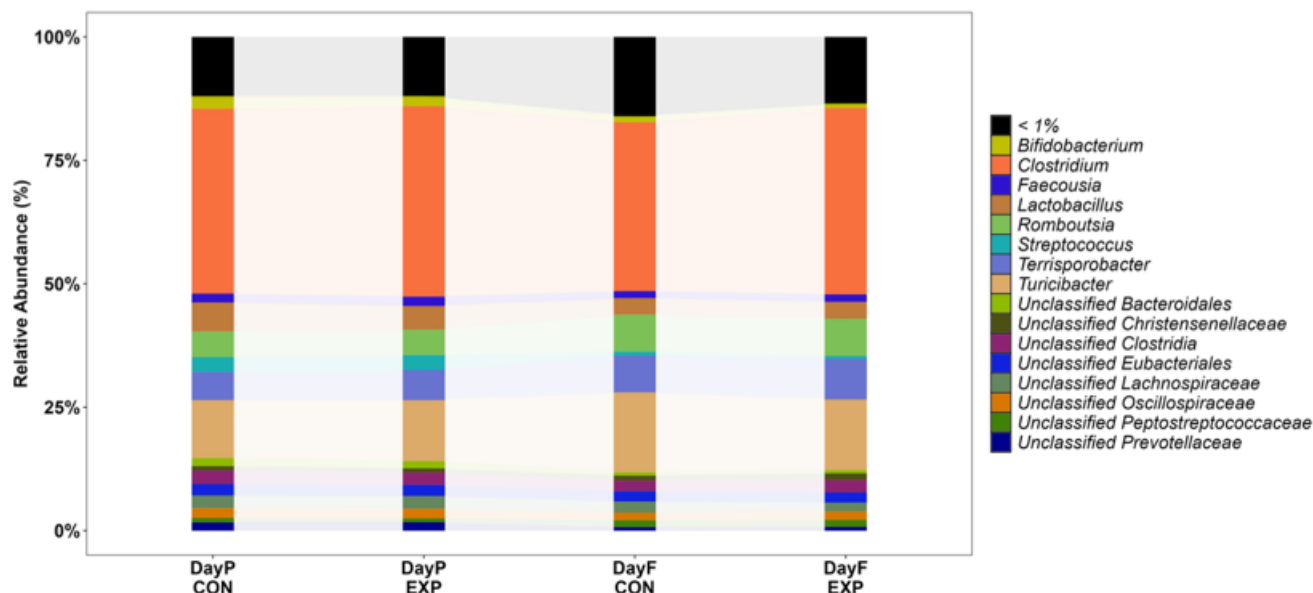


Figure 8: FS2: Genus-level bacterial relative abundance profile of multi-parity sows, showing taxa with relative abundance greater than 1%.

## 4. DISCUSSION

### 4.1 Focal Study 1

What a pig eats during pregnancy can have lasting effects - not only her own performance, but also on the development of her piglets' gut microbiome and overall health [17-19]. In this study, we initially analyzed the effect of an EP119-supplemented diet on maternal and piglet gut microbiome by monitoring the changes from baseline to the day after farrowing (Day  $\div$ X to Day  $\div$ Z) (**Sub-study 1**). In a subset of these sows we extended the study by including samples from the third parity (Day P and Day F). , Thus, we analyzed the differences in gut microbiome from baseline to late gestation gilts to first-parity sows to pregnant sows and to third-parity sows (Day  $\div$ X to Day F) (**Sub-study 2**).

#### 4.1.1 Sub-study 1

Inter-individual differences may strongly influence the gut microbiome composition in longitudinal studies [20]. In this sub-study, at baseline, we observed a significant difference in gut microbiome community composition between CON and EXP (PCoA based on Bray-Curtis dissimilarity). After accounting for the inter-individual variation, the difference between CON and EXP diets became apparent over sampling days. This may indicate the complex interplay between host-related factors and dietary influences in shaping the gut microbiome.

In the EXP, compared to baseline, the abundance of *Clostridium* genus showed a significant upward trend at the late gestation and first-parity, whereas it remained relatively stable in the CON. The rise in abundance of this genus indicates a potential response to EP119 supplementation. Studies show that fermented feed can either increase [21] or decrease [22] *Clostridium* abundance. This genus includes several beneficial bacteria such as *Clostridium butyricum*, which can contribute gut homeostasis by promoting short-chain fatty acid (SCFA) production [23,24], while some of its members are also pathogenic, such as *Clostridium perfringens* [25]. Therefore, interpreting changes at the genus level alone is insufficient to fully understand this shift.

Feeding pigs with lactic acid bacteria fermented feed can increase the abundance of putatively beneficial bacteria, *Lactobacillus* spp., in the intestines [26]. But a 2010 study [27] showed that in young pigs, this feeding approach raised *Lactobacillus* abundance in the ileum, while lowering it in the caecum, indicating that changes can differ by gut region. We observed a significantly lower relative abundance of *Lactobacillus* at baseline (i.e. before EP119 introduction) and late gestation in the EXP compared to CON. Interestingly, when the changes in its abundance were assessed within each group, both groups showed a marked decrease in its abundance the day after farrowing compared to baseline (Day  $\div$ X vs Day  $\div$ Z). This parallel trend may indicate that the observed reduction may not be solely attributable to dietary difference (e.g. difference between gestation feed and lactation feed). Sows'

immune systems also change significantly during the same period, which could also explain this change.

Several studies report that fermented feed can lower *Streptococcus* abundance: Tang et al. (2022) [28] observed a significant decrease in growing pigs fed fermented complete feed. Another study also highlighted that feed fermentation with *Lactobacillus* may reduce *Streptococcus* levels [29]. Additionally, piglets fed a basal diet supplemented with fermented feed revealed a marked drop in *Streptococcus* abundance [30]. In this sub-study, in the late gestation gilts (Day  $\div$ Y), the abundance of *Streptococcus* increased in CON but reduced in EXP. Within each group, a sharp significant decline was observed the day after farrowing compared to baseline and late gestation.

As previously shown, maternal nutrition with a fermented diet may modulate the gut microbiome in a way that confers protection against intestinal inflammation in the piglets [18]. Moreover, feeding sows with fermented rapeseed meal during gestation and lactation can reduce diarrhea and improve health indicators in their piglets [31]. Unlike these studies, no significant change in piglet gut microbiome diversity and composition or the abundance of the diarrhea-associated *Clostridium perfringens* was observed with the EP119-supplemented maternal diet compared to control piglets.

#### 4.1.2 Sub-study 2

When we monitored a subset of FS1 pigs from gilts to third-parity sows, we did not observe inter-individual variation between CON and EXP at baseline. The changes in gut microbiome community composition between groups were observed only in the late gestation gilts and the first-parity sows. Again, during these periods, an increase in *Clostridium* and a trend towards a decrease in *Lactobacillus* were observed in EXP compared to the CON groups. For genus *Streptococcus*, its abundance was lower in EXP only in the late gestation when comparing pregnant gilts on EXP feed compared to CON.

After sampling from first-parity sows (Day  $\div$ Z), there was a short-term usage of benzoic acid, due to a breach of protocol beyond control of AU. Therefore, faeces samples were not collected from the second-parity sows. After this time gap, comparisons of groups at Day P and Day F in the third-parity sows showed no differences in gut microbiome diversity and community composition, as well as in abundance of these genera between CON and EXP. Whether this is due to the short usage of benzoic acid, the smaller sample size in FS1 sub-study 2 or other reasons is not possible to determine.

Overall, the two FS1 sub-studies indicated that EXP lead to significant, but rather modest changes in the gut microbiome for first parity sows compared to CON – but this effect was not seen for third parity sows. Possibly due to the short-term use of benzoic acid between parity 1 and 2.

## 4.2 Focal Study 2

A recent study reports that while fermented feed induced selective shifts in the GM, most genera remained unaffected in multiparous late-gestation sows [32]. Our study shows no significant changes in gut microbiome in multi-parity sows from pregnancy to farrowing completed compared to the control group. One possible explanation could possibly be that the gut microbiome of multi-parity sows might tend to be more stable than first parity sows.

## 5. CONCLUSION

This study highlights that the EP119-supplemented diet selectively influenced maternal gut microbiome in late gestation gilts and first parity sows, with increased abundance of *Clostridium*. Given this genus includes both beneficial and harmful bacteria, species-level analysis may help clarify the relevance of this shift. Despite lower levels of *Lactobacillus* and *Streptococcus* in the late gestation gilts fed an EP119-supplemented diet, within-group declines in first-parity sows compared to baseline in both groups suggest that this may not be solely due to dietary intervention. Maternal feeding with EP119-supplemented diet had no notable impact on the first-parity sows' piglets gut microbiome and the abundance of the pathogen bacterium *Clostridium perfringens*. There was no difference in the gut microbiome of multi-parity pregnant and farrowed sows between the EXP and CON groups.

## 6. FUTURE CONSIDERATIONS

In Focal Study 1 and Focal Study 2, the percentage of EP119 in EXP sow diets was different in the gestation and farrowing sections (6% and 9%, respectively). Our analyses did not consider the differences in EP119 ratio across the EXP diets, as the study design does not allow us to isolate this effect. It cannot be ruled out that this variation could potentially affect the gut microbiome during gestation and farrowing. Therefore, future analysis could account for this to provide further information on the effect of EP119-supplemented diet on gut microbiome.

Studies point to the interplay between the parity and fecal microbiome [33,34], as well as to the influence of feed additives and maternal parity on offspring gut microbiome [35]. The D4.4 mainly focused on gut microbiome diversity and composition across groups at each parity to assess the EP119-supplemented diet effect. Future analysis could consider monitoring the changes in gut microbiome between parities within the EXP group.

In addition, differences in the pig gut microbiome across breeds are well-documented in the literature [36,37]. In focal study 2, our analysis did not consider the breed, again because the study design did not allow us to isolate this effect. In this context, future analysis could also consider this factor in gut microbiome analysis of focal study 2 sows.

This deliverable supports Key Exploitable Results 4 (KER4) and aligns with the specific objectives 6 (SO6) of task 4.3 through showing the effect of EP119 on the sow gut microbiome.

## 7. ACKNOWLEDGMENTS

We would like to thank laboratory technician Emilie Gaal, laboratory technician trainees Danuta J. Kotkiewicz-Szulia and Isidora Cale, and former post-doc Zhuqing Xie from University of Copenhagen for their assistance in the laboratory work, as well as to thank laboratory technician Denitsa Vladimirova Stefanova from University of Copenhagen for her technical assistance in the sequencing process and generation of the ASV table.

We also would like to thank the farmer and farm staffs for this trial and their assistance, particularly with tasks such as faeces sample collection.

## REFERENCES

1. Shuai C, Chen D, Yu B, Luo Y, Zheng P, Huang Z, Yu J, Mao X, Yan H, He J. Effect of fermented rapeseed meal on growth performance, nutrient digestibility, and intestinal health in growing pigs. *Anim Nutr*. 2023 Jul 16;15:420-429.
2. Corino C, Modina SC, Di Giancamillo A, Chiapparini S, Rossi R. Seaweeds in Pig Nutrition. *Animals (Basel)*. 2019 Dec 12;9(12):1126.
3. Wlazio Ł, Nowakowicz-Dębek B, Ossowski M, Łukaszewicz M, Czech A. Effect of Fermented Rapeseed Meal in Diets for Piglets on Blood Biochemical Parameters and the Microbial Composition of the Feed and Faeces. *Animals (Basel)*. 2022 Oct 28;12(21):2972.
4. Satessa GD, Tamez-Hidalgo P, Kjærulff S, Vargas-Bello-Pérez E, Dhakal R, Nielsen MO. Effects of Increasing Doses of Lactobacillus Pre-Fermented Rapeseed Product with or without Inclusion of Macroalgae Product on Weaner Piglet Performance and Intestinal Development. *Animals (Basel)*. 2020 Mar 27;10(4):559.
5. Hui Y, Tamez-Hidalgo P, Cieplak T, Satessa GD, Kot W, Kjærulff S, Nielsen MO, Nielsen DS, Krych L. Supplementation of a lacto-fermented rapeseed-seaweed blend promotes gut microbial- and gut immune-modulation in weaner piglets. *J Anim Sci Biotechnol*. 2021 Jul 20;12(1):85.
6. Choi H, Kim SW. Dietary Intervention of Benzoic Acid for Intestinal Health and Growth of Nursery Pigs. *Animals (Basel)*. 2024 Aug 18;14(16):2394.
7. Andersen-Civil AIS, Myhill LJ, Büdeyri Gökgöz N, Engström MT, Mejer H, Zhu L, Zeller WE, Salminen JP, Krych L, Lauridsen C, Nielsen DS, Thamsborg SM, Williams AR. Dietary proanthocyanidins promote localized antioxidant responses in porcine pulmonary and gastrointestinal tissues during *Ascaris suum*-induced type 2 inflammation. *FASEB J*. 2022 Apr;36(4):e22256.

8. R. Team, RStudio: Integrated Development Environment for R, <https://www.rstudio.com/>.
9. R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
10. McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE. 2013;8(4): e61217.
11. Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, O'Hara R, Solymos P, Stevens M, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista H, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill M, Lahti L, McGlinn D, Ouellette M, Ribeiro Cunha E, Smith T, Stier A, Ter Braak C, Weedon J (2022). vegan: Community Ecology Package. R package version 2.6-4.
12. Martinez Arbizu P (2017). pairwiseAdonis: Pairwise Multilevel Comparison using Adonis. R package version 0.4.1.
13. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Soft. [Internet]. 2015 Oct. 7 [cited 2025 Jun. 21];67(1):1-48. Available from: <https://www.jstatsoft.org/index.php/jss/article/view/v067i01>
14. Hartig F (2022). \_DHARMA: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models\_. R package version 0.4.6.
15. Fox J and Weisberg S (2019). An {R} Companion to Applied Regression, Third Edition. Thousand Oaks CA: Sage.
16. Lenth R (2023). \_emmeans: Estimated Marginal Means, aka Least-Squares Means\_. R package version 1.8.5.
17. Law K, Johnston LJ, Urriola PE, Gomez A. Maternal Programming of Nursery Pig Performance and Gut Microbiome through Live Yeast Supplementation. Animals. 2024; 14(6):910.
18. Wang C, Wei S, Liu B, Wang F, Lu Z, Jin M, Wang Y. Maternal consumption of a fermented diet protects offspring against intestinal inflammation by regulating the gut microbiota. Gut Microbes. 2022 Jan-Dec;14(1):2057779.
19. Tancredi A, Matthijs T, Cox E. *et al.* From mother to piglet: the lasting influence of the maternal microbiome. *anim microbiome* 7, 52 (2025).
20. Wang, X., Tsai, T., Deng, F. *et al.* Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome* 7, 109 (2019).
21. Tang X, Liu X, Zhang K. Effects of Microbial Fermented Feed on Serum Biochemical Profile, Carcass Traits, Meat Amino Acid and Fatty Acid Profile, and Gut Microbiome Composition of Finishing Pigs. Front Vet Sci. 2021 Nov 4;8:744630.
22. Zhang M, Yang Z, Wu G, Xu F, Zhang J, Luo X, Ma Y, Pang H, Duan Y, Chen J, Cai Y, Wang L, Tan Z. Effects of Probiotic-Fermented Feed on the Growth Profile, Immune Functions, and Intestinal Microbiota of Bamei Piglets. Animals (Basel). 2024 Feb 17;14(4):647.
23. Wu J, Wang J, Lin Z, Liu C, Zhang Y, Zhang S, Zhou M, Zhao J, Liu H, Ma X. Clostridium butyricum alleviates weaned stress of piglets by improving intestinal immune function and gut microbiota. Food Chem. 2023 Mar 30;405(Pt B):135014.
24. Fu J, Wang T, Xiao X, Cheng Y, Wang F, Jin M, Wang Y, Zong X. Clostridium Butyricum ZJU-F1 Benefits the Intestinal Barrier Function and Immune Response Associated with Its Modulation of Gut Microbiota in Weaned Piglets. Cells. 2021 Mar 2;10(3):527.
25. Ruiz VL, Bersano JG, Carvalho AF, Catroxo MH, Chiebao DP, Gregori F, Miyashiro S, Nassar AF, Oliveira TM, Ogata RA, Scarcelli EP, Tonietti PO. Case-control study of pathogens involved in piglet diarrhea. BMC Res Notes. 2016 Jan 11;9:22.
26. Bunte S, Grone R, Keller B, Keller C, Galvez E, Strowig T, Kamphues J, Hankel J. Intestinal Microbiota of Fattening Pigs Offered Non-Fermented and Fermented Liquid Feed with and without the Supplementation of Non-Fermented Coarse Cereals. Microorganisms. 2020 Apr 27;8(5):638.
27. Tajima K, Ohmori H, Aminov RI, Kobashi Y, Kawashima T. Fermented liquid feed enhances bacterial diversity in piglet intestine. Anaerobe. 2010 Feb;16(1):6-11.
28. Tang X, Zhang K, Xiong K. Fecal Microbial Changes in Response to Finishing Pigs Directly Fed With Fermented Feed. Front Vet Sci. 2022 Jul 22;9:894909.
29. Zhang D, Ji H, Wang S, Liu Y, Chen M, Liu H. 2023. Lactobacillus-driven feed fermentation regulates microbiota metabolism and reduces odor emission from the feces of pigs. mSystems 8:e00988-23.
30. Yang Y, Yan G, Meng X, Wang X, Zhao Z, Zhou S, Li G, Zhang Q, Wei X. Effects of *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed on growth performance and gut microbiota of nursery pigs. Front Vet Sci. 2022 Dec 12;9:1076906.
31. Grela ER, Czech A, Kiesz M, Wlazło Ł, Nowakowicz-Dębek B. A fermented rapeseed meal additive: Effects on production performance, nutrient digestibility, colostrum immunoglobulin content and microbial flora in sows. Anim Nutr. 2019 Dec;5(4):373-379.
32. Sheng H, Liu C, Li Z, Wang P, Wang L, Jin S, Li X, Yuan L, Chang J, Yin Q, Zhu Q, Lu F. Effects of fermented diet on reproductive performance and fecal microbiota for late-gestation sows. Front Nutr. 2025 May 2;12:1502193.
33. Law, K., Garcia, E.R.M., Berry ASF, Pierdon MK, Misisic AM, Sullivan MC, O'Brien K, Chen Y, Murray SJ, Ramharack LA, Baldassano RN, Parsons TD, Beiting DP. Remodeling of the maternal gut microbiome during pregnancy is shaped by parity. Microbiome. 2021 Jun 27;9(1):146.
34. Zhang, D., Ji, H., Wang, S. *et al.* Parity changed fecal microbiota of sows and its correlation with milk long-chain fatty acid profiles. Appl Microbiol Biotechnol 108, 4 (2024).
35. Law K, Garcia ERM, Hastad C, Murray D, Urriola PE, Gomez A. Interactions between maternal parity and feed additives drive the composition of pig gut microbiomes in the post-weaning period. J Anim Sci Biotechnol. 2024 Mar 3;15(1):33.

36. Bergamaschi M, Tiezzi F, Howard J, Huang YJ, Gray KA, Schillebeeckx C, McNulty NP, Maltecca C. Gut microbiome composition differences among breeds impact feed efficiency in swine. *Microbiome*. 2020;8(1):110.
37. Wang C, Wei S, Chen N, Xiang Y, Wang Y, Jin M. Characteristics of gut microbiota in pigs with different breeds, growth periods and genders. *Microb Biotechnol*. 2022;15(3):793-804.

## DATA SETS

- Raw 16S rRNA gene amplicon sequencing data will be uploaded to NCBI Sequence Read Archive (SRA), and the accession number for public access will be included in the manuscript to be published.