12- Month PhD Report
December 2021

PhD 13- VIMOGUT

Responsible Partner: WBVR

Important Notes

- The full 9M report will be included in the Summary Progress Report due in September of each year.

- The full 12M report will be included in a new WP6 deliverable due in March of 2021/2022.

- A 'Summary' of each PhD project will be compiled from these 12M reports, and included in the Periodic report in February of each year.

    - The summary will be compiled from the content of the following sections :
        - Summary (approx. 500 words)
        - Overview of PhD progress
        - Deliverables and Milestones

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

1. Summary

The VIMOGUT project consists of *in vivo* analysis of the microbiome in the caecum of healthy broilers and the set-up of an *in vitro* model to study the transmission dynamics of antimicrobial resistance in caecal microbial communities. In the first period of 2021, the PhD student has worked on both aspects of the project.

Further data analysis was performed for the in vivo component to complete the first manuscript titled "Succession in the caecal microbiota of developing broilers colonised by Extended-spectrum β-lactamase-producing Escherichia coli", which was submitted in September 2021. The collection of additional broiler caecal samples at farms was postponed due to COVID-19. Preparations were arranged with the hope to perform the sampling at the end of 2021. However, due to the Avian influenza outbreaks in the Netherlands, the sampling at the broiler farms had to be postponed once more until further notice, hopefully, the first quarter of 2022.

The *in vitro* chicken caecum model was first tested in 2020, after which further optimisation was necessary. Challenges with the oxygen sensors were under investigation and solved. The initial tests in the semi-automated in vitro chicken have been run for ~8 days. Analyses of data collected (real-time process monitoring and 16S rRNA gene analysis) have shown reproducibility and reliability of the system between experiments. Inoculation and intervention experiments have been delayed due to a hardware failure in the *in vitro* system secondary to a power failure at WBVR in October 2021. Applikon engineers are currently working on this issue.

2. Overview of the PhD project progress

In WP 1, the data analysis from the first experiment has been finalised. The student has written the results into a manuscript submitted to a peer-reviewed journal.
For WP2, the *in vitro* chicken caecum model was adjusted and optimised to minimise the presence of oxygen in the culture media, underlined by the high presence of anaerobic bacteria in the microbiome. Tests to monitor the presence of oxygen in the system were performed with a different set of sensors. The first experiments used two feed additive phytochemicals as an intervention strategy to test the system. 16S rRNA genes sequencing and microbiome analyses were performed for different test runs.

In preparation for the planned experiments, an approach of fluorescent labelling of plasmids has been selected, which was previously carried out at the University of Copenhagen. A short-term mission proposal was submitted for the student to visit this research group to create her own fluorescently labelled plasmids efficiently. The PhD student was granted the STM and will start her work in May 2022.

3. Progress of the research performed in the PhD project and key scientific results

Before the start of VIMOGUT, a number of broiler caecal samples was collected, on which 16S rRNA gene sequencing was performed. For WP1, additional samples of this collection were sequenced for VIMOGUT to allow proper statistical analysis of the data set. Challenges in the analysis arose due to a difference in 16S rRNA genes amplification between these datasets. It was impractical to analyse both datasets together due to the nature of the research question and the possible bias influencing the results of the microbiome downstream analysis. Precautions have been made to prevent this problem from occurring in any other datasets generated during VIMOGUT.

From the data generated in VIMOGUT, a manuscript has been prepared and submitted and is currently under peer reviewing. In this study we describe the ESBL-*E.coli* prevalence and successional dynamics of the caecal microbiome of developing broilers in a commercial flock during their production life (age 0 to 35). Broilers were discriminated as ESBL-*E.coli* colonised or not by selective culturing. Using 16S rRNA gene sequencing,

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

we compared the richness, evenness and composition of the cecal microbiota of both broiler groups and assessed the combined role of age and ESBL status on the microbiota.

We have observed a significant linear trend in the proportions of ESBL-*E.coli* throughout the broilers' production round; X2 (1, N= 12) = 28.4, p < .001 (Fig 1). Over time, the microbial richness was consistently higher in non colonised broilers, but significant differences between both groups were found exclusively on day three; Wilcoxon rank-sum test, p = .016 (Fig 2). Multivariate analysis showed no explanatory power of ESBL status, while age explained 14% of the compositional variation of the caecal microbiome, F (2, 66) = 6.47, p = .001 (Fig. 3).

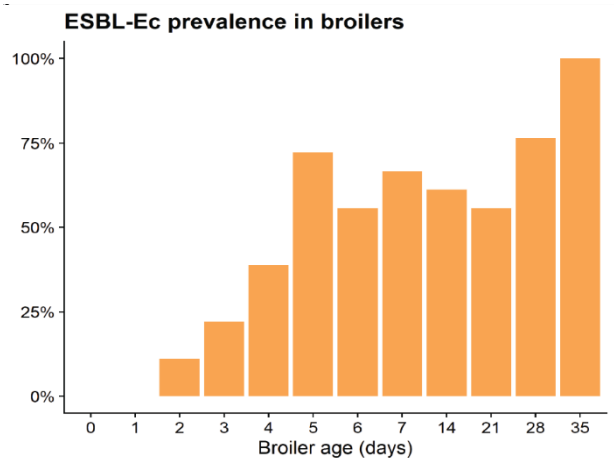

Figure 1. ESBL-*E.coli* prevalence throughout the broiler production round.

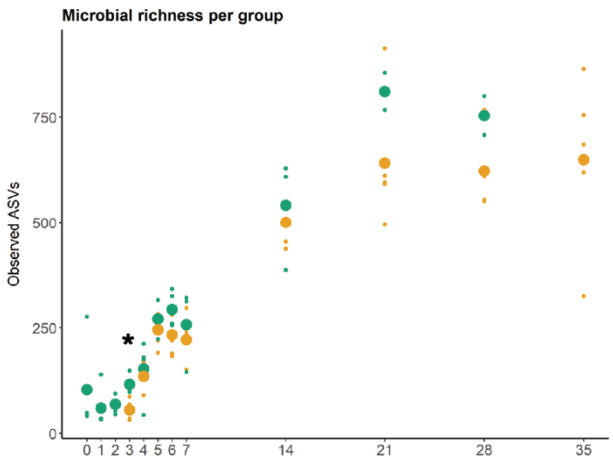

Figure 2. Comparison of microbial richness between ESBL-*E.coli* colonised and non-colonised broilers.

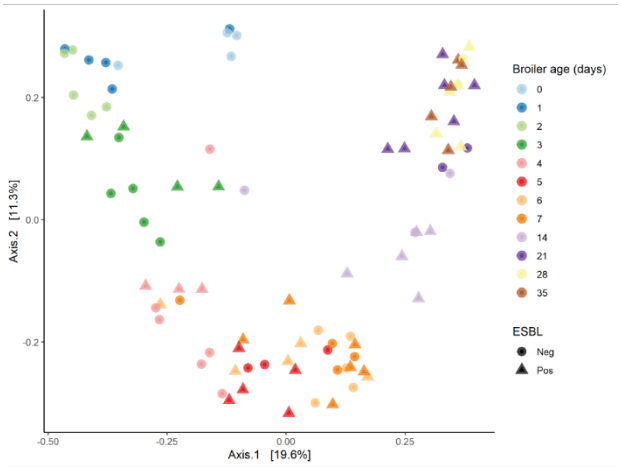

Figure 3. Principal coordinate analyses based on Bray Curtis dissimilarity metrics (BC-PCoA). Changes in the caecal microbial community composition over time (day 0-35).

In conclusion, we have assessed the role of ESBL-producing *E.coli* in the successional dynamics of the cecal microbiome in developing broilers and showed that the presence of ESBL-producing *E.coli* is associated with mild but consistent reductions in alpha diversity and transient microbial compositional differences. We also reported the clonal spread of ESBL-*E.coli* and point to the farm environment as a likely source for ESBLs.

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

For WP2, an in vitro chicken caecum model was set up. The model was challenged by difficulties using the oxygen sensors for which the calibration to a completely anaerobic environment appears to be inaccurate. Replacement with new sensors did not help overcome this problem, and tests with different sensors (e.g. redox) were performed.

Additional optimisation of the system included the addition of a second gas inlet that creates an overlay of nitrogen on top of the culture media in the bioreactor to prevent any oxygen from dissolving into the culture. Two experiments were performed in which phytochemical feed additives were added to the culture at 3 days after the start of the experiment. After DNA isolation, 16S rRNA genes amplicon sequencing was performed, and the data was analysed. In both experiments, the relative abundance of phyla of the in vitro microbiome was relatively stable and consisted primarily of anaerobic bacteria, confirming that the in vitro system can maintain the main broilers caecal microbial communities (Fig. 4). The control bioreactors in which no intervention was added showed similar stabilisation of the microbiome after 3 days, suggesting that any interventions can be started from the time point.

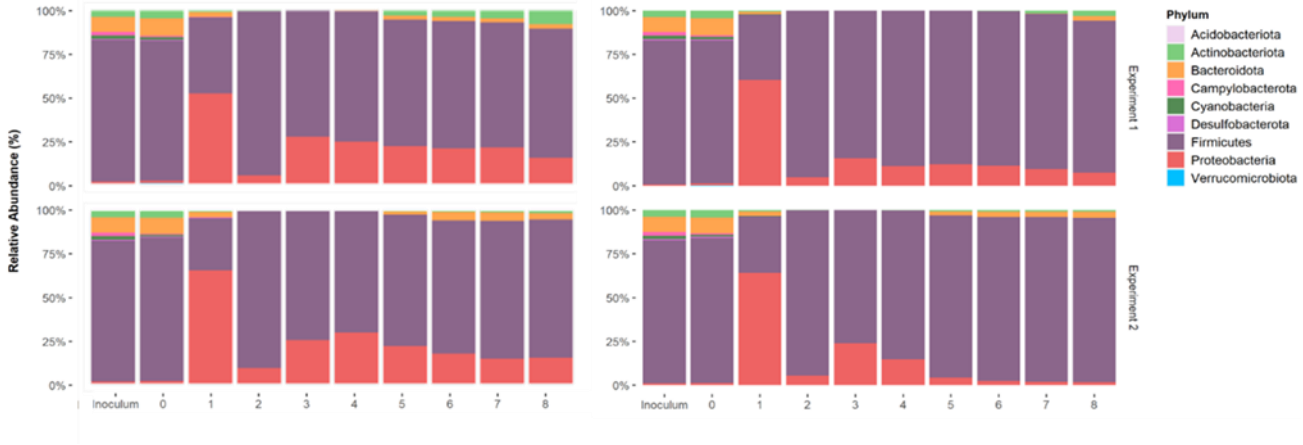

Figure 4. Relative abundance of different phyla measured in the in vitro caecal microbiome. The numbers below the bar indicate the experiment timeline (when the sample was acquired). The two upper plots represent the experiment 1, the lower plots are for experiment 2. The left panels represent the control reactors, while the right panels show the effects of two phytochemicals which are added to the bioreactors from day 3.

The chicken caecal in vitro system is in continuous optimisation. In the previous months, an experiment was performed to evaluate the effect of gassing type on the in vitro caecal microbiome composition. Maintaining adequate anaerobic conditions is essential to simulate the chicken caecal microbiome. In this experiment, both bioreactors were supplied with nitrogen (N2) for eight days to create an anaerobic environment; one bioreactor was supplied with N2 continuously via sparger (into the media) and the other bioreactor via an overlay (on the top of the media).
Preliminary results showed that the type of gassing could influence the relative abundance of the in vitro chicken caecal composition (Fig. 5). However, no significant differences in microbiome composition were found between the two types of gassing. Additional analyses are currently being perfomed.

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

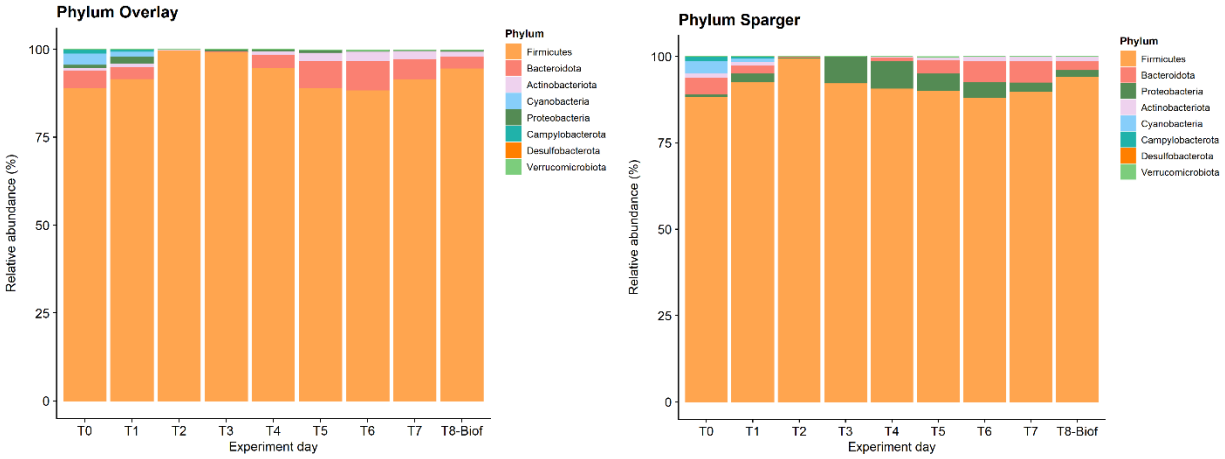

Figure 5. Differences in phyla relative abundance observed between overlay and sparger gassing. N2 delivery via sparger stimulated the grow of specific taxa of the phylum *Proteobacteria* compared to overlay.

4. Progress of the research project: milestones and deliverables

Deliverables

PhD Project Reference	Deliverable number	Deliverable name	Delivery date from Annual Work Plan	Actual Delivery Date	If not achieved: Forecast achievement date	Comments
	D1	Manuscript on preliminary findings for the relationship between chicken gut microbiome maturation and ESBL colonisation.	36		45	Challenges in data analysis delayed the preparation of the manuscript.
	D2	Manuscript on relationship between chicken gut microbiome maturation and ESBL colonisation over several farms.	48		56	Farm visits could not be planned in the first half of 2021 due to COVID-19.

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

Milestones

PhD Project Reference	Milestone number	Milestone name	Delivery date from Annual Work Plan	Achieved (Yes / No)	If not achieved: Forecast achievement date	Comments
	M6	Perform initial test runs on in vitro gut model to determine CFU for reliable ESBL colonisation.	36		48	Delayed due to closure of lab facilities in 2020.
	M7	Write manuscript on initial results relationship between chicken gut microbiome maturation and ESBL gut colonisation.	36		45	See deliverable 1.
	M8	Perform 16S sequencing and analysis of caecal samples from OH-EJP VIMOGUT.	42		52	Samples were not collected yet due to the inability to visit broiler farms during an outbreak of avian influenza in the Netherlands.
	M9	Write manuscript on relationship between chicken gut microbiome maturation and ESBL colonisation over several farms.	48		56	See deliverable 2.

5. Soft skills and Continuing Professional Development training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Peer review discussion meeting	Communication	12/01/21	GSS-WUR
Metagenomics webinar (DADA2)	Data analysis	21/01/21	Loop Genomics
Scicom IG meeting	Communication	16/02/21	WUR
Imposter syndrome	Personal development	24/02/21	
7th OHEJP cogwheel workshop	Science	25/02/21	CACTUS
Global Tricycle Surveillance ESBL *E.coli*	Science	03/03/21	WHO
Applications of -omics technologies in poultry health and productivity : where are we now ?	Science	22/04/21	*IHSIG*

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

Designing and attractive and effective poster	Communication	17/05/21	PE&RC - WSG
Effective and efficient verbal communication in academia and beyond	Communication	26/05/21	PE&RC - WSG
Mindful productivity for PhD Candidates	Personal development	28/05/21	PE&RC - WSG
Multivariate analysis course	Statistical analysis	23 – 25, 28 – 29 June 2021	PE&RC – WSG
RMarkdown	Data analysis	5-6 July 2021	VLAG - WSG

6. Publications and additional outputs

Publications

One manuscript has been submitted for publication. A preprint is available at https://doi.org/10.21203/rs.3.rs-929974/v1.

7. Remarkable outcomes

Currently not applicable.

8. Impact & relevance

The initial in vivo 16s data set analysis has been performed with Dr. Stephanie Jurburg at iDiv Germany. The positive collaboration in the VIMOGUT project has led to the preparation of a new collaborative project which started in 2021.

The *in vitro* gut model experiment to compare the effects of phytochemicals described above has been conducted collaborating with Dr. Adam Roberts at Liverpool School of Tropical Medicine. The PhD student of Dr. Roberts, Mr. William Hutton visited WBVR at the end of 2020 for one month to perform this experiment. The collaboration has boosted the implementation of the *in vitro* model. Furthermore, a consortium led by Dr. Roberts has submitted a pre-proposal and is now preparing a full proposal for the JPI-AMR call in which the *in vitro* gut model at WBVR is an important component.

As part of the work for VIMOGUT, the PhD has engaged with the research group of Prof. Luca Guardabassi at the University of Copenhagen to receive technical assistance in constructing fluorescently labelled plasmids, as the group previously mentioned described it in the literature. As such, she has been granted a short-term mission to visit Prof. Guardabassi's group for nine weeks in 2022. The fluorescently labelled plasmids will be utilised in the *in vitro* model to study the host range of ESBL-encoding plasmids.

9. Follow-up of the recommendations and comments in previous review(s) by the Ethics Advisors

Currently not applicable.

10. Impact of COVID-19 crisis on the project

Tasks or Subtasks	Milestones and Deliverables	Associated budget

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

Name of Task or Subtask	End date according to AWP 2020	Expected end date due to crisis	Associated Milestone or Deliverable	Deadline according to AWP 2020	New proposed deadline	Reason for delay	Budget that will not be spent	Budget that will be spent with delay
			D2	48	56	Farm visits could not be planned in the first half of 2021 due to COVID-19 and not in the second half of 2021 due to an outbreak of Avian Influenza. This sampling is now planned for May-Sept 2022.	-	-
			D3	60	68	Lab facilities were closed for several months in 2020.	-	-
			M6	36	48	Lab facilities were closed for several months in 2020.	-	-
			M8	42	50	Farm visits could not be planned in the first half of 2021 due to COVID-19.	-	-
			M9	48	56	Farm visits could not be planned in the first half of 2021 due to COVID-19 and not in the second half of 2021 due to an outbreak of Avian Influenza. This sampling is now planned for May-Sept 2022.	-	-

Comments: The COVID-19 crisis has affected the feasibility of meeting the deadlines of the milestones. The work on the *in vitro* model has had significant delays in Y3 due to the temporary closure of the laboratory facilities and the postponement of an essential course to commence this part of the work properly, but progress has since been made and most of the work will be delivered by the end of the project.

For the *in vivo* work, sampling at farms has been delayed until the PhD student has been vaccinated against COVID-19. Preparations are made to start this work in Q3/Q4 of 2021.

11. List of critical risks

Description of risk	Yes/No
Loss of PhD supervisor(s)	No
Loss of technical training staff delaying progress of the work	No
Delay in work plan execution	Yes
Conflicts between the collaborative partners that support the PhD	No
Lack of commitment between the collaborative partners that support the PhD	No
Delay in duties, tasks or reporting	No
Poor working relationships within the PhD project team	No
Change in PhD student circumstances requiring temporary leave	No
Other risks (please describe)	No

Additional information: Delay in work plan execution', see 'Impact of COVID-19 crisis on the project'

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

12. Interactions with on-going JRPs/JIPs or with external (EU or national) relevant projects or initiatives such as national action plans (AMR, Zoonoses etc.), OHEJP stakeholders, national and international surveillance programmes.

Currently not applicable.

13. List of dissemination and communication activities

Name of the activity:	*OH-EJP 3-minute thesis competition at OH-EJP ASM 2021*		
Date:	*10-06-21*		
Place:	*Online*		
Specify the Dissemination and Communication activities linked to the One Health EJP project for each of the following categories			
	Yes / No		*Yes / No*
Organisation of a Conference		***Participation to a Conference***	*Yes*
Organisation of a Workshop		***Participation to a Workshop***	
Press release		***Participation to an Event other than a Conference or a Workshop***	
Non-scientific and non-peer-reviewed publication (popularised publication)		***Video/Film***	
Exhibition		***Brokerage Event***	
Flyer		***Pitch Event***	*Yes*
Training		***Trade Fair***	
Social Media		***Participation in activities organized jointly with other H2020 projects***	
Website		***Other***	
Communication Campaign (e.g. Radio, TV)			
Specify the estimated number of persons reached, in the context of this dissemination and communication activity), in each of the following categoriesS			
	Number		*Number*
Scientific Community (Higher Education, Research)	***1000***	***Media***	
Industry		***Investors***	
Civil Society		***Customers***	
General Public		***Other***	

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830

Policy Makers			

Name of the activity:	*Poster presentation at OHEJP ASM 2021*
Date:	*09-06-21 / 11-06-21*
Place:	*Online*

Specify the Dissemination and Communication activities linked to the One Health EJP project for each of the following categories

	Yes / No		*Yes / No*
Organisation of a Conference		***Participation to a Conference***	*Yes*
Organisation of a Workshop		***Participation to a Workshop***	
Press release		***Participation to an Event other than a Conference or a Workshop***	
Non-scientific and non-peer-reviewed publication (popularised publication)		***Video/Film***	
Exhibition		***Brokerage Event***	
Flyer		***Pitch Event***	
Training		***Trade Fair***	
Social Media		***Participation in activities organized jointly with other H2020 projects***	
Website		***Other***	
Communication Campaign (e.g. Radio, TV)			

Specify the estimated number of persons reached, in the context of this dissemination and communication activity), in each of the following categoriesS

	Number		*Number*
Scientific Community (Higher Education, Research)	***1000***	***Media***	
Industry		***Investors***	
Civil Society		***Customers***	
General Public		***Other***	
Policy Makers			

This document is part of the European Joint Programme One Health EJP. This Programme has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830