# AVANT – Alternatives to Veterinary ANTimicrobials

# Deliverable 4.3

# Effects of feeding strategies on gut health

**Due Date: Q4 2023 (M48)**
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**Dissemination Level: PU**
**Lead beneficiary: WR**
**Main contact: WR**
**Other contributors: SFR, UCPH**

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| Document Control Information | |
|---|---|
| **Title** | *Effects of feeding strategies on gut health* |
| **Scope / purpose of deliverable** | *Analysis of digesta, faecal and blood plasma samples related to gut health and functionality of Task 4.1, 4.2 and 4.3. Analysis includes the metabolome (SCFA/VFA) in faeces (targeted) and in plasma samples (untargeted); microbiome analysis in intestinal digesta samples.* |
| **Expected outcomes / contribution to impact** | *The results of the analysis will provide more in dept insight into the effects and mode of action of dietary interventions imposed on lactating sows and post-weaning piglets to reduce post-weaning diarrhea and support gut and animal health in piglets, thereby reducing the use of antibiotics in pig husbandry.* |
| **Editor** | *Soumya Kanti Kar & Alfons Jansman* |
| **Dissemination level** (select one, as in DoA) | ☐ **CO** Confidential (please provide Published Summary)<br>☒ **PU** Public |
| **Target audience** | *Researchers working in the domain of pig nutrition and intestinal health*<br>*Pig veterinarians*<br>*Animal feed sector*<br>*Pig farmers* |
| **IPRs underlined** | *No* |
| **Datasets underlined** | *Yes. Metabolomics and metagenomics data.* |

| **Date** | **Version** | ***Change/Comment*** |
|---|---|---|
| 01/07/2024 | 1 | *First edition* |
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# Abbreviations

| | |
|---|---|
| GCMS | Gas Chromatography-Mass Spectrometry |
| LCMS | Liquid Chromatography-Mass Spectrometry |
| SCFA | Short Chain Fatty Acid |
| SFR | Schothorst Feed Research |
| UCPH | University of Copenhagen |
| VFA | Volatile Fatty Acid |
| WR | Wageningen Research |



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# 1 Publishable Summary

As part of WP4, three animal experiments were carried out as described in tasks 4.1, 4.2 and 4.3. Most of the associated results were evaluated and reported in part in deliverable 4.1 and 4.2. Based on these results, there is sufficient information to include the interventions in the options for further testing under field conditions in WP5 “Intervention assessment on clinical efficacy under field conditions”. Major part of the analysis of digesta, faecal and blood plasma samples related to gut health and functionality of tasks 4.1, 4.2 and 4.3, e.g. microbiome analysis in intestinal digesta samples, metabolome in intestinal digesta and plasma samples, SCFA in faeces, has been performed. This resulted in generation of metabolomics and metagenomics data. The data obtained will be stored and shared according to the AVANT data management plan (see T9.5) following the F.A.I.R. principles (findability, accessibility, interoperability and reuse).

Currently, the ~omics data are shared with the beneficiaries and the data are stored in their data infrastructure, with a backup copy being created. The responsible parties and data owners plan to deposit the ~omics data obtained here in suitable open-source repositories, adhering to the common practice of declaring data availability in reputable scientific journals where they intend to publish the results. Appropriate further analysis of these ~omics data will be performed and related to other results obtained in the different studies. The outcomes will be reported and published accordingly as detailed in the
scientific publication plan.

The primary impact indicator is the effectiveness of the interventions, specifically the feeding strategy, which primarily influences animal health and welfare. The objectives are to:

- Reduce pre- and post-weaning diarrhea and related mortality
- Increase body weight gain and feed efficiency in piglets in the post weaning phase by preventing diarrhea and improving gut functionality
- Identify biomarkers in metabolome profiles and in the gut microbiome related to gut health and functionality

Achieving these goals is critical to the AVANT project as it lays the foundation for novel dietary strategies that improve the health and welfare of pigs. This in turn will reduce the reliance on antimicrobials in the pig production chain.



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# 2 Introduction

Work Package 4 (WP4) encompassed three distinct animal experiments detailed in Tasks 4.1, 4.2, and 4.3, with initial outcomes documented in deliverables 4.1 and 4.2. Extensive analytical work was conducted on digesta, fecal, and blood plasma samples collected from these tasks. This involved detailed microbiome analysis of intestinal digesta, metabolome profiling of intestinal digesta and plasma samples, and analysis of short-chain fatty acids (SCFA) in faecal samples. These analyses were pivotal in generating comprehensive metabolomics and metagenomics datasets.

The primary objective of WP4 was to enhance understanding and improve gut health and functionality in relation to dietary interventions in the studied subjects. A key focus was on identifying biomarkers indicative for intestinal health and functionality and to animal health and resilience in more general, crucial for optimizing well-being and overall performance of pigs.

The findings from WP4 not only advance our knowledge of dietary strategies to support animal health but also aim to reduce the reliance on antimicrobials in pig production. This dual approach ensures that our research is scientifically rigorous and translates into practical benefits for welfare and sustainability of the pig husbandry sector.

# 3 Results

In the AVANT project, WR served as the central hub for conducting analytical procedures, including metabolomics, while the UCPH handled the metagenomics. To ensure uniformity in sample collection and storage, particularly for those intended for metabolomics analysis, we developed standard operating procedures (SOPs, see appendix) as part of the AVANT initiative.

On the collected samples both targeted and untargeted approach for metabolite detection was engaged (see **Table 1**). In the targeted approach, eight VFA/SCFA's were quantitatively detected using GCMS. In the untargeted approach, semi-polar compounds were detected using LCMS and polar compounds were detected by GCMS in a relative quantitation manner. For metagenomics, 16S rRNA amplicon sequencing was performed using Illumina Mi-Seq platform.

**Table 1**: Overview of metabolomics performed at samples across different tasks in work package 4.

| WP | Lead | Theme | Targeted metabolomics (SCFA/VFA) | Untargeted metabolomics |
|---|---|---|---|---|
| 4.1 | WR | Feeding strategies in weaned piglets | Done | Done |
| 4.2 | SFR | Feeding strategies in farrowing room | Done | Not requested |
| 4.3 | SFR | E.coli challenge | Done | Done |

Comprehensive procedures, will be detailed in the methods and materials (M&M) section including the necessary statistical analyses and discussions of the results in the associated publications.

Metabolomics and metagenomics data were generated from relevant biological samples obtained from individual pigs in the trials. **Table 2** offers a snapshot of the metagenomics and metabolomics profiles from samples collected in Tasks 4.1, 4.2, and 4.3.



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**Table 1**: Brief summary of metabolomics and metagenomics data derived from samples across different tasks in work package 4.

| Omics catagory | Work Package (WP) | Task (T) | Theme | Lead | Contributor | Catagory of pig | Sample type | Measures | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| Metabolomics | WP 4 | T4.2 | Feeding strategies in farrowing room | SFR | WR | Sow | Faeces | VFA/SCFA | 120 faeces samples; 40 sows divided in 5 treatments i.e. 8 sow/treatment > faecal samples collected in 3 time points |
| Metagenomics | WP 4 | T4.2 | Feeding strategies in farrowing room | SFR | UCPH | Sow | Rectal swabs | 16S rRNA amplicon seq | 120 rectal swabs; 40 sows divided in 5 treatments i.e. 8 sow/treatment > collected in 3 time points |
| Metagenomics | WP 4 | T4.2 | Feeding strategies in farrowing room | SFR | UCPH | Suckling piglets | Rectal swabs | 16S rRNA amplicon seq | 480 rectal swabs in total; 4 swabs/litter x 8 litters; 5 treatments; 3 time points |
| Metabolomics | WP 4 | T4.2 | Feeding strategies in farrowing room | SFR | WR | Suckling piglets | Faeces | VFA/SCFA | Faecal samples from two time points i.e D9 & D27; Results is delivered for D9- 117 faecal samples; D27- 162 (114 + 48) faecal samples |
| Metabolomics | WP 4 | T4.3 | E.coli challenge | SFR | WR | Weaned piglets | Ileal & colon digesta | VFA/SCFA | 70 ileal and colon samples (in total 140 samples*)\| 10 piglets divided in 7 [* 4 samples each from ileum and colon were excluded from analysis, as eaters were placed with non-eaters]\|overall 132 samples |
| Metabolomics | WP 4 | T4.3 | E.coli challenge | SFR | WR | Weaned piglets | Plasma | Untargeted metaboltes | 70 plasma samples \| Metabolite extraction- LCMS: smei-polar compounds; GCMS: polar compounds \| Metabolites detected (approx.): semipolar 1200; polar 150 |
| Metagenomics | WP 4 | T4.3 | E.coli challenge | SFR | UCPH | Weaned piglets | Ileal & colon digesta | 16S rRNA amplicon seq | Total 140 digesta samples \| One time points: D9 post-weaning\| Two locations: Ileum & colon \| Seven treatments with n=10\| |
| Metagenomics | WP 4 | T4.3 | E.coli challenge | SFR | UCPH | Weaned piglets | Rectal swabs | 16S rRNA amplicon seq | Total 70 rectal swabs \| One time points: D9 post-weaning\| Seven treatments with n=10\| |



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Continued Table 2

| Omics catagory | Work Package (WP) | Task (T) | Theme | Lead | Contributor | Catagory of pig | Sample type | Measures | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| Metabolomics | WP 4 | T4.1 | Feeding strategies in weaned piglets | WR | WR | Weaned piglets | Plasma | Untargeted metaboltes | 144 plasma samples \|Metabolite extraction- LCMS: smei-polar compounds; GCMS: polar compounds \| Metabolites detected (approx.): semipolar 1200; |
| Metagenomics | WP 4 | T4.1 | Feeding strategies in weaned piglets | WR | UCPH | Weaned piglets | Ileal & colon digesta | 16S rRNA amplicon seq | Total 288 digesta samples\| Two time points: D13 & D34 \| Two locations: Ileum & colon \| Six treatments with n=24\| Sequencing performed successfully on: 134 samples in ileum and 143 samples in colon; such drop out are usual |

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# 4 Conclusions

The analysis of digesta, fecal, and blood plasma samples related to gut health and functionality in Tasks 4.1, 4.2, and 4.3 included detailed metabolome profiling of intestinal digesta and plasma, SCFA analysis in faeces, and microbiome analysis of intestinal digesta. These comprehensive analyses have generated extensive metabolomics and metagenomics data, which have already been shared with the project beneficiaries to further link them to other results obtained in the studies and on the longer run facilitate further collaboration. Towards this, efforts are underway to prepare manuscripts detailing the findings for submission to peer-reviewed scientific journals, in accordance with the publication plan. These manuscripts will not only document the methodologies and results but also discuss the implications of the findings in the context of feeding strategies and improvement of intestinal health in piglets.

The generated ~omics data is currently further being analysed and integrated with existing results to gain deeper insights into the health and functionality of the gut in pigs. This integrative approach aims to uncover potential biomarkers and elucidate the underlying mechanisms that support gut health and overall welfare of pigs.

Additionally, significant efforts are being made to ensure that the ~omics data adhere to the F.A.I.R. principles (Findable, Accessible, Interoperable, and Reusable). This initiative aims to enhance the usability and accessibility of the data, thereby maximizing its value to the AVANT project and the broader scientific community. By making the data F.A.I.R., we hope to facilitate further research, promote transparency, and enable other researchers to build upon our findings, ultimately advancing the field of animal health and nutrition.



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# 5 Appendix

1. Pdf file: SOP_Metabolomics_WR_SkK



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# Blood plasma collection and preparation for metabolomics analysis from pigs (for untargeted metabolomics)

## Materials:

- Plasma collection tubes, Vacutainer or Vacuette) containing anti-coagulant
  Plasma Anticoagulant Guidelines:
  - Best Results: EDTA (K2, K3, Na; avoid Li).
  - For Metabolomics studies, avoid Citrate.
  - For all studies, never include multiple anticoagulant sample types in the same experiment.
- Eppendorf™ Storage Boxes OR boxes with similar specifications (including height of box as mentioned for eppendrof storage boxes) in relevance to the tube you are considering.
- Eppendorf Safe-Lock Tubes: 2 ml
- Liquid nitrogen for flash-freeze
- Dry ice for transport
- -80ºC freezer for storage

## Plasma Samples:

**1. Volumes of 750 µL plasma in 2 ml Eppendorf tubes, preferably in Duplo's. Sample collection should be conducted in the following manner:**

**Plasma:** Collect whole blood in tubes (e.g. Vacutainer or Vacuette) containing anti-coagulant and follow tube manufacturer's processing instructions.

NB: proceed with step 2 asap, don't leave plasma tubes at room temperature without processing for prolonged period as this can affect the quality of the sample.

**2. Immediately aliquot the collected plasma into eppendrof tubes and flash-freeze (liquid nitrogen). Store samples at -80°C until shipment.** N.B: store the samples in dry-ice after the flash-freeze step, and can be transported from the experimental unit in dry-ice until they can be stored in -80°C.

## Blanc controls:

**Please, provide us 3 eppendrof tubes filled with water instead of blood, as blanc controls (coagulant compound peaks). To prepare this, just replace "whole blood" with either- distilled or MiliQ water and follow the same instructions as provided for plasma sample.**

## Labeling Tips:

Please use cryo labels for labelling each samples so that it can withstand the storage conditions in liquid nitrogen and –80°C. Please consider to place an extra layer of transparent adhesive

tape over the labels in each sample tubes to secure the labels intact in both liquid nitrogen and -80°C. Send electronic version of the sample list (e.g. Excel list) to soumya.kar@wur.nl.

Additionally write an unique number on top of container lid with a black label marker. Underline all numbers with 6 and 9. This unique number should correspond to e.g. the row number of that sample in the electronic sample list (e.g. Excel). If label is lost, we might trace back from this written number. Black marker (dry) usually resist liquid nitrogen.

For labelling please fill-in the following:

Work Package (WP)_Trial number (as described in the implementation section in the AVANT full proposal)_Initial of participating institute/organization
Date of collection (dd-mm-yy)_type of sample (i.e. blood plasma)
Three digit unique ID (kindly avoid multiples of 10's like 110, 120, 130, 140 etc.)_Aliquot number (e.g. use numbers 1 to 5)_Animal ID (e.g. your unique animal ID)

Say, Schothorst Feed Research has collected sample on 1st of Aug 2020 of animal trial number T4.2 from animal with ID S44, S48 and S88, then the labeling would be:

WP4_T4.2_SRF
01-08-20_blood plasma
101_1_S44

WP4_T4.2_SRF
01-08-20_blood plasma
102_1_S48

WP4_T4.2_SRF
01-08-20_blood plasma
103_1_S88

N.B: The principle investigator of the corresponding animal trial will be responsible for maintenance of the metadata.

# Digesta or Faecal sample collection and preparation for metabolomics analysis in pigs (for SCFA analysis)

## Materials:

- Appropriate cryogenic sample tubes that can contain 5 mg of digesta or faecal smaples
- Appropriate storage boxes that can accommodate the cyogenic sample tubes
- Liquid nitrogen for flash-freeze
- Dry-ice
- Weight balance (for small unit measures)

## Digesta or Faecal sample:

**1. 5g (wet weight) of digesta or fecal material is preferable for analysis. Collect the measured amount of sample and store them immediately in sample tubes. Whenever possible, samples must be done in duplos.**

NB: immediately proceed with step 2, don't leave the tubes containing samples at room temperature without processing for prolonged period as this can affect the quality of the sample.

**2. Immediately flash-freeze the eppendrof tubes containing the samples in liquid nitrogen. Store samples at -80°C until shipment.** N.B: store the samples in dry-ice after the flash-freeze step, and can be transported from the experimental unit in dry-ice until they can be stored in -80°C.

## Labeling:

Please use cryo labels for labelling each samples so that it can withstand the storage conditions at –80°C. Please consider to place an extra layer of transparent adhesive tape over the labels in each sample tubes to secure the labels intact. Send electronic version of the sample list (e.g. Excel list) to soumya.kar@wur.nl.

Additionally write an unique number on top of container lid with a black label marker. Underline all numbers with 6 and 9. This unique number should correspond to e.g. the row number of that sample in the electronic sample list (e.g. Excel). If label is lost, we might trace back from this written number. Black marker (dry) usually resist liquid nitrogen.

For labelling please fill-in the following:

Work Package (WP)_Trial number (as described in the implementation section in the AVANT full proposal)_Initial of participating institute/organization
Date of collection (dd-mm-yy)_type of sample (i.e. blood plasma)
Three digit unique ID (kindly avoid multiples of 10's like 110, 120, 130, 140 etc.)_Aliquot number (e.g. use numbers 1 to 5)_Animal ID (e.g. your unique animal ID)

Say, Schothorst Feed Research has collected sample on 1st of Aug 2020 of animal trial number T4.2 from animal with ID S44, S48 and S88, then the labeling would be:

WP4_T4.2_SRF
01-08-20_digesta/faeces
101_1_S44

WP4_T4.2_SRF
01-08-20_ digesta/faeces
102_1_S48

WP4_T4.2_SRF
01-08-20_ digesta/faeces
103_1_S88

N.B: The principle investigator of the corresponding animal trial will be responsible for maintenance of the metadata.