Relationship between intestinal microbiota composition and growth performance in piglets

Further datamining on studies in piglets carried out within the framework of the Public Private Partnership Feed4Foodure

Dirkjan Schokker, Alfons Jansman, Michiel Kleerebezem and Monique Wolvekamp

Report 1144

WAGENINGEN
UNIVERSITY & RESEARCH

Relationship between intestinal microbiota composition and growth performance in piglets

Further datamining on studies in piglets carried out within the framework of the Public Private Partnership Feed4Foodure

Dirkjan Schokker[1], Alfons Jansman[1], Michiel Kleerebezem[1,2], and Monique Wolvekamp[1]

1 Wageningen Livestock Research

2 Wageningen University, Host-Microbe Interactomics

This research was conducted by Wageningen Livestock Research, commissioned and funded by the Ministry of Agriculture, Nature and Food Quality, within the framework of the Public Private Partnership 'Breed&Feed4Food' (TKI-AF-14215; project number BO-47-001-008) and supported by "Vereniging Diervoederonderzoek Nederland" (VDN).

Wageningen Livestock Research
Wageningen, March 2019

Report 1144

WAGENINGEN
UNIVERSITY & RESEARCH

Schokker, D., A.J.M. Jansman, M. Kleerebezem, M.C.J. Wolvekamp, 2019. *Relationship between intestinal microbiota composition and growth performance in piglets; Further datamining on studies in piglets carried out within the framework of the Public Private Partnership Feed4Foodure.* Wageningen Livestock Research, Report 1144.

This report can be downloaded for free at https://doi.org/10.18174/469020 or at www.wur.nl/livestock-research (under Wageningen Livestock Research publications).

P.O. Box 338, 6700 AH Wageningen, The Netherlands, T +31 (0)317 48 39 53,
E info.livestockresearch@wur.nl, www.wur.nl/livestock-research. Wageningen Livestock Research is part of Wageningen University & Research.

Wageningen Livestock Research Report 1144

Table of contents

Summary

This research was focused on investigating the relationship between the intestinal microbiota composition in individual piglets and their growth performance, indicated by their increase in body weight relative to their body weight on a previous time-point. The age varied between 1 day old piglets to 63 days old piglets (approximately 9 weeks). In this research we used multiple microbiota datasets acquired from four animal experiments. Because in these studies most data were available for the microbiota composition in the jejunum, growth performance data were related to the composition in this intestinal segment.

The study showed a large variation in the jejunal microbiota composition within and between the animal experiments involved. Despite the large diversity in sampling time of the samples collected and the relatively low number of animals of which data were available, the data were employed to investigate whether potential relationships between the microbiota genus composition in jejunum and the relative body weight gain could be detected, using data on individual piglets. In the trials evaluated, significant microbiota signatures could be identified that correlated to the performance parameter(s), here relative body weight gain, however no bacterial group was consistently associated to this performance parameter.

Data mining efforts showed that size of the dataset (number of animals involved) is critical to identify relationships between the microbiota composition and growth performance. In our attempt, we merged two animal experiments of a similar experimental design to increase the number of animals. The strongest explanatory variable for the variation in the jejunal microbiota composition was the experiment rather than e.g. the time-point of sampling (age of the pig), implying that combining these small intestinal microbiota data of different experiments to increase the number of animals for the discovery of microbiota signatures correlated to the weight gain parameters was not effective for pigs.

Future research aiming to detect legitimate microbiota signatures that correlate with zootechnical performance parameters will require experiments in which substantially higher number of pigs are used, which is echoing the conclusions reached by various scientists that search for disease or other phenotype associated microbiota signatures in the human population. Another improvement in future approaches could be to focus on faecal rather than jejunal microbiota, which is more practically feasible, especially when the number of animals has to be drastically increased. However, we have to make sure that we account for dietary composition and the potential effect of ingredients on the fermentable fraction reaching the hindgut. Additionally, determination of the microbiota functional blueprint by shotgun metagenome sequencing rather than its species composition analysis is able to decipher complex interactions that occur in the gut in more detail, again, an approach that was shown to be fruitful in human microbiota research in health and disease.

1 Introduction

1.1 Linking microbiota profile to performance parameters

In pigs a few studies have been published in which microbiota profiles are associated to performance parameters, i.e. feed efficiency (FE) or growth/body weight. A study by Verschuren *et al*. [1] showed the relationship between the faecal microbial composition and FE in individual growing-finishing pigs. A distinct faecal microbiota composition was observed in pigs on different diets (corn/soybean meal or wheat/barley/by-products) and in males vs. females. For diet only two operational taxonomic units (OTUs) ($P = 0.02$) were needed to separate the groups, whereas for gender 18 OTUs ($P = 0.04$) were necessary. In addition, pigs fed the wheat/barley/by-products diet showed differences in FE, which was associated with 17 OTUs in males ($P = 0.02$) and to 7 OTUs in females ($P = 0.01$). This shows that microbiota profile(s) can be associated to performance parameters. In another study by McCormack *et al*. [2] the residual feed intake (RFI; a metric for FE) was linked to the faecal microbiota composition in pigs. Although no differences in microbial diversity were observed between the RFI groups (high/medium/low), some RFI-associated compositional differences were revealed, principally among members of *Firmicutes*, and predominantly in faeces at slaughter. In particular, microbial species associated with a leaner and "healthier" host (e.g. *Christensenellaceae*, *Oscillibacter*, *Cellulosilyticum*) were enriched in low RFI pigs, indicative for high FE pigs. Yang *et al*. [3] showed a tendency of association ($P = 0.07$) between FE and faecal microbiota in pigs. Moreover, two enterotype-like groups were observed and the authors identified 31 OTUs which were mainly annotated to the bacteria related to the metabolism of dietary polysaccharides. In addition to these more efficiency related studies, a study was performed by Han *et al*. [4] investigating the alterations in microbiota composition at different stages in life (i.e. day 10, 21, 63, 93, and 147). From the obtained results, they inferred that age, composition of diet, and weaning experience, are critical components for the development of swine faecal microbiota. An earlier study by this group [5] investigated the link between body weight and the microbiota profiles on a selected group of animals based on their body weight (high or low). They observed a significantly higher diversity in the pigs that were heavier compared to the lighter group. Furthermore, when focusing on the underlying biology of these significant bacterial groups, the pathways "NLR signalling pathway" and "xenobiotics biodegradation and metabolism" were observed and could be linked to growth performance, i.e. immune signalling.

Besides linking the faecal microbiome to performance parameters, studies have also been performed in which the small and large intestinal microbiome composition were associated to feed conversion ratio (FCR) and FE (by RFI). Quan *et al*. [6] investigated the gut (ileum, caecum, and colon) microbiome of pigs with contrasting FCRs and identified 11 up to 55 OTUs with significantly different relative abundances. These results suggested that the OTUs in the cecum and colon of the high FCR pigs might have a greater ability to utilize dietary polysaccharides and dietary protein compared to low FCR pigs, and the SCFAs and indolic compounds produced by microbial fermentation might improve porcine feed efficiency and promote intestinal health. Another study by Vigors *et al*. [7] investigated pre-selected bacteria to phenotype pigs differing in FE (by RFI), in a basal state and they have extracted ileal and colonic explants and challenged those with lipopolysaccharide (LPS). The low RFI pigs had increased *lactobacillus* spp. in the caecum compared to high RFI pigs ($P < 0.05$). Interestingly, there was an interaction between RFI and LPS for multiple cytokines in colon, with the low RFI group having consistently lower gene expression in the colon following the LPS challenge, compared to the high RFI group.

In literature, to our knowledge not many publications have been focusing on small intestinal microbiota linked to body weight characteristics. However these studies mentioned here show it is possible to link the gut microbiome, small or large intestinal or faecal, to important indicators of performance, i.e. body weight, FCR, or FE (RFI).

1.2 Knowledge gap

Most of the published studies mentioned in the previous paragraph focus on the extremes of the phenotype of interest, FCR and/or FE (by RFI), and delivered inconsistent results with respect to (beneficial) microbiota. More specifically, different bacterial genera were identified to be associated with a specific phenotypic contrast in different studies. In addition, most of these studies involve single animal experiments or at best a few batches ran on the same farm, implying that the observed results may be study specific and thereby possibly explaining the different results.

In the present study, we investigated four different pig studies from the VDI programme in which both microbiota composition was determined by 16S rRNA-based composition profiling (V3/V4 region), and individual performance parameters were recorded (i.e. absolute and relative daily body weight gain). After acquiring the microbiota profiles at the jejunal level for each of the four studies, we combined these data to investigate whether overlap exists in the observed bacterial genera related to the RBWG.

It should be noted, that the acquired datasets from the VDI program were not intended to provide information on the relationship between intestinal microbiota composition and the growth performance, but were initially designed to learn more about the relationship between selected dietary interventions and responses of the intestinal microbiota, the immune system and development of immune competence.

2 Material and Methods

2.1 Data acquisition

Data were acquired from four studies in the 'Voeding, Darmgezondheid en Immuniteit' (VDI) programme which was carried out between 2012-2016 as part of the Feed4Foodure Public Private Partnership. In Table 1 the four studies are depicted from which data were available on the growth performance and intestinal microbiota composition of individual animals. In these datasets, individual microbiota profiles of jejunum were available from pigs in all experimental groups, as well as a set of performance parameters, i.e. relative body weight gain in a certain period (RBWG). RBWG was calculated by dividing the BW at sacrifice with the BW of a previous time-point. For example, BW at day 7 divided by the birth weight or BW at 14 days after weaning divided by the BW at weaning.

Table 1 *Overview of studies with pigs from the VDI programme from which data were used for the present study on the relationship between growth performance and intestinal microbiota composition.*

Short Title	Title	Total number of animals[1]	Intervention	Time-points	Microbiota composition
VDI-2[2]	Maternally administered amoxicillin and the effect on the offspring	41	Amoxicillin (sow)	1, 7, W[3], W+4 days, W+28 days	jej
VDI-5.1 & VDI-5.2	Zinc oxide as model intervention in weaned pigs	29 & 46	High dosage of Zinc-oxide	W+14, W+23, W+35	jej and ile
VDI-12	Maternal/neonatal interventions (*MCFA, BG, GOS*) and the effects on the offspring/piglets	52	Medium-chain fatty acids, beta-glucans, or Galactooligosaccharides	1 and W+3	jej, ile, or col

[1] The number of animals with individual microbiota data

[2] VDI refers to the 'Voeding, darmgezondheid, en immuniteit' (VDI) programme, and the number to the specific sub-project.

Detail information, like specific diets, can be found in the respective reports, listed in references as A (VDI-2), B (VDI-5.1), C (VDI-5.2), and D (VDI-12).

[3] W refers to weaning and was approximately 28 days of age.

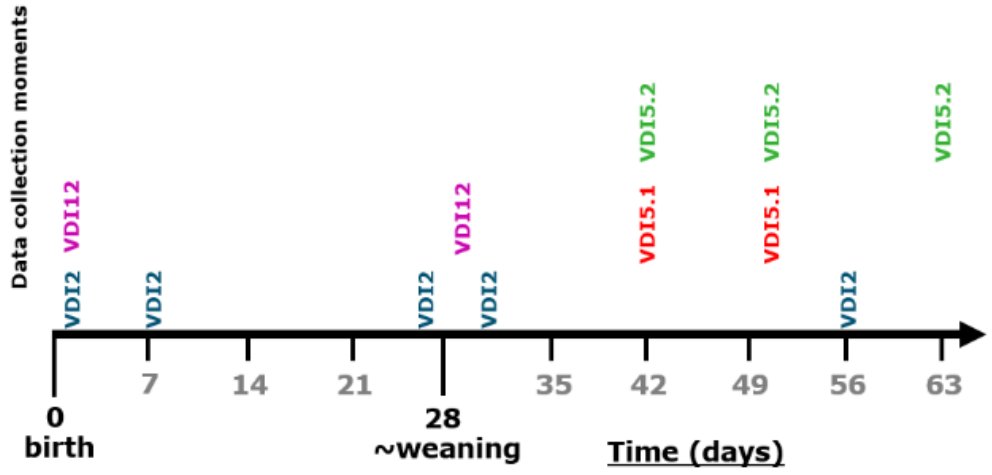

Figure 1 *Schematic overview of the time points (days after birth) at which piglets were sampled in each sub-project. On the horizontal arrow the time from birth (day 0) up till day 63 of age is depicted. The sub-projects included were VDI2 (blue), VDI5.1 (red), VDI5.2 (green), and VDI12 (magenta).*

2.2 Data analysis

Cleaning and structuring data
For each study a metadata file was created based upon the unique piglet identifier and subsequently the average daily gain (ADG) and relative body weight gain (RBWG) for the relevant periods were calculated. Thereafter, the related microbiota composition data from jejunum at genus level were linked to each piglet by its identifier.

Data analyses
CANOCO (http://www.canoco5.com, windows release 5.10) software was used to analyse the data. CANOCO is a popular program for multivariate statistical analysis using ordination methods in the field of ecology.

Heatmaps were generated using R (version 3.5.0). Heatmaps are graphical representation of data where the individual values contained in a matrix are represented as colours. A heatmap was generated to visualize the RBWG values of animals and the accompanying values of multiple bacterial genera of interest. The scatterplots representing the bacterial genera of interest that contributed the most to the RBWG were also generated within the R environment.

From previous analyses on the different microbiota datasets, it was already observed that age of the animal generated high variation. Therefore, current results are presented per study: VDI-2 (4.1), VDI5.1 (4.2), VDI5.2 (4.3), VDI12 (4.4). Within each study, the data of the individual piglet at the time-point of sacrifice was used for further analyses, i.e. performance parameter (RBWG) and microbiota profiles in jejunum digesta (genus level).

For each study, the descriptive statistics were calculated to investigate the variation (i.e. range) in the parameters of interest. From this it was concluded that there is variation in the RBWG within the different studies as well as within the different time-points within a study. This was also performed for the microbiota data by employing a Bray-Curtis dissimilarity; $BCij = 1 - 2Cij \,/\, Si + Sj$, where Cij is the sum of the lesser values for only those species in common between both sites. Si and Sj are the total number of specimens counted at both sites, this will generate a number between 0 and 1. If 0, the two sites share all the same species; if 1, they do not share any species. For example a Bray-Curtis dissimilarity of 0.21, can be referred to as a Bray-Curtis dissimilarity percent of 21%.

Thereafter four consecutive steps have been taken to analyse the statistical data per time-point in a given study:

1. A redundancy analysis (RDA) was performed with RBWG as constrained variable. RDA is a method to extract and summarise the variation in a set of response variables that can be explained by a set of explanatory variables. More accurately, RDA is a direct gradient analysis technique which summarises linear relationships between components of response variables that are "redundant" with (i.e. "explained" by) a set of explanatory variables. The total variance of the data set, partitioned into constrained and unconstrained variances, is a standard result. This result shows how much variation in your response variables was redundant with the variation in the explanatory variables. If the constrained variance is much higher than the unconstrained variance, the analysis suggests that much of the variation in the response data may be accounted for by the explanatory variables. If, however, there is a large proportion of unconstrained variation (i.e. variation in the response matrix that is non-redundant with the variation in the explanatory matrix), then the results should be interpreted with caution as only a small amount of the variation in your response matrix is displayed.
2. The bacterial genera were plotted as vectors in the RDA and the bacterial genera with the highest (absolute) values on the X-coordinate, top 10% and above absolute 0.5, were selected for further analysis.
3. A heatmap was generated to visualize which bacterial genera were most likely to have a significant association with RBWG. In addition, the root-mean-squared-error (RMSE) and mean absolute error (MAE) were calculated for the bacterial genera, to identify the bacterial most associated to the RBWG.

4. Based on the lowest values of the RMSE (MAE), a scatterplot of RBWG (x-axis) and average relative contribution of the bacterial genera (y-axis) was generated, and subsequently the linear trend line, R^2, and P-value, were calculated between the bacterial genera of interest and the RBWG. This analysis gave more insight into the association of the individual bacterial genera and the RBWG.

3 Results

3.1 VDI-2

3.1.1 Descriptive statistics for VDI-2

For specific periods within a study, the descriptive statistics were calculated for average daily gain (ADG), relative body weight (RBWG), as well as for the Bray–Curtis dissimilarity of the associated jejunal microbiota data (Table 2). These values were calculated because they give insight in the variation within a study. The variation of these given parameters are used to identify associations between the RBWG and the (jejunal) microbiota profiles. In other words, are certain microbiota profiles associated with high or low RBWG.

Table 2 *Descriptive statistics of the VDI-2 study.*

	Period*	N	Mean	Min	Max	sd
ADG[1]	0-7	13	*169*	*0*	*245*	*62*
ADG	0-W	9	*248*	*146*	*341*	*75*
ADG	W-W4	10	*110*	*-50*	*225*	*92*
ADG	W-W28	9	*350*	*245*	*463*	*69*
RBWG[2]	0-7	13	188	146	219	20
RBWG	0-W	9	626	372	990	221
RBWG	W-W4	10	105	98	112	5
RBWG	W-W28	9	186	156	223	18
Bray[3]	7	13	0.29	0.11	0.59	0.12
Bray	W	9	0.42	0.12	0.84	0.22
Bray	W4	10	0.43	0.07	0.99	0.26
Bray	W28	9	0.52	0.05	0.95	0.26

* Shows the period (in days) used for calculation of the parameter, where day 0 is birth and W is weaning (d 28) (W4 means 4 days post-weaning).

[1] Average daily gain, in grams per day.

[2] Relative body weight gain as percentage.

[3] Short for Bray–Curtis dissimilarity, units are an index.

3.1.2 Period, birth till day 7

Step 1

A redundancy analysis (RDA) was performed with RBWG as constrained variable. RDA is a method to extract and summarise the variation in a set of response variables that can be explained by a set of explanatory variables. More accurately, RDA is a direct gradient analysis technique which summarises linear relationships between components of response variables that are "redundant" with (i.e. "explained" by) a set of explanatory variables.

The total variance of the data set, partitioned into constrained and unconstrained variances, is a standard result. This result shows how much variation in your response variables was redundant with the variation in the explanatory variables. If the constrained variance is much higher than the unconstrained variance, the analysis suggests that much of the variation in the response data may be accounted for by the explanatory variables. If, however, there is a large proportion of unconstrained variation (i.e. variation in the response matrix that is non-redundant with the variation in the explanatory matrix), then the results should be interpreted with caution as only a small amount of the variation in your response matrix is displayed.

The RDA output we generated is a so called tri-plot (Figure 2), where samples (open circles), bacterial genera (blue arrows), and the constrained variable (red arrow) are plotted together in one space. The explanatory variable explains 13.9% of the variance (P = 0.06). In humans, it has been established that the gut microbiome is heritable, as well as a heritable variance component for 210 operational taxonomic units (OTUs) in faecal samples, which explained on average 22.7% of the observed total variance [8]. Another study in humans linked the gut microbiome to cytokine production and this resulted in the overall percentage of cytokine variation explained by species composition of the gut microbiome ranged from 0.4% to 9.7% [9]. Another study showed approximately 10% of the total variance on the microbiota composition was ascribed to diet [10]. In conclusion, the percentage of explained variance in the present study is in line with values in similar studies in humans. The variance component in the present study could even be overestimated due to the low number of animal samples in the studies involved in the data analysis in the present study.

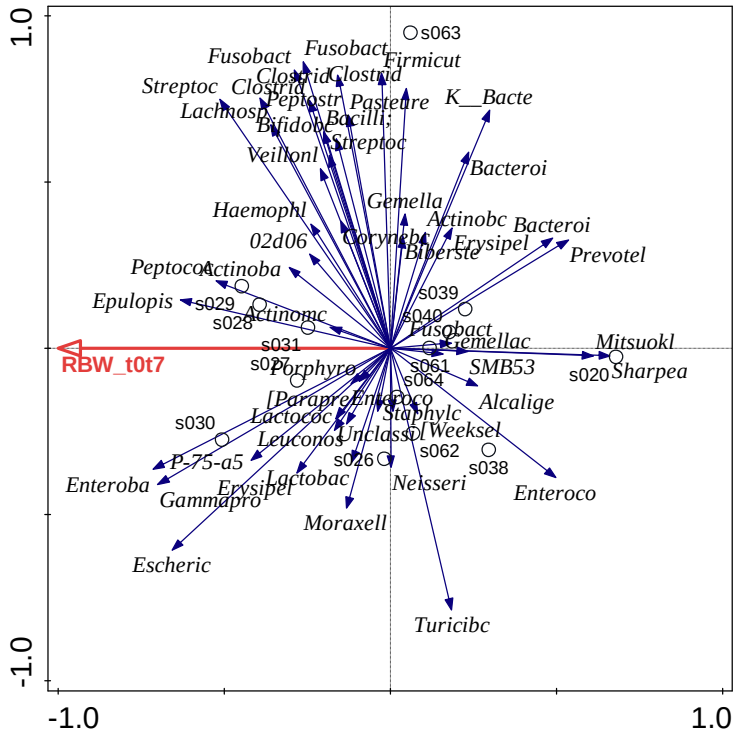

Figure 2 *RDA with RBWG 0-7 as constrained variable (explanatory variables account for 13.9%, P = 0.06). Circles represent piglet samples, the angles among the blue arrows denote the degree of correlation of a given bacterial genera with the RBWG 0-7 (red arrow). In addition, positively correlated variables are shown as arrows pointing in the same direction as the RBWG 0-7 (red arrow), negatively correlated variables pointing in opposite directions.*

Step 2

Based on the RDA for each bacterial genera the X-coordinate was calculated, i.e. higher X-coordinate values denote higher correlation to the RBWG 0-7. In Table 3, the top 10% of highest X-coordinates values above 0.4 or below -0.4 are shown. These bacterial genera were used as input for further analysis.

Table 3 *Selection of bacterial genera associated with RBWG 0-7 based upon the X-coordinate.*

Bacterial genera	X-coordinate
Enterobacteriaceae; Other	-0.71
Gammaproteobacteria; Other; Other	-0.70
Escherichia	-0.66
Epulopiscium	-0.63
Peptococcus	-0.53
Streptococcaceae; Other	-0.51
Bacteroidia; Other; Other	0.49
Enterococcus	0.50
Prevotella	0.54
Mitsuokella	0.61
Sharpea	0.66

* Other means the classifier algorithm used could not come to a consensus above a specific threshold for a certain taxonomic level.

Step 3

To visualize the associations between the RBWG and the bacterial genera a heatmap was generated (Figure 3). In this figure a dendrogram (tree diagram) was calculated on the columns (by hierarchical clustering), to cluster (group) the different variables with similar patterns. Here, the variables RBWG 0-7 and *Streptococcea*.Other are highly similar and placed adjacent to each other in the tree.

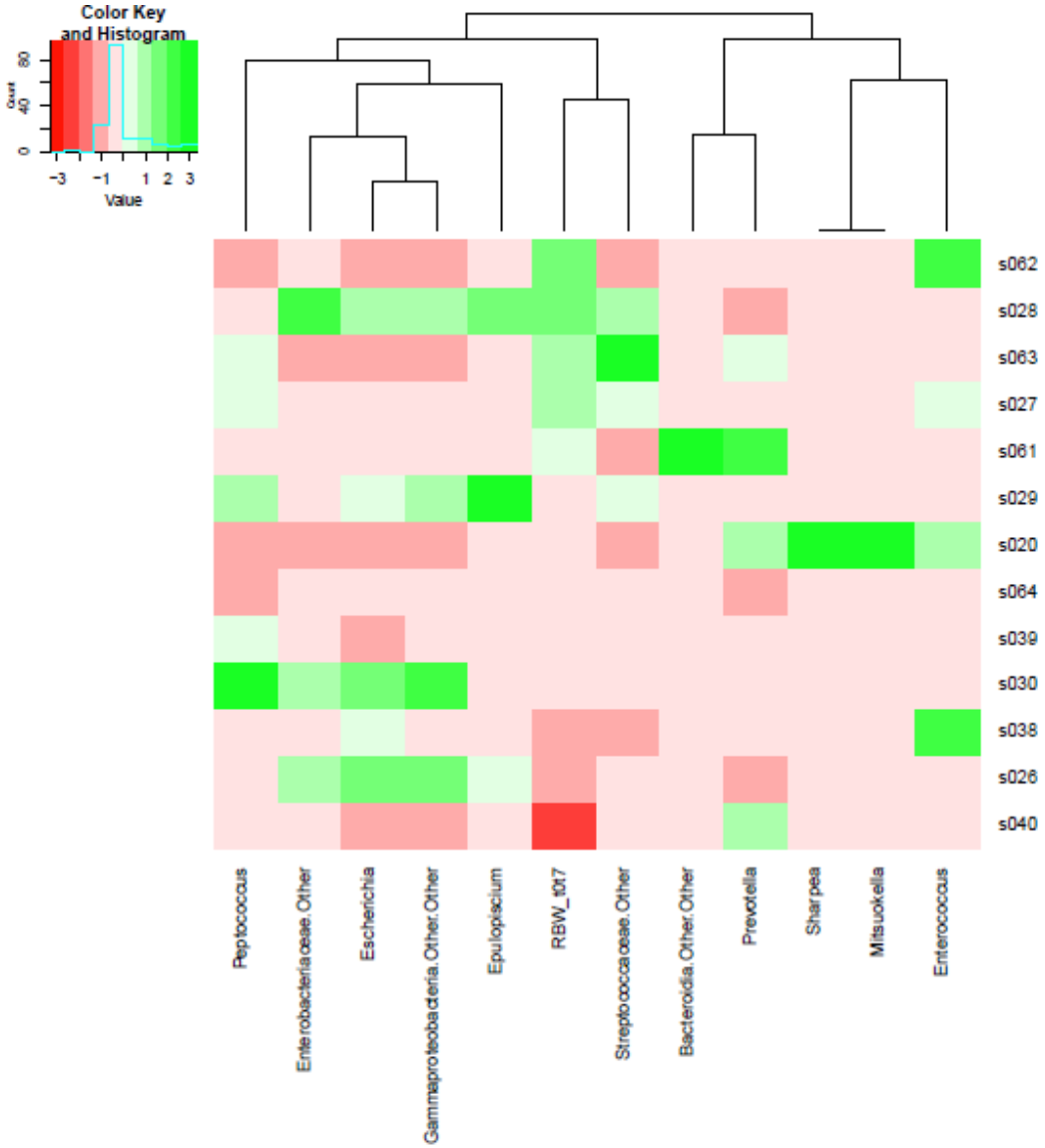

Figure 3 *Heatmap with RBWG 0-7, where columns represent the selected bacterial genera and RBWG 0-7. Rows depict the values for an individual pig sample, i.e. average relative contribution of the bacterial genera or RBWG. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

For all selected bacterial genera, the root-mean-squared-error (RMSE) and mean absolute error (MAE) were calculated, to identify the bacterial genera most associated to the RBWG 0-7. In other words, the significance of effect(s) visualized in the heatmap was calculated.

Based on the lowest RMSE (MAE) values, a scatterplot of RBWG (x-axis) and average relative contribution of the bacterial genera (y-axis) was generated. Subsequently the linear trend line, R^2, and P-value were calculated only for the bacterial genera of interest and the RBWG. The plots are only shown when the P-value was below 0.05. This analysis gives more insight into the association of the individual bacterial genera and the RBWG.

Table 4 *Measuring the difference between RBWG 0-7 and bacterial genera.*

Bacterial genera	RMSE[1]	MAE[2]
Enterobacteriaceae; Other*	1.21	1.02
Gammaproteobacteria; Other; Other	1.28	1.05
Escherichia	1.44	1.12
Epulopiscium	1.27	0.93
Peptococcus	1.38	1.09
Sharpea	1.43	1.03
Bacteroidia; Other; Other	1.38	1.01
Enterococcus	1.24	0.95
Prevotella	1.37	1.04
Mitsuokella	1.42	1.02
Streptococcaceae; Other	1.02	0.70

[1]Root-mean-squared error, [2]Mean absolute error

*Other means the classifier algorithm used could not come to a consensus above a specific threshold for a certain taxonomic level, in the above table it was twice for both the family and genus level.

Hereafter, all the results for other studies and age periods will be presented similar to the tables and figures in this section (3.1.2 Period, birth till day 7).

3.1.3 Period: Birth till weaning

The period of interest is birth (day 0) till weaning (approximately day 28).

Step 1

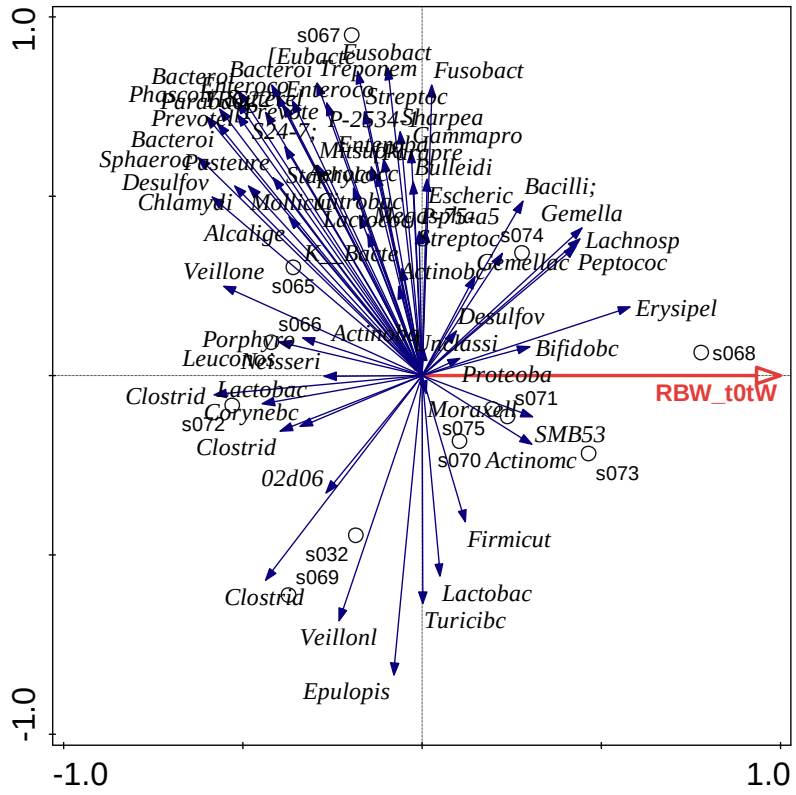

Figure 4 *RDA with RBWG 0-W (birth to weaning) as constrained variable (explanatory variables account for 12.0%, P = 0.21).*

Step 2

Table 5 *Selection bacterial genera associated with RBWG 0-W based upon the X-coordinate.*

Bacterial genera	X-coordinate
Lactobacillaceae	-0.45
Mollicutes; f__	-0.48
Bacteroidia; Other; Other	-0.51
Prevotella	-0.52
YRC22	-0.52
Chlamydia	-0.52
Veillonellaceae; Other	-0.55
Phascolarctobacterium	-0.57
Bacteroidia; f__;	-0.57
Clostridium	-0.58
Desulfovibrio	-0.59
Parabacteroides	-0.60
Sphaerochaeta	-0.63
Erysipelotrichaceae; Other	0.58
Gemella	0.45
Lachnospiraceae; Other	0.44

Step 3

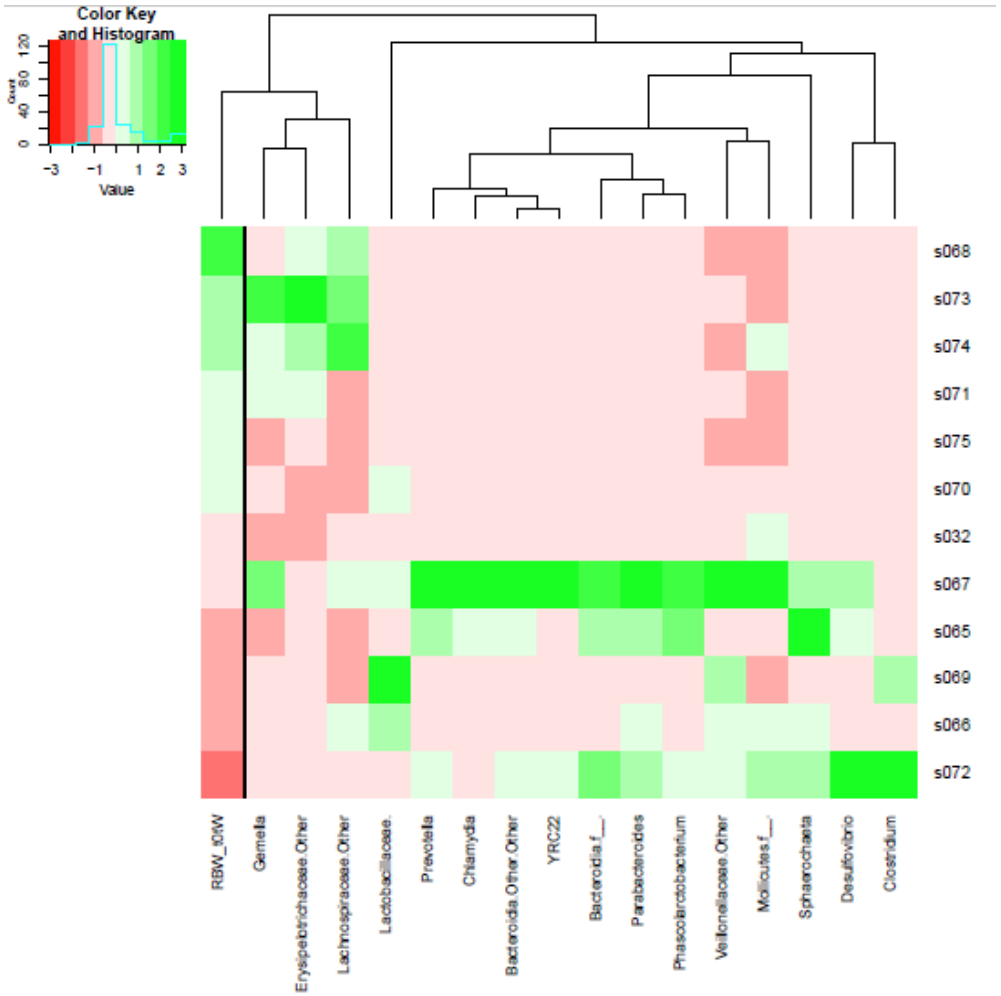

Figure 5 *Heatmap with RBWG 0-W (birth to weaning). On the x-axis are the selected bacterial genera and the y-axis depicts the individual pig samples. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

Table 6 *Measuring the difference between RBWG 0-W and bacterial genera.*

Bacterial genera	RMSE1	MAE2
Lactobacillaceae;	1.62	1.30
Mollicutes;f__;	1.60	1.31
Bacteroidia;Other;Other	1.52	1.22
Prevotella	1.56	1.28
YRC22	1.54	1.25
Chlamydia	1.51	1.23
Veillonellaceae;Other	1.61	1.39
Phascolarctobacterium	1.63	1.38
Bacteroidia;f__;	1.64	1.38
Clostridium	1.67	1.30
Desulfovibrio	1.69	1.36
Parabacteroides	1.65	1.42
Sphaerochaeta	1.68	1.42
Erysipelotrichaceae;Other	0.90	0.72
Gemella	1.04	0.85
Lachnospiraceae;Other	0.95	0.82

[1]Root-mean-squared error, [2]Mean absolute error

3.1.4 Period: Weaning till 4 days post-weaning

The period of interest is weaning (approximately day 28) till 4 days post-weaning.

Step 1

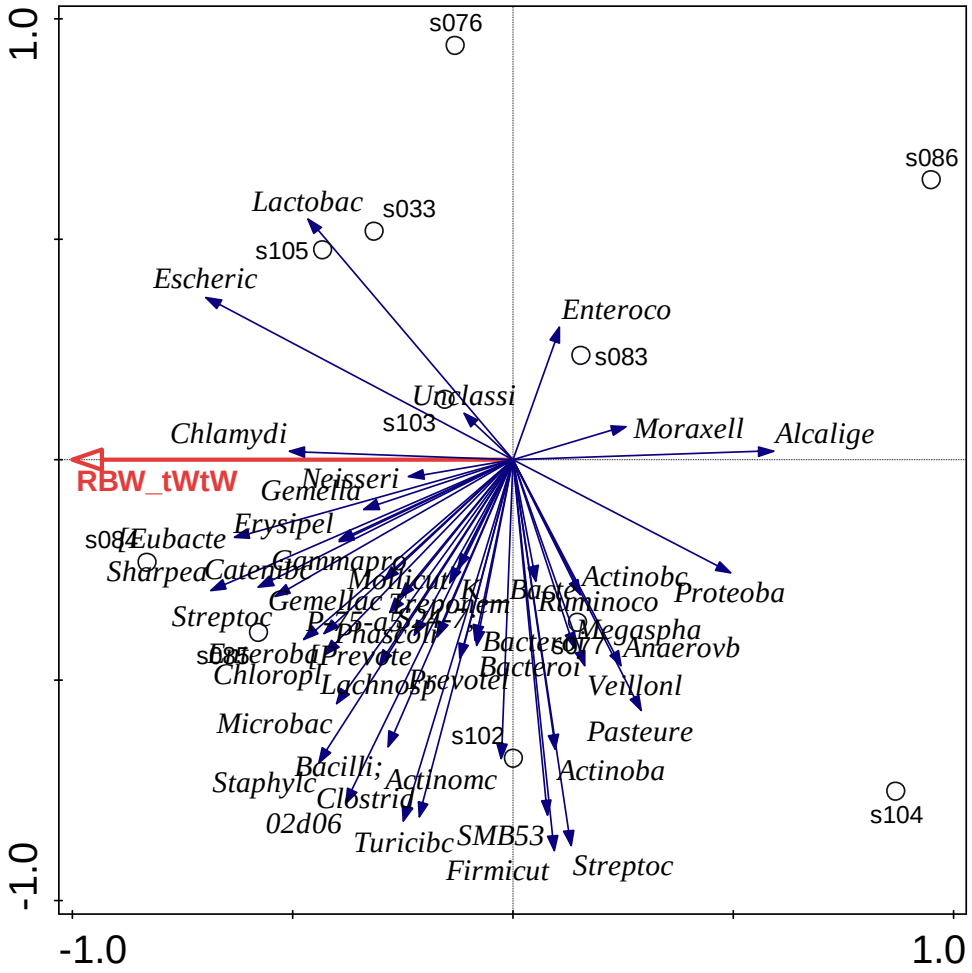

Figure 6 *RDA with RBWG W-W4 as constrained variable (explanatory variables account for 12.1%, P=0.37).*

Step 2

Table 7 *Selection bacterial genera associated with RBWG W-W4 based upon the X-coordinate.*

Bacterial genera	X-coordinate
Escherichia	-0.70
Sharpea	-0.69
[Eubacterium]	-0.63
Catenibacterium	-0.58
Streptococcaceae;Other	-0.54
Chlamydia	-0.51
Proteobacteria;Other;Other;Other	0.50
Alcaligenaceae;Other	0.59

Step 3

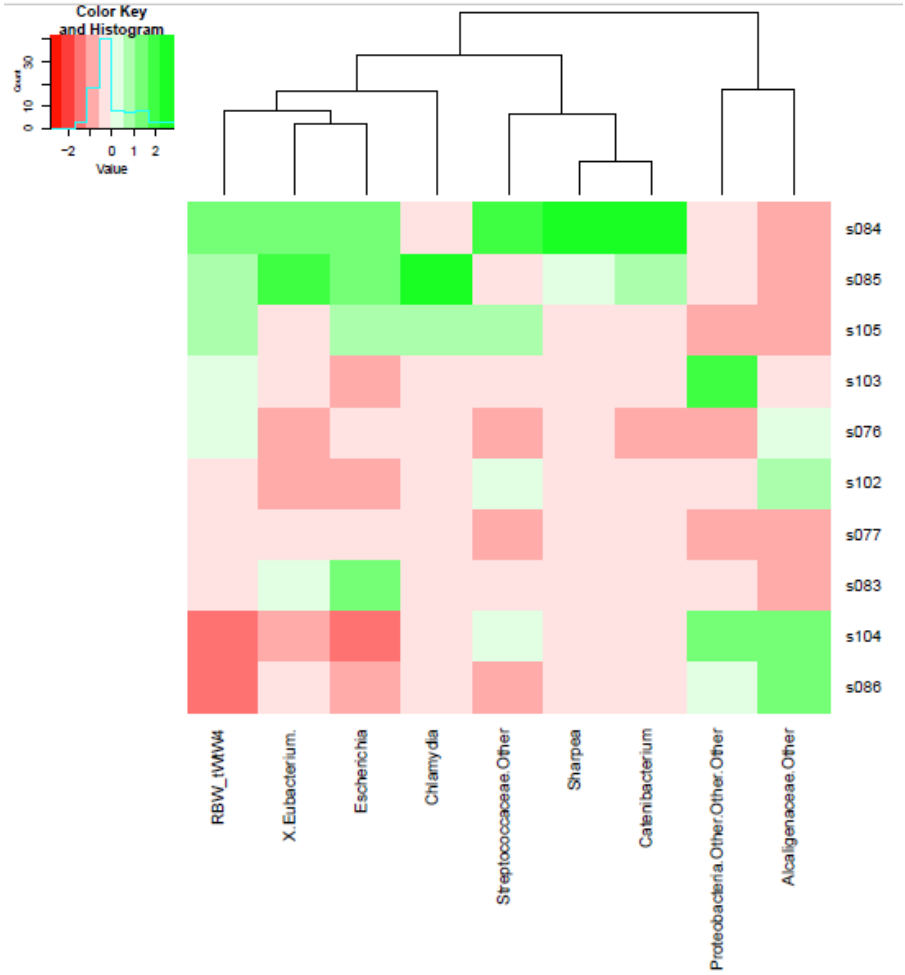

Figure 7 *Heatmap with RBWG 0-W. On the x-axis are the selected bacterial genera and the y-axis depicts the individual pig samples. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

Table 8 *Measuring the difference between RBWG W-W4 and bacterial genera.*

Bacterial genera	RMSE[1]	MAE[2]
Escherichia	0.73	0.56
Sharpea	0.88	0.75
X.Eubacterium.	0.81	0.70
Catenibacterium	0.82	0.67
Streptococcaceae.Other	0.89	0.77
Chlamydia	1.01	0.81
Proteobacteria.Other.Other.Other	1.61	1.36
Alcaligenaceae.Other	1.76	1.46

[1]Root-mean-squared error, [2]Mean absolute error

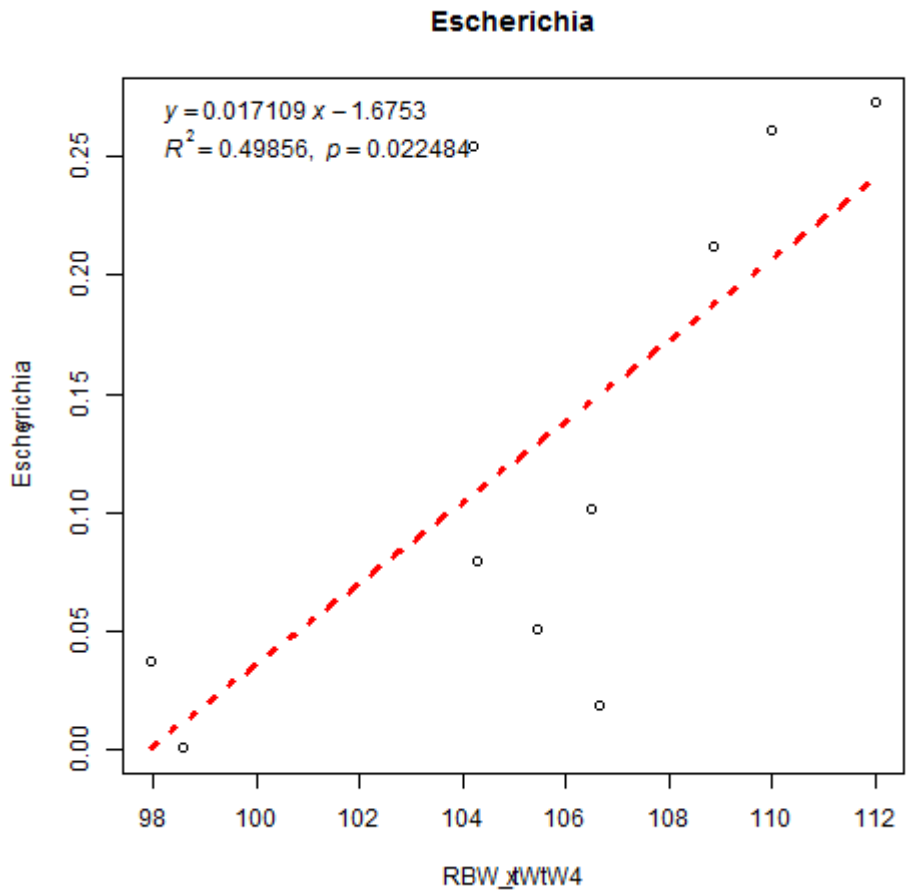

Figure 8 *Scatterplot of RBWG W-W4 and Escherichia. A linear trend-line (red dashed line) was calculated, with an R^2 of 50% and a P-value of 0.02.*

3.1.5 Period: 4 days post-weaning till 28 days post-weaning

The period of interest is 4 days post-weaning till 28 days post-weaning.

Step 1

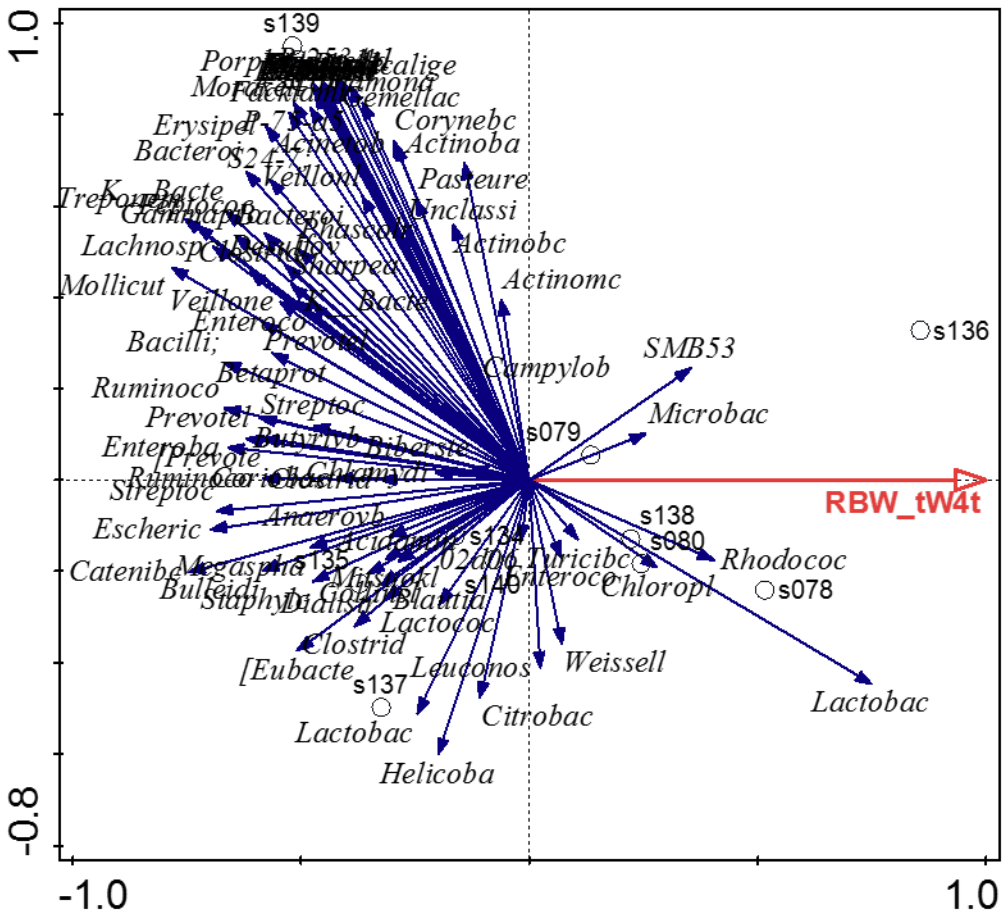

Figure 9 *RDA with RBWG W4-W28 as constrained variable (explanatory variables account for 22.8%, P=0.10).*

Step 2

Table 9 *Selection bacterial genera associated with RBWG W4-W28 based upon the X-coordinate.*

Bacterial genera	X-coordinate
Mollicutes;	-0.78
Treponema	-0.76
Catenibacterium	-0.75
Peptococcus	-0.73
Escherichia	-0.70
Lachnospiraceae;Other	-0.69
Streptococcus	-0.69
Ruminococcaceae;Other	-0.67
Bacilli;Other;Other	-0.67
k__Bacteria;Other;Other;Other;Other	-0.67
Enterobacteriaceae;Other	-0.66
Bacteroidia;	-0.65
Bacteroidia;Other;Other	-0.62
Butyrivibrio	-0.62
Clostridia;Other;Other	-0.61
Lactobacillus	0.75

Step 3

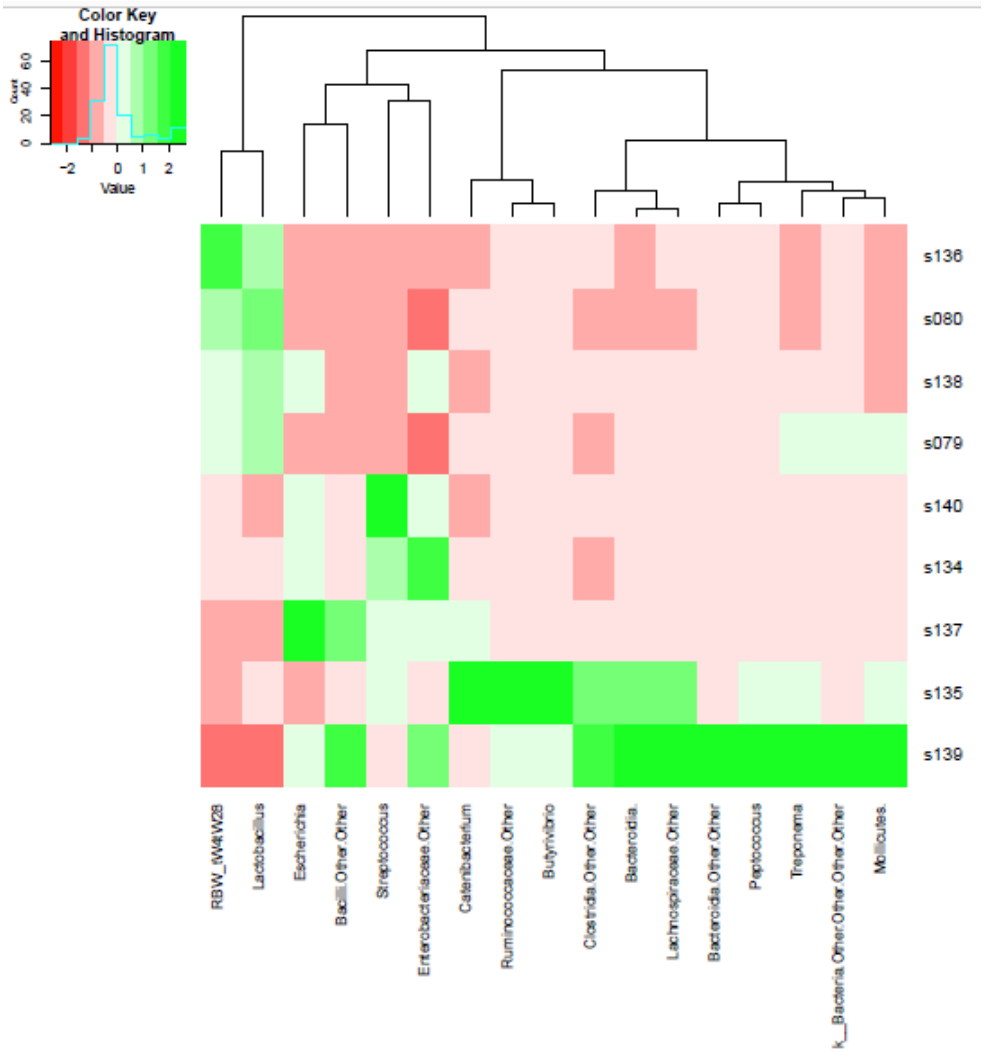

Figure 10 *Heatmap with RBWG W4-W28. On the x-axis are the selected bacterial genera and the y-axis depicts the individual pig samples. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

Table 10 *Measuring the difference between RBWG W4-W28 and bacterial genera.*

Bacterial genera	RMSE[1]	MAE[2]
Mollicutes.	1.70	1.22
Treponema	1.71	1.24
Catenibacterium	1.66	1.30
Peptococcus	1.66	1.19
Escherichia	1.62	1.28
Lachnospiraceae.Other	1.70	1.29
Streptococcus	1.60	1.45
Ruminococcaceae.Other	1.63	1.23
Bacilli.Other.Other	1.72	1.36
k__Bacteria.Other.Other.Other.Other	1.64	1.12
Enterobacteriaceae.Other	1.70	1.42
Bacteroidia.	1.69	1.28
Bacteroidia.Other.Other	1.63	1.13
Butyrivibrio	1.60	1.17
Clostridia.Other.Other	1.70	1.31
Lactobacillus	0.58	0.45

[1]Root-mean-squared error, [2]Mean absolute error

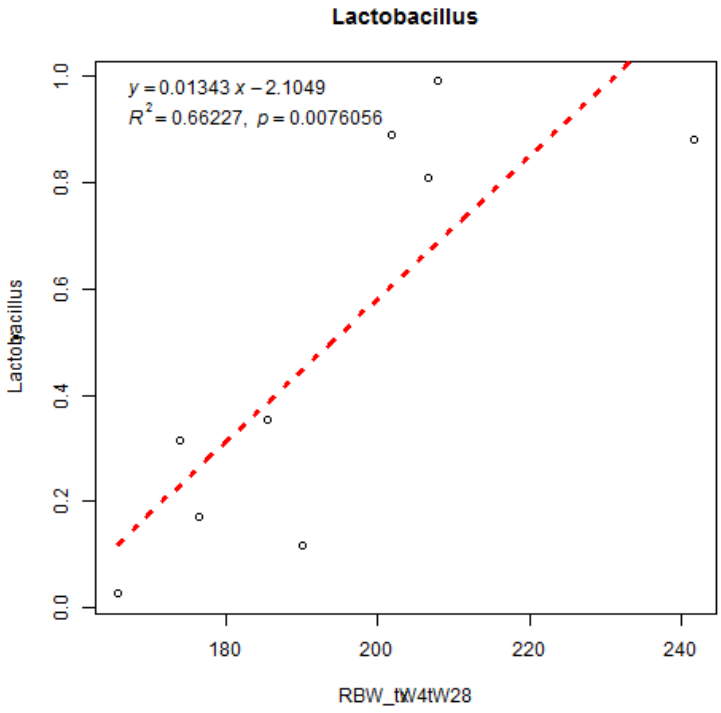

Figure 11 *Scatterplot of RBWG W4-W28 and Lactobacillus. A linear trend-line (red dashed line) was calculated, with an R^2 of 66% and a P-value of 0.007.*

3.2 VDI-5.1

For specific periods within the study, the descriptive statistics were calculated for average daily gain (ADG), relative body weight (RBWG), as well as for the Bray–Curtis dissimilarity of the associated jejunal microbiota data (Table 11).

Table 11 *Descriptive statistics of the VDI-5.1 study.*

	Period*	N	Mean	Min	Max	sd
ADG[1]	W-W14	5	213	123	279	66
ADG	W14-W23	12	472	249	633	109
ADG	W23-W35	12	476	333	600	84
RBWG[2]	W-W14	5	139	123	152	12
RBWG	W14-W23	12	137	122	150	8
RBWG	W23-W35	12	142	132	156	6
Bray[3]	W-W14	5	x	x	x	x
Bray	W14-W23	12	0.29	0.01	0.75	0.19
Bray	W23-W35	12	0.34	0.04	0.71	0.19

* Shows the period (in days) used for calculation of the parameter, where day 0 is birth and W is weaning (W4 means 4 days post-weaning)

[1] Average daily gain, in grams per day

[2] Relative body weight gain as percentage

[3] Short for Bray–Curtis dissimilarity, units are an index

3.2.1 Period: Weaning till 14 days post-weaning

The period of interest is weaning till 14 days post-weaning.

Step 1

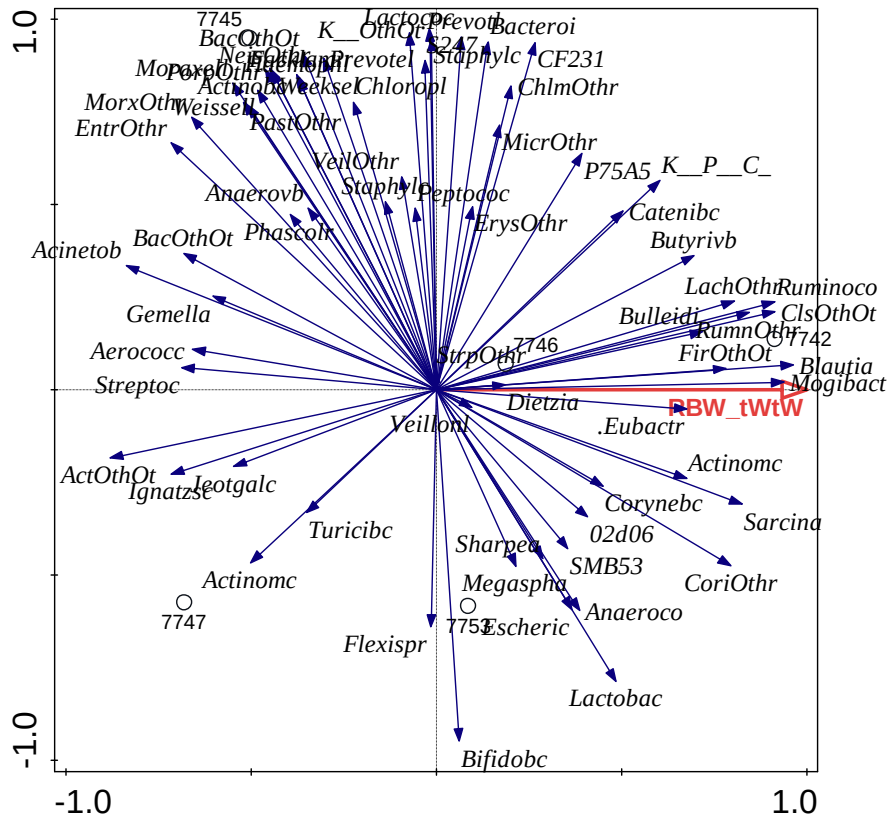

Figure 12 *RDA with RBWG W-W14 as constrained variable (explanatory variables account for 25.0%, P=0.42).*

Step 2

Table 12 *Selection bacterial genera associated with RBWG W-W14 based upon the X-coordinate*

Bacterial genera	X-coordinate
Actinobacteria.Other.Other	-0.88
Acinetobacter	-0.84
Lachnospiraceae.Other	0.81
Sarcina	0.83
Ruminococcaceae.Other	0.85
Ruminococcus	0.91
Clostridia.Other.Other	0.92
Mogibacterium	0.94
Blautia	0.96

Step 3

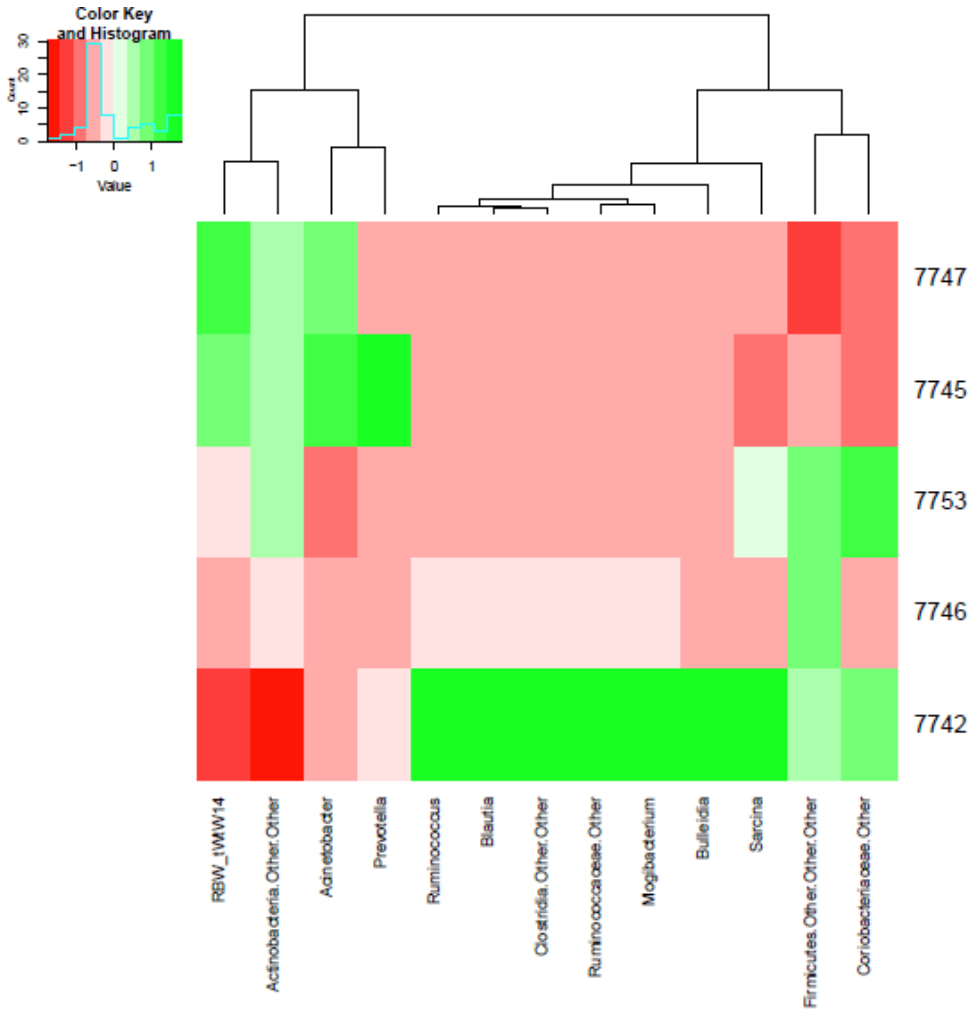

Figure 13 *Heatmap with RBWG W-W14. On the x-axis are the selected bacterial genera and the y-axis depicts the individual pig samples. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

Table 13 *Measuring the difference between RBWG W-W14 and bacterial genera.*

Bacterial genera	RMSE[1]	MAE[2]
Acinetobacter	0.50	0.49
Actinobacteria.Other.Other	0.47	0.42
Coriobacteriaceae.Other	1.68	1.50
Sarcina	1.71	1.37
Ruminococcaceae.Other	1.70	1.30
Firmicutes.Other.Other.Other	1.70	1.62
Clostridia.Other.Other	1.72	1.34
Ruminococcus	1.72	1.38
Mogibacterium	1.72	1.31
Blautia	1.73	1.35

[1]Root-mean-squared error, [2]Mean absolute error

3.2.2 Period: 14 till 23 days post-weaning

The period of interest is 14 days post-weaning till 23 days post-weaning.

Step 1

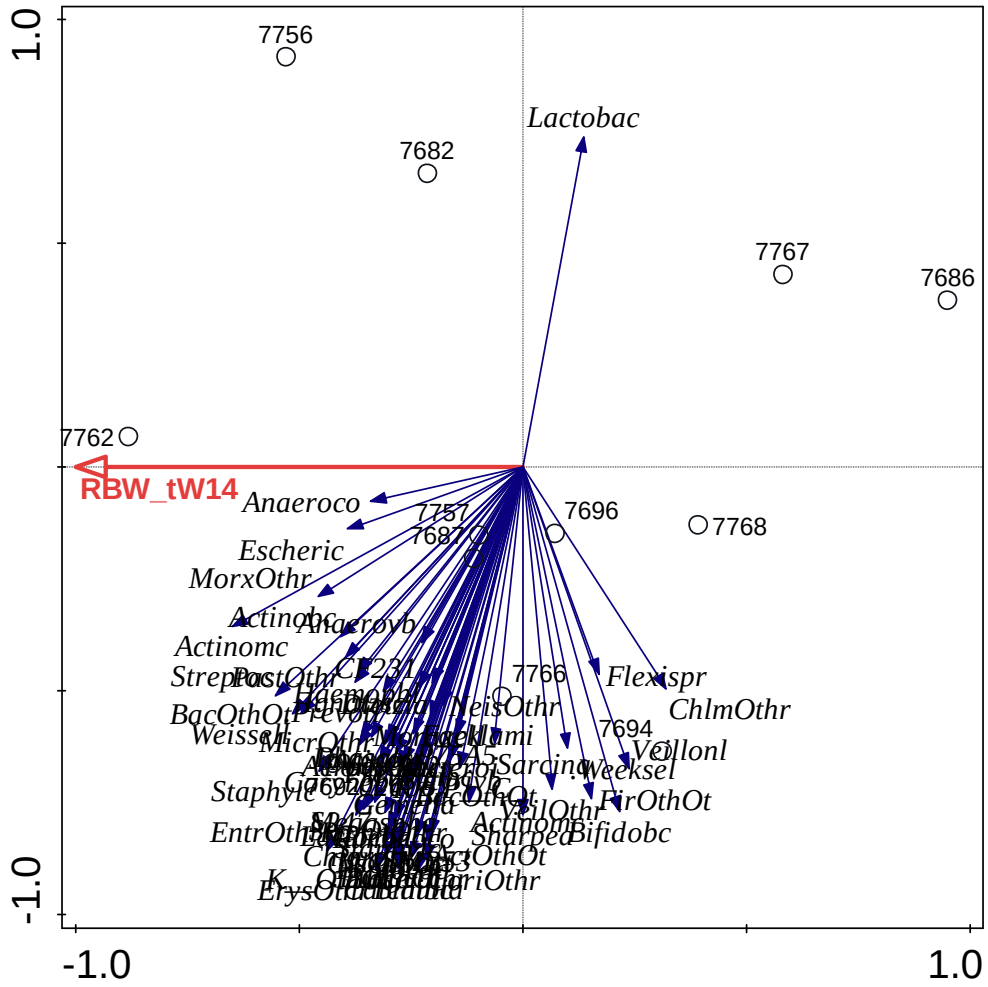

Figure 14 *RDA with RBWG W14-W23 as constrained variable (explanatory variables account for 10.7%, P=0.31).*

Step 2

Table 14 *Selection bacterial genera associated with RBWG W14-W23 based upon the X-coordinate.*

Bacterial genera	X-coordinate
Actinomycetaceae.	-0.65
Streptococcus	-0.55
Weissella	-0.51
Bacilli.Other.Other	-0.50
Moraxellaceae.Other	-0.46
Staphylococcus	-0.46
Enterococcaceae.Other	-0.44
Actinobacillus	-0.41

Step 3

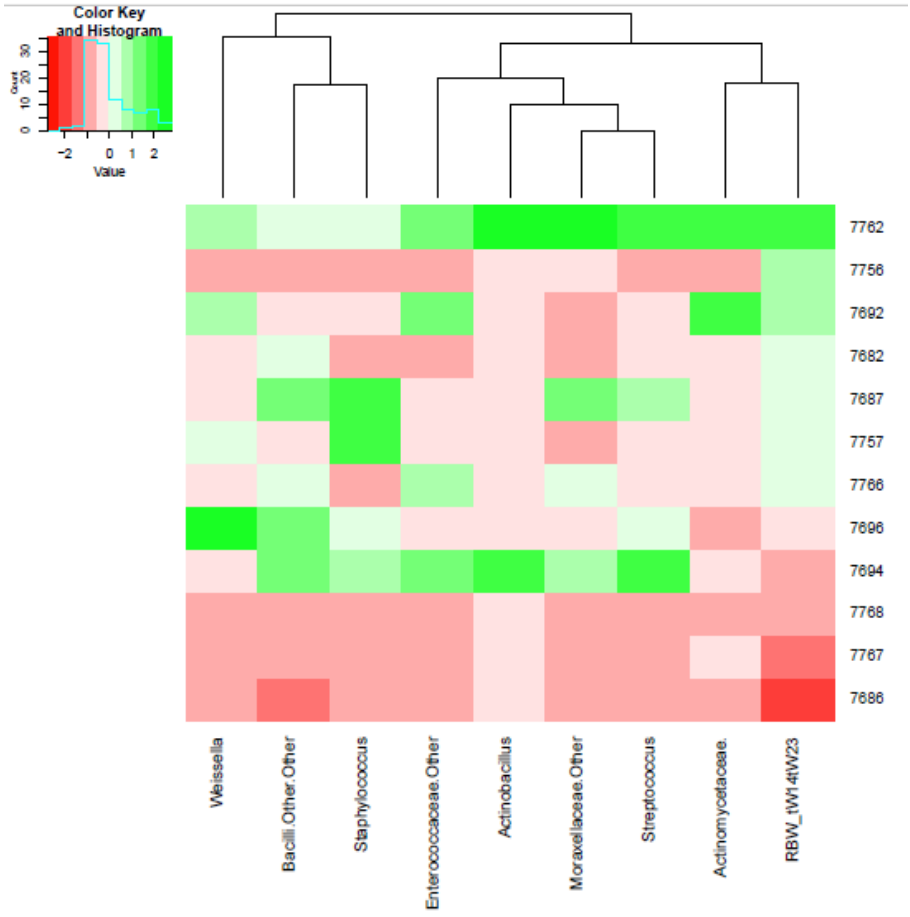

Figure 15 *Heatmap with RBWG W14-W23. On the x-axis are the selected bacterial genera and the y-axis depicts the individual pig samples. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

Table 15 *Measuring the difference between RBWG W14-W23 and bacterial genera*

Bacterial genera	RMSE[1]	MAE[2]
Actinomycetaceae.	0.82	0.68
Streptococcus	1.07	0.80
Weissella	1.15	0.86
Bacilli.Other.Other	1.18	0.95
Moraxellaceae.Other	0.99	0.84
Staphylococcus	1.17	1.04
Enterococcaceae.Other	1.06	0.81
Actinobacillus	1.08	0.91

[1]Root-mean-squared error, [2]Mean absolute error

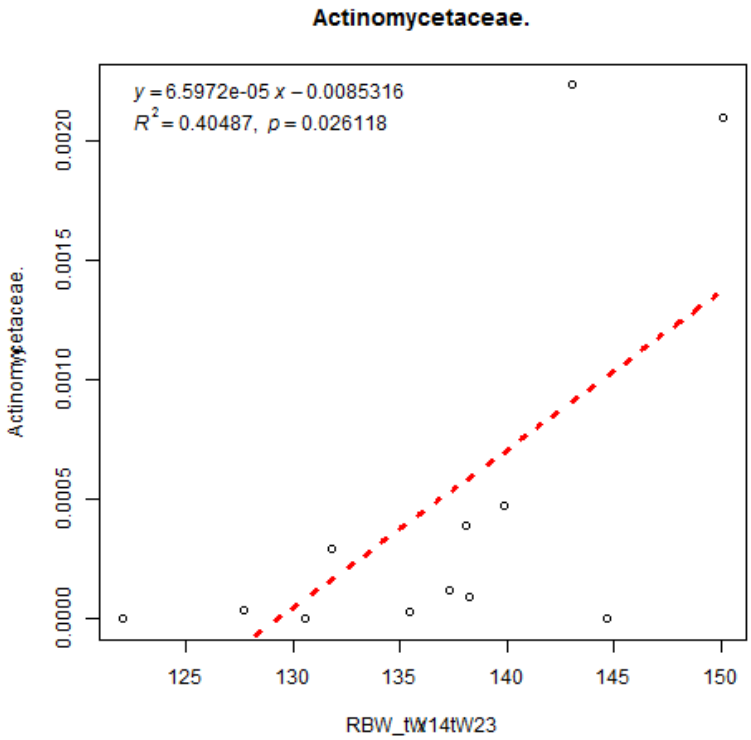

Figure 16 *Scatterplot of RBWG W14-W23 and Actinomycetaceae. A linear trend-line (red dashed line) was calculated, with an R^2 of 40% and a P-value of 0.026.*

3.2.3 Period: 23 till 35 days post-weaning

The period of interest is 23 days post-weaning till 35 days post-weaning.

Step 1

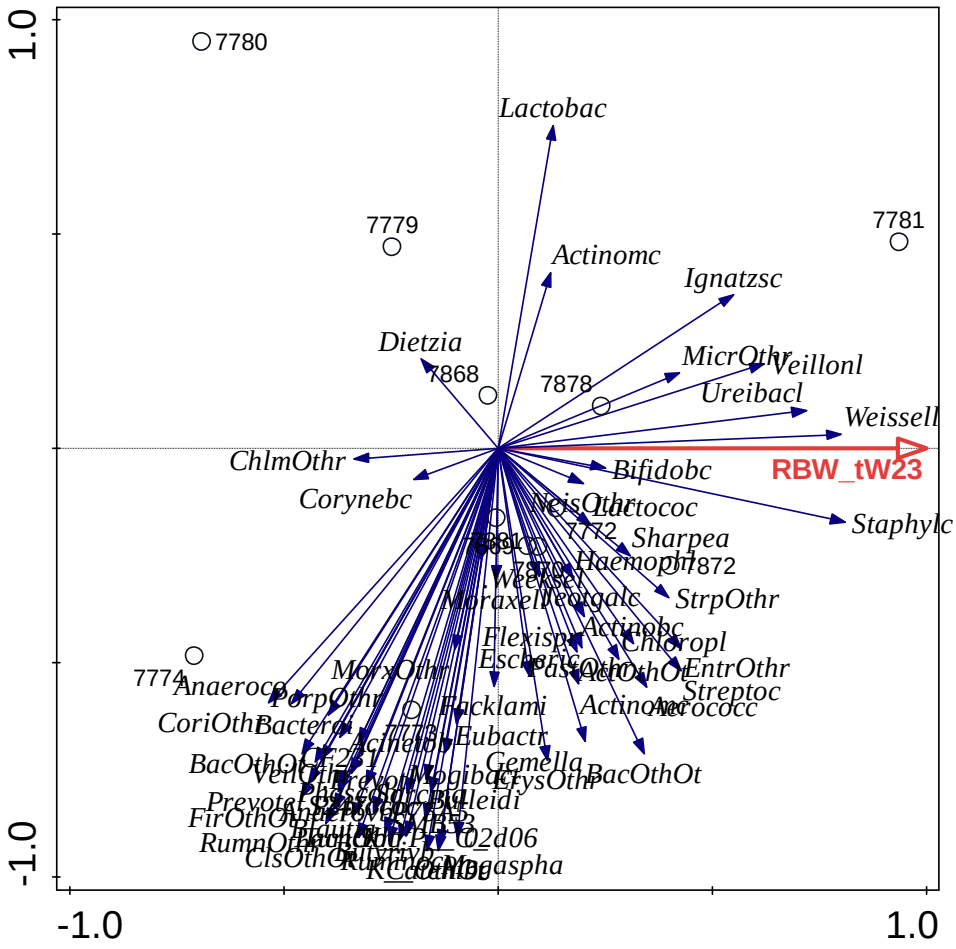

Figure 17 *RDA with RBWG W23-W35 as constrained variable (explanatory variables account for 14.1%, P=0.12).*

Step 2

Table 16 *Selection bacterial genera associated with RBWG W23-W35 based upon the X-coordinate.*

Bacterial genera	X-coordinate
Coriobacteriaceae.Other	-0.54
Anaerococcus	-0.48
Firmicutes.Other.Other.Other	-0.46
Veillonellaceae.Other	-0.46
Prevotella	-0.44
Bacteroidia.Other.Other	-0.43
Microbacteriaceae.Other	0.42
Streptococcus	0.43
Enterococcaceae.Other	0.43
Ignatzschineria	0.55
Veillonella	0.62
Ureibacillus	0.72
Weissella	0.80
Staphylococcus	0.81

Step 3

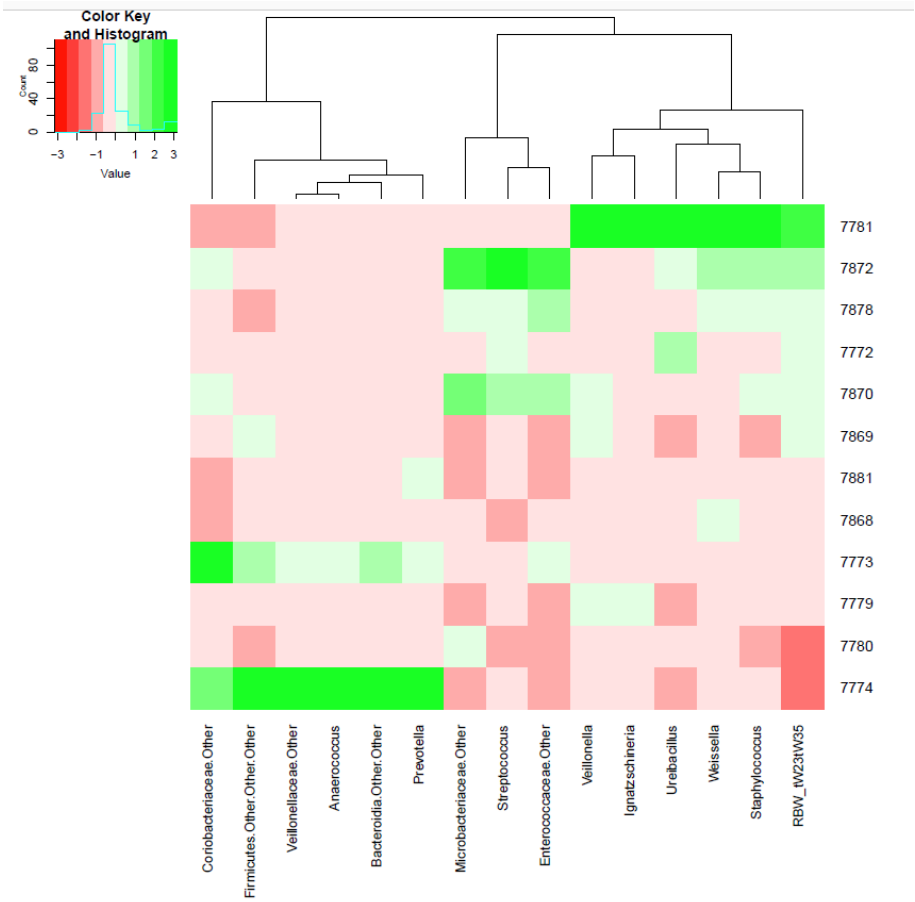

Figure 18 *Heatmap with RBWG W23-W35. On the x-axis are the selected bacterial genera and the y-axis depicts the individual pig samples. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

Table 17 *Measuring the difference between RBWG W23-W35 and bacterial genera.*

Bacterial genera	RMSE1	MAE2
Coriobacteriaceae.Other	1.63	1.25
Anaerococcus	1.67	1.16
Firmicutes.Other.Other.Other	1.67	1.11
Veillonellaceae.Other	1.67	1.15
Prevotella	1.67	1.16
Bacteroidia.Other.Other	1.68	1.16
Microbacteriaceae.Other	1.08	0.86
Streptococcus	1.06	0.76
Enterococcaceae.Other	1.07	0.82
Ignatzschineria	0.82	0.70
Veillonella	0.75	0.69
Ureibacillus	0.65	0.58
Weissella	0.60	0.48
Staphylococcus	0.59	0.49

[1]Root-mean-squared error, [2]Mean absolute error

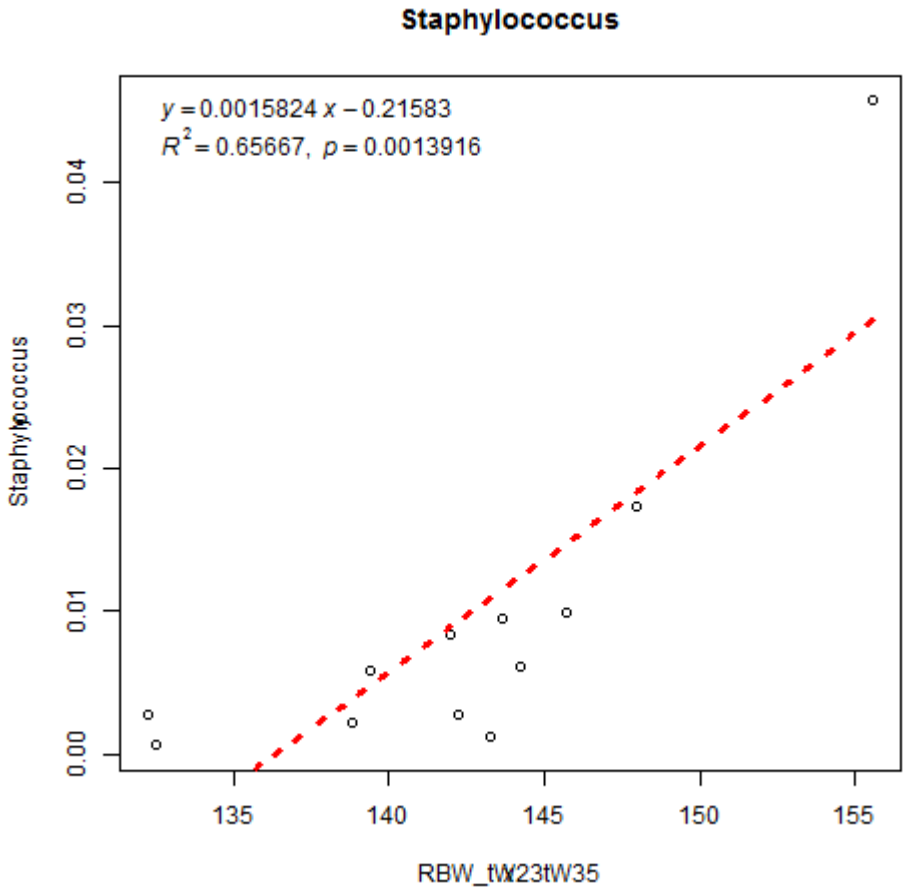

Figure 19 *Scatterplot of RBWG W23-W35 and Staphylococcus. A linear trend-line (red dashed line) was calculated, with an R^2 of 66% and a P-value of 0.001.*

3.3 VDI-5.2

For specific periods within the study, the descriptive statistics were calculated for average daily gain (ADG), relative body weight (RBWG), as well as for the Bray–Curtis dissimilarity of the associated jejunal microbiota data (Table 4).

Table 18 *Descriptive statistics of the VDI-5.2 study.*

	Period*	N	Mean	Min	Max	Sd
ADG[1]	W-W14	16	276	201	411	57
ADG	W14-W23	30	403	225	533	80
RBWG[2]	W-W14	16	151	139	169	10
RBWG	W14-W23	30	131	118	143	7
Bray	W	16	0.24	0.01	0.45	0.11
Bray[3]	W14	30	0.10	0.01	0.24	0.05

* Shows the period (in days) used for calculation of the parameter, where day 0 is birth and W is weaning (W4 means 4 days post-weaning)

1 Average daily gain, in grams per day

2 Relative body weight gain as percentage

3 Short for Bray–Curtis dissimilarity, units are an index

3.3.1 Period: Weaning till 14 days post-weaning

The period of interest is weaning (approximately day 28) till 14 days post-weaning.

Step 1

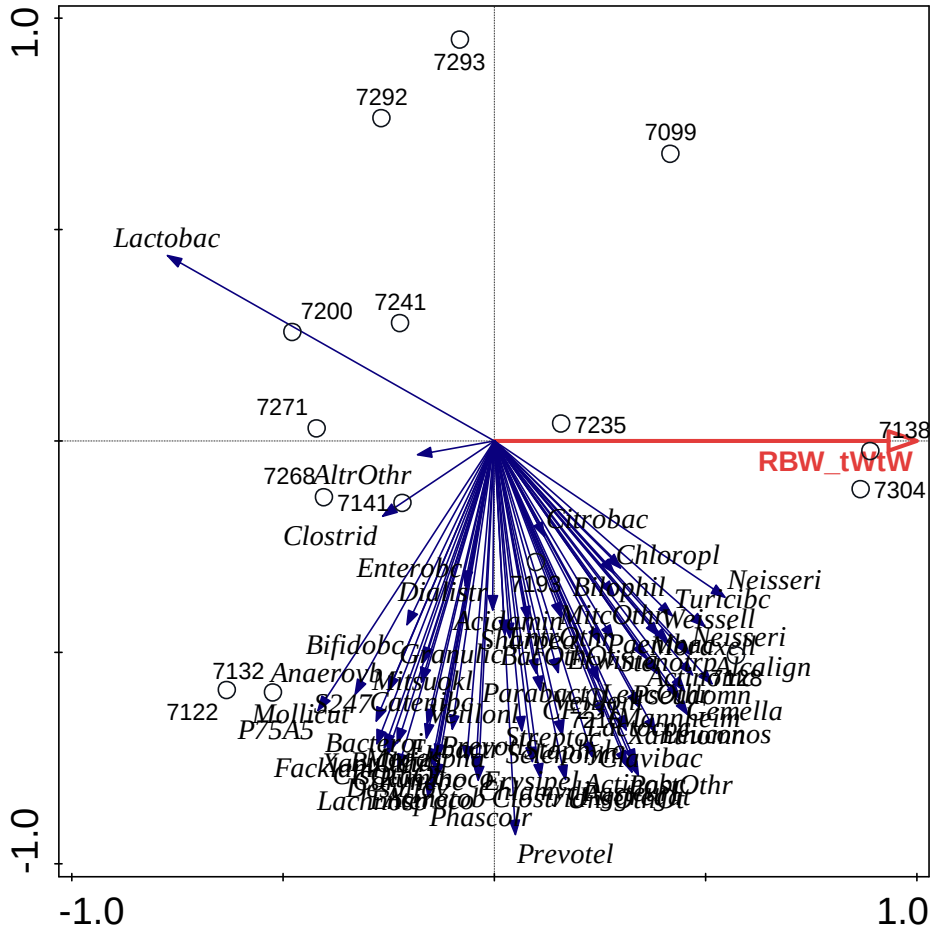

Figure 20 *RDA with RBWG W-W14 as constrained variable (explanatory variables account for 9.4%, P=0.21).*

Step 2

Table 19 *Selection bacterial genera associated with RBWG W23-W35 based upon the X-coordinate.*

Bacterial genera	X-coordinate
Lactobacillus	-0.77
Gemella	0.45
Xanthomonadaceae.	0.46
Neisseria	0.46
Pseudomonas	0.47
Moraxella	0.50
Alcaligenaceae.	0.52
Neisseriaceae.	0.55

Step 3

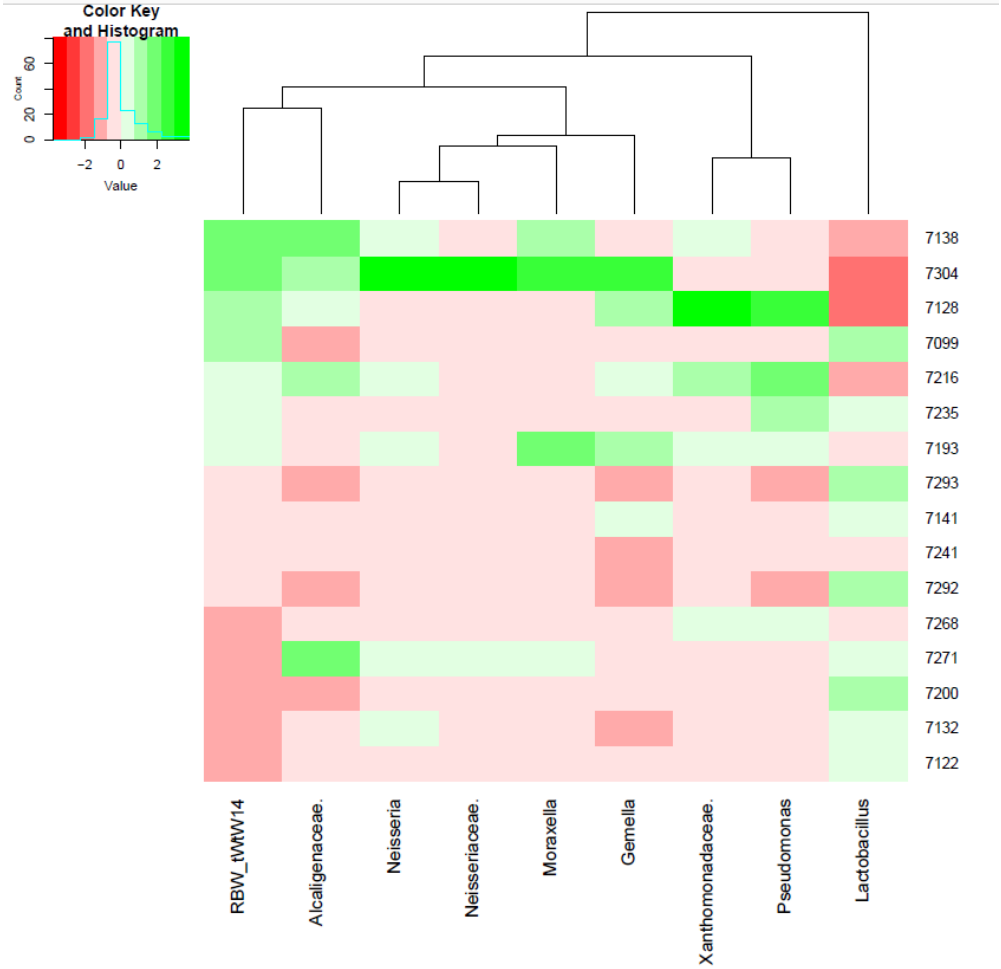

Figure 21 *Heatmap with RBWG W-W14. On the x-axis are the selected bacterial genera and the y-axis depicts the individual pig samples. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

Table 20 *Measuring the difference between RBWG W-W14 and bacterial genera.*

Bacterial genera	RMSE1	MAE2
Lactobacillus	1.76	1.44
Gemella	0.92	0.74
Xanthomonadaceae	1.04	0.82
Neisseria	0.95	0.75
Pseudomonas	1.04	0.82
Moraxella	0.92	0.77
Alcaligenaceae	0.92	0.71
Neisseriaceae	0.98	0.79

[1]Root-mean-squared error, [2]Mean absolute error

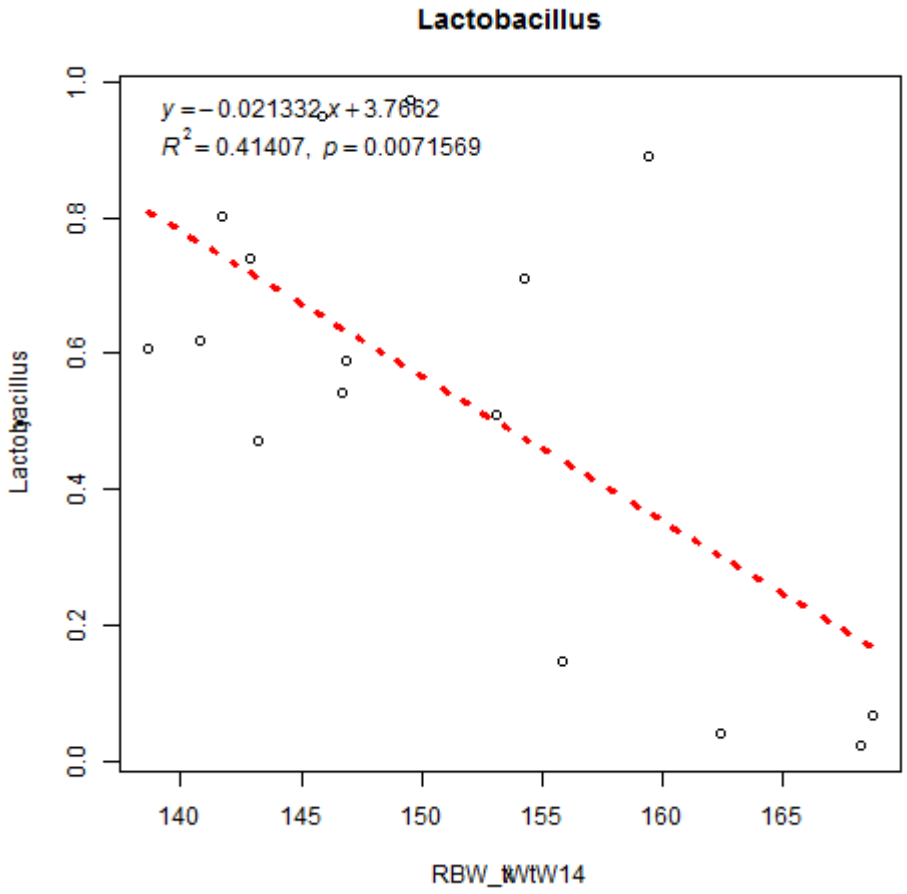

Figure 22 *Scatterplot of RBWG W23-W35 and Lactobacillus. A linear trend-line (red dashed line) was calculated, with an R^2 of 41% and a P-value of 0.007.*

3.3.2 Period: 14 till 23 days post-weaning

The period of interest is 14 days post-weaning till 23 post-weaning.

Step 1

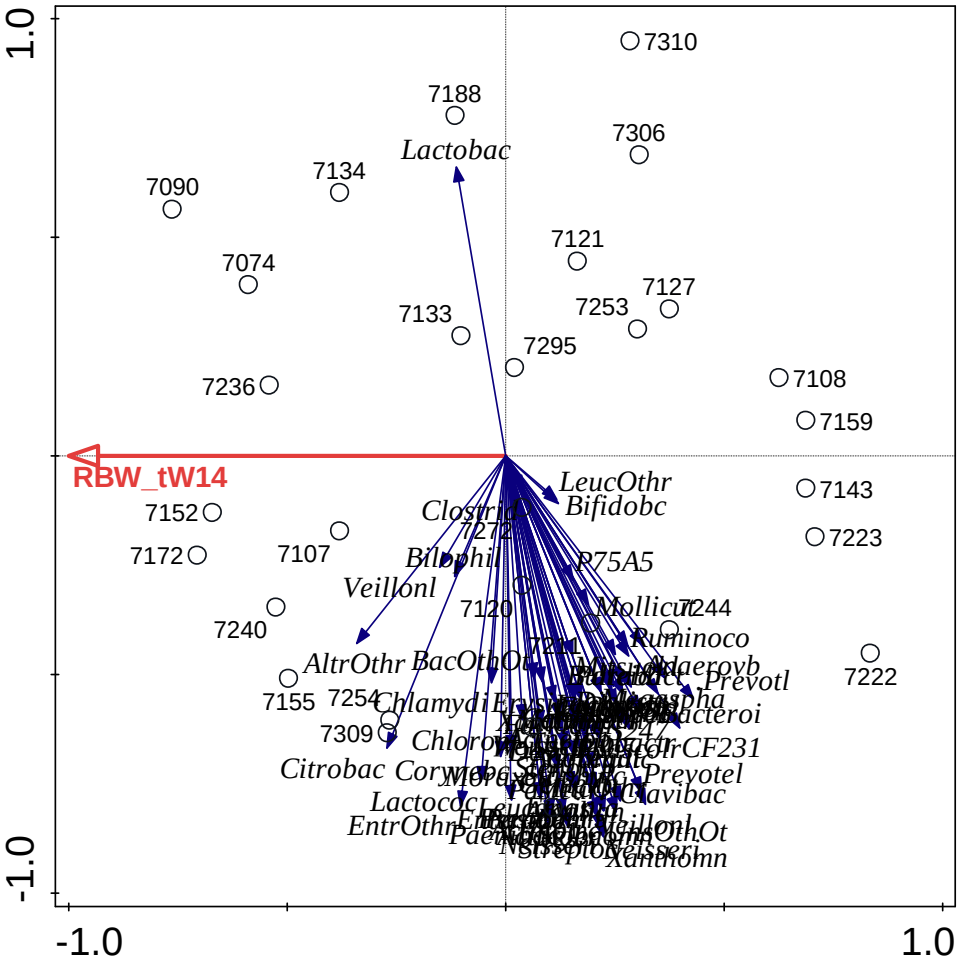

Figure 23 *RDA with RBWG W-W14 as constrained variable (explanatory variables account for 3.7%, P=0.32).*

Only one of the bacterial genera had a substantial contribution to the phenotype, namely *Prevotella* (0.43). However, the average relative contribution was very low as well as the correlation (data not shown).

3.4 VDI-12

For specific periods within the study, the descriptive statistics were calculated for average daily gain (ADG), relative body weight (RBWG), as well as for the Bray–Curtis dissimilarity of the associated jejunal microbiota data (Table 5).

Table 21 *Descriptive statistics of the VDI-12 study.*

	Period*	N	Mean	Min	Max	sd
ADG[1]	0-1	26	31	-110	200	71
ADG	0-W3	26	245	191	327	35
ADG	W-W3	26	124	-50	275	95
RBWG[2]	0-1	26	102	92	116	5
RBWG	0-W3	26	621	462	829	91
RBWG	W-W3	26	106	98	116	5
Bray	1	26	0.25	0	0.53	0.10
Bray[3]	W3	26	0.13	0	0.42	0.11

* Shows the period (in days) used for calculation of the parameter, where day 0 is birth and W is weaning (W4 means 4 days post-weaning)

1 Average daily gain, in grams per day

2 Relative body weight gain as percentage

3 Short for Bray–Curtis dissimilarity, units are an index

3.4.1 Period: Birth till day 1

The period of interest is birth (day 0) till day 1.

Step 1

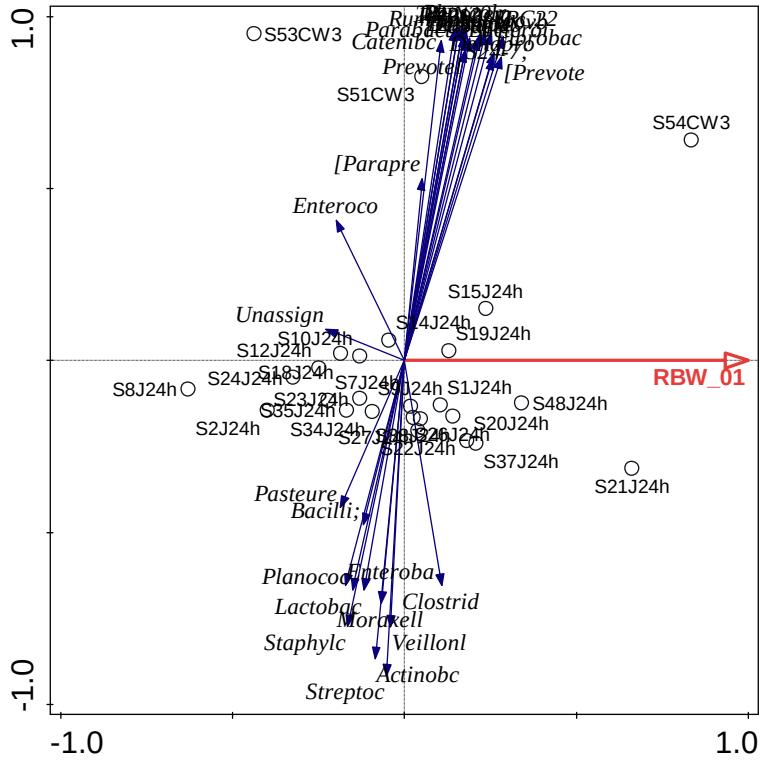

Figure 24 *RDA with RBWG 0-1 as constrained variable (explanatory variables account for 3.2%, P=0.42).*

None of the bacterial genera had a substantial contribution to the RBWG, all x-coordinate values were below 0.4 (or above -0.4).

3.4.2 Period: Weaning till 3 days post-weaning

The period of interest is weaning (approximately day 28) till 3 days post-weaning.

Step 1

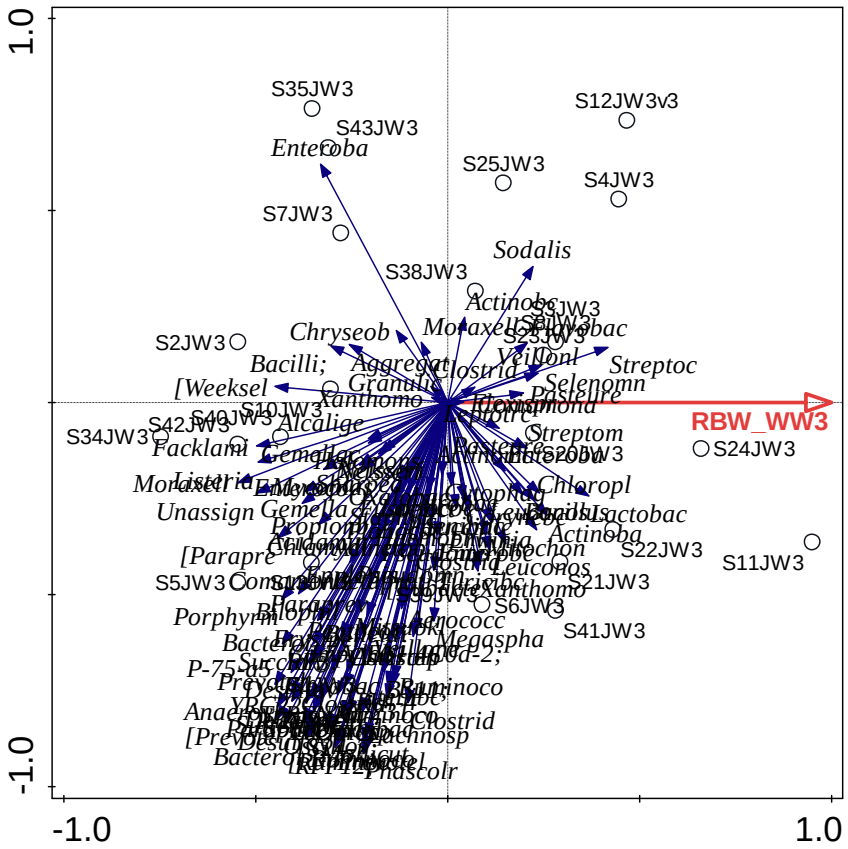

Figure 25 *RDA with RBWG W-W3 as constrained variable (explanatory variables account for 8.0%, P=0.05).*

Step 2

Table 22 *Selection bacterial genera of the VDI-12 study based upon the X-coordinate.*

Bacterial genera	X-coordinate
Moraxellaceae;	-0.55
Facklamia	-0.50
Unassigned;Other;Other;Other;Other	-0.50
Listeria	-0.50
p-75-a5	-0.45
[Weeksellaceae];	-0.45
Sutterella	-0.44
[Prevotella]	-0.44
[Paraprevotellaceae];	-0.44
Porphyromonas	-0.43
Succinivibrio	-0.43
Desulfovibrio	-0.43
Anaerovibrio	-0.41
Desulfovibrionaceae;	-0.41
Deltaproteobacteria;	-0.41
Streptococcus	0.42

Step 3

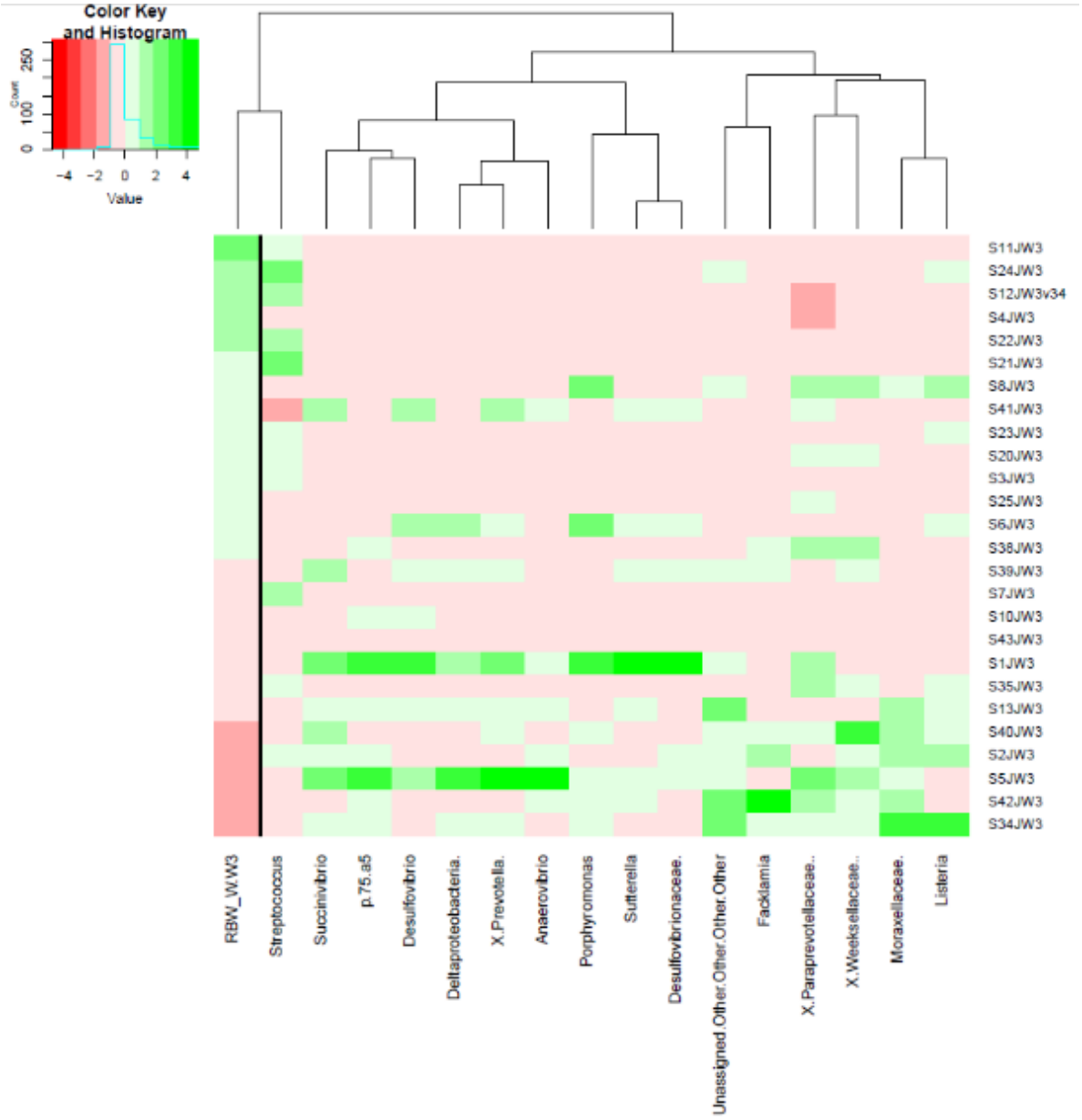

Figure 26 *Heatmap with RBWG W-W3. On the x-axis are the selected bacterial genera and the y-axis depicts the individual pig samples. Red indicates low values, whereas green indicates high values of the respective data source, RBWG or average relative contribution of a certain bacterial genera.*

Step 4

Table 23 *Measuring the difference between RBWG W-W3 and bacterial genera.*

Bacterial genera	RMSE1	MAE2
Streptococcus	0.96	0.76
Desulfovibrionaceae	1.53	1.14
Porphyromonas	1.54	1.29
Sutterella	1.57	1.17
Desulfovibrio	1.60	1.31
Deltaproteobacteria	1.61	1.26
Prevotella	1.61	1.21
Anaerovibrio	1.61	1.18
Weeksellaceae	1.64	1.31
Facklamia	1.64	1.18
Succinivibrio	1.65	1.36
p.75.a5	1.66	1.29
Paraprevotellaceae	1.67	1.33
Listeria	1.68	1.31
Unassigned; Other Other Other Other	1.72	1.38
Moraxellaceae	1.77	1.38

[1]Root-mean-squared error, [2]Mean absolute error

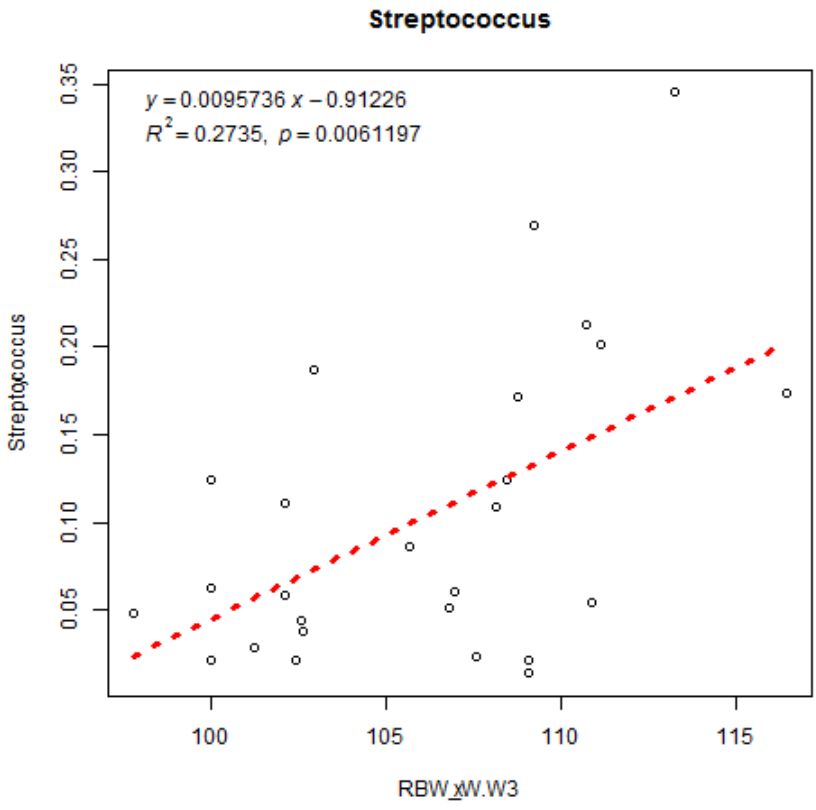

Figure 27 *Scatterplot of RBWG W-W3 and Streptococcus. A linear trend-line (red dashed line) was calculated, with an R^2 of 27% and a P-value of 0.006.*

3.5 Combining of multiple studies based upon data of a single time-point

To gain more statistical power, data of two studies were combined in which a similar day of sampling was applied as well as a similar dietary intervention. Here, the VDI-5.1 and VDI-5.2 samples of day 23 post-weaning were combined. This resulted in an overlap of 177 bacterial genera between the two datasets (Figure 28). These 177 bacterial genera were used as input to explore whether the variation of RBWG was independent of study. Figure 29 shows a clear separation of samples, where VDI 5.1 samples are located on the right, whereas VDI5.2 samples are on the left. This separation in the x-axis shows that the factor study induced major part of the variation in jejunal microbiota composition. Nevertheless, it is possible to separate the samples based on RBWG in the y-axis direction (as shown by the downward facing red arrow). In conclusion, it is possible to merge different microbial datasets, however the variation was too large between the datasets, i.e. bacterial genera differed, for useful interpretation to study the association with RBWG.

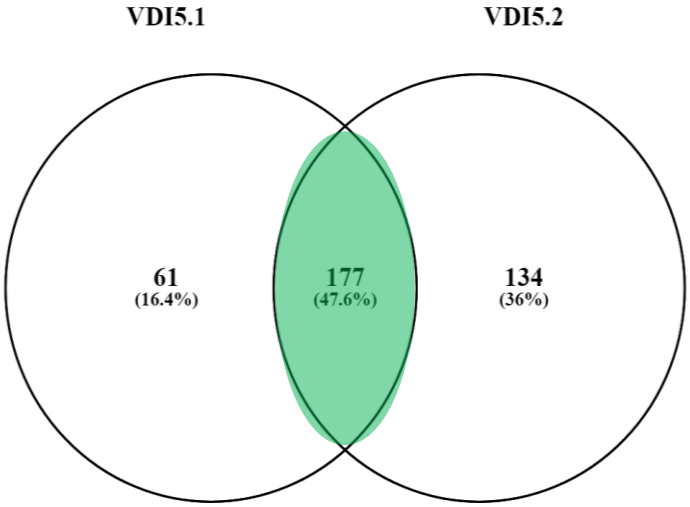

Figure 28 *Venn-diagram of the number of bacterial genera unique to studies VDI5.1 (61 genera), VDI5.2 (134 genera), and its overlap (177 genera).*

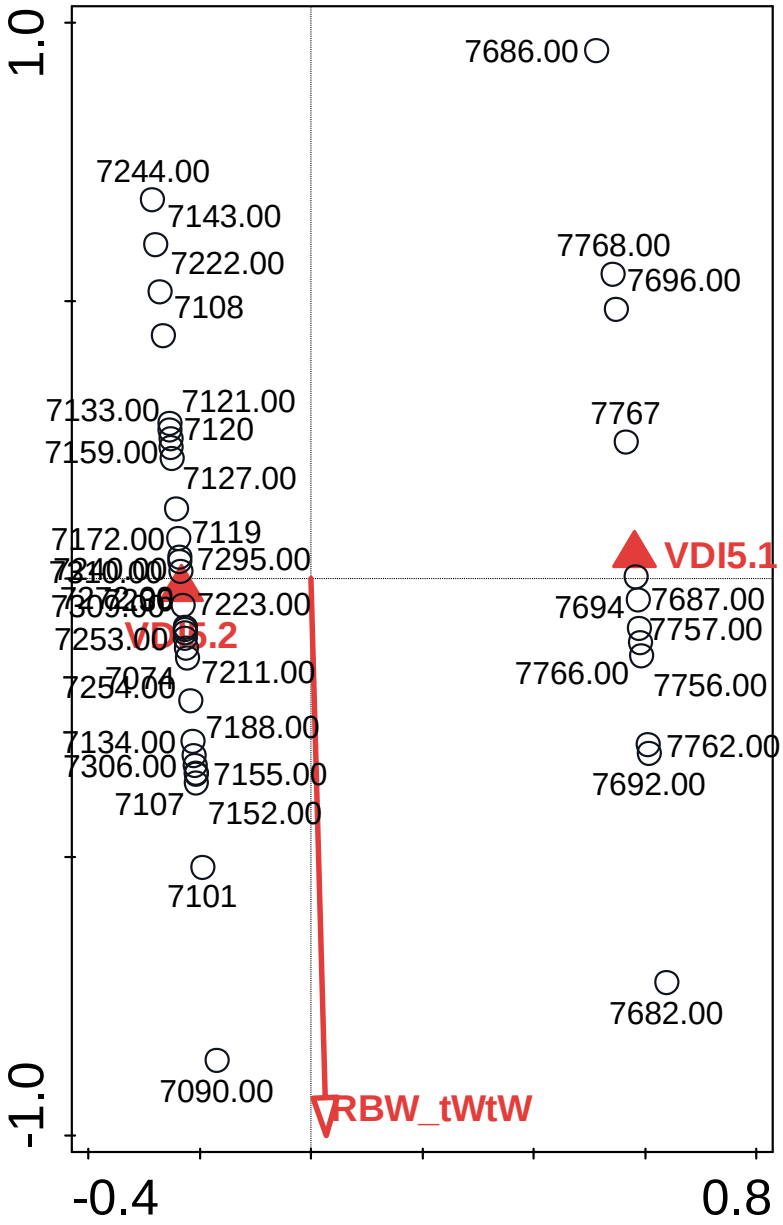

Figure 29 *RDA of piglets from two different studies (VDI5.1 and VDI5.2) sampled at the same time after weaning (day 23).*

3.6 Microbial groups related to variation in relative growth

Complete results are shown in Appendix 1. In the current paragraph we focus on the bacterial genera that were observed in two or more studies, irrespective of their positive or negative association with the RBWG (see Table 24).

Table 24 *Overlap of bacterial genera in jejunal digesta associated with RBWG between studies VDI2, VDI5.1, and VDI5.2.*

Bacterial genera	Study 1	Study 2	Study 3	Study 4
Bacilli.Other.Other	VDI2.W4-W28*	VDI5.1.W14-W23		
Bacteroidia.Other.Other	VDI2.W4-W28	VDI5.1.W23-W35	VDI2.0-7*	VDI2.0-W
Bacteroidia.	VDI2.W4-W28	VDI2.0-W		
Catenibacterium	VDI2.W4-W28	VDI2.W-W4		
Chlamydia	VDI2.0-W	VDI2.W-W4		
Clostridia.Other.Other	VDI2.W4-W28	VDI5.1.W-W14		
Coriobacteriaceae.Other	VDI5.1.W23-W35	VDI5.1.W-W14		
Desulfovibrio	VDI12.W-W3	VDI2.0-W		
Enterobacteriaceae.Other	VDI2.0-7	VDI2.W4-W28		
Enterococcaceae.Other	VDI5.1.W14-W23	VDI5.1.W23-W35		
Escherichia	VDI2.0-7	VDI2.W4-W28	VDI2.W-W4	
Firmicutes.Other.Other.Other	VDI5.1.W23-W35	VDI5.1.W-W14		
Gemella	VDI2.0-W	VDI5.2.W-W14		
Lachnospiraceae.Other	VDI2.W4-W28	VDI2.0-W		
Lactobacillus	VDI5.2.W-W14	VDI2.W4-W28		
Mollicutes.	VDI2.W4-W28	VDI2.0-W		
Peptococcus	VDI2.0-7	VDI2.W4-W28		
Prevotella	VDI2.0-W	VDI5.1.W23-W35	VDI2.0-7	
Ruminococcaceae.Other	VDI2.W4-W28	VDI5.1.W-W14		
Sharpea	VDI2.0-7	VDI2.W-W4		
Staphylococcus	VDI5.1.W14-W23	VDI5.1.W23-W35		
Streptococcaceae.Other	VDI2.W-W4	VDI2.0-7		
Streptococcus	VDI2.W4-W28	VDI5.1.W14-W23	VDI5.1.W23-W35	VDI12.W-W3
Veillonellaceae.Other	VDI5.1.W23-W35	VDI2.0-W		
Weissella	VDI5.1.W14-W23	VDI5.1.W23-W35		

* Green depicts a positive association to RBWG, meaning a higher average relative abundance correlates with a higher RBWG, whereas red depicts a negative association to RBWG. Note, a match was scored and depicted here when the names, based on their taxonomy, exactly matched.

In Table 24 the results are shown when integrating the different significant bacterial genera per study and time-point combination. For each significant bacterial genera we investigated if it was present in other comparisons as well, in total 25 bacterial genera were significant in two or more study time-points. Generally, overlap of bacterial genera is within the VDI-2 study, i.e. between different time-periods within this study, similarly for the VDI-5.1 study. Or between time-points of these two studies, e.g. the bacterial genus *Veillonellaceae.* Other time-period W23-35 in the VDI5.1 study and time-period 0-W in the VDI2 study. The VDI-5.2 study is represented two times by *Gemella* and *Lactobacillus*, whereas the VDI-12 study is only once represented, by the genus *Streptococcus*.

4 Discussion

In this study it was shown that it is possible to associate microbiota composition at genus level in the jejunum of piglets in the pre- and post-weaning phase to performance parameters (Relative Body Weight Gain; RBWG) in individual animals. For this we have re-used existing data from previous studies that were executed within the framework of the Public Private Partnership Feed4Foodure.

It is important to realise that these studies were not designed to identify an association between the intestinal microbiota composition and growth performance. It has been established that the gut microbiota is associated to many diseases in humans [11], as well as body weight (BMI) [12], and that it is involved in orchestrating energy homeostasis [13]. The four studies used as input for the current report contain recorded data on individual weight and weight gain as well as jejunal microbiota composition at the day of sacrifice. Although it was tried to narrow down data to an overall microbiota signature, one must keep in mind that the microbiota composition is driven by many different factors. For example, the intestinal segment of choice is an important factor. Small intestinal microbiota composition is very different from the microbiota composition in the large intestine [14]. In the analyses performed for the current report only relationships with the jejunal microbiota could be investigated, because data on microbiota composition were only available for this segment for all individual animals. In addition to the intestinal segment being an important source for variation in the microbiota composition, the age of the animal also contributes to the variation. Colonization starts immediately after birth, and during early life the microbiota composition is influenced by environmental conditions [15-21].

Batch effect(s) also contribute to the variation, here batch refers to individual experiments. Other confounding factors that can influence the microbiota in the gut are the diet composition, which can be different per experiment or the different (animal) genetics used. Another aspect that is different between the experiments is the age of the piglets at sampling and the number of animals sacrificed to acquire the small intestinal microbiomes. The latter is an important issue, because for each experiment the days of sacrifice were tailored to support the particular hypothesis of that study. In addition, because these studies were interested in putative changes of the small intestinal microbiota, animals needed to be sacrificed. Thus, longitudinal studies of an individual animal were not performed and the only effect that could be investigated was the effect of treatment at a particular time point.

Despite these limitations that arose from the experimental setups, it was possible to identify different bacterial genera in jejunal digesta that showed an association with RBWG. For most study-time-points (age of piglets at which samples were taken) RBWG associated bacterial genera were identified and their significant correlation with RBWG was illustrated (with high R^2). The most obvious results were found for *Lactobacillus* (VDI-2.W4W28) and *Staphylococcus* (VDI-5.1.W23W35), which both emerged as RBWG-correlated with p-values below P=0.05 and R^2 coefficients of approximately 0.66. However, the average relative contribution (ARC) for *Staphylococcus* in the jejunal microbiota was only 1-5%, and thereby not different from many of the bacterial genera associated to RBWG. Contrary, *Lactobacillus* had an ARC ranging from approximately 20% for the piglets with low RBWG, and an ARC up to 90% in piglets with a high RBWG. This positive correlation is likely study-specific, because a significantly negative correlation of *Lactobacillus* in study VDI5.2 (W-W14) with an R^2 of 0.41 and ARC ranging from 10% to 80% was observed. In most cases, low ARC values were observed for significantly correlating bacterial genera. Another observation is that single bacterial genera are not likely to explain the complex RBWG-phenotype. This is shown by the explained variation in the RDA models, ranging from 3 to 25%. Although these generated values are promising, the results suggest that the contribution of the microbiota to the RBWG is a community effect rather than a consequence of the presence or absence of single bacterial genera. It is possible to go beyond the genera level and zoom in to the species level or the strain level, however more sophisticated and reliable tools and/or methods for taxonomic profiling are necessary [22]. In addition, 16S sequencing is more cost effective compared to shotgun metagenome sequencing, the latter is able to detect novel species as well as detecting fungi and viruses. To overcome the limitations of microbiome species composition analysis (i.e., no direct linkage to the functional composition), shotgun metagenome sequencing is the

alternative. This type of analysis enables to investigate microbiome signature relatedness with performance from a microbiome-function perspective, providing a much higher resolution in these analysis that can overcome problems of function-redundancy at species level and other confounding factors that remain undiscovered by 16S based analyses.

Overall it can be concluded that it is possible to associate bacterial signatures at genera level from the small intestine to a phenotype such as growth performance in individual piglets. However, no consistent and significant microbiota signatures could be identified that correlated to the weight gain parameters used. Moreover, these conclusions need to be taken with caution, related to the low number of animals included in these analyses. Future studies dedicated to unravel intestinal microbiome relationships with RBWG or other performance parameters would require a much higher number of animals involved to overcome these limitations. Generating an estimate of the number of animals needed for future studies is difficult, because it heavily depends on the parameters of interest and its variation. To enable longitudinal studies that correlate microbiome and performance, faecal samples would be preferred. However, not much is known about which gut segment has the greatest association to growth performance. Moreover, contrary to jejunal microbiomes, faecal microbiome signatures associated with performance can more readily be translated to a practically applicable measurement. In addition, to avoid identifying experiment, location (farm), or season specific microbial signatures that associate with performance values, it would be recommendable to expand such sample surveys to a number of different locations, and over an extended period.

References

1. Verschuren, L.M.G., et al., Fecal microbial composition associated with variation in feed efficiency in pigs depends on diet and sex. J Anim Sci, 2018. **96**(4): p. 1405-1418.
2. McCormack, U.M., et al., Exploring a Possible Link between the Intestinal Microbiota and Feed Efficiency in Pigs. Appl Environ Microbiol, 2017. **83**(15).
3. Yang, H., et al., Unraveling the Fecal Microbiota and Metagenomic Functional Capacity Associated with Feed Efficiency in Pigs. Front Microbiol, 2017. **8**: p. 1555.
4. Han, G.G., et al., Tracing of the fecal microbiota of commercial pigs at five growth stages from birth to shipment. Sci Rep, 2018. **8**(1): p. 6012.
5. Han, G.G., et al., Evaluating the association between body weight and the intestinal microbiota of weaned piglets via 16S rRNA sequencing. Appl Microbiol Biotechnol, 2017. **101**(14): p. 5903-5911.
6. Quan, J., et al., A global comparison of the microbiome compositions of three gut locations in commercial pigs with extreme feed conversion ratios. Sci Rep, 2018. **8**(1): p. 4536.
7. Vigors, S., et al., The Effect of Divergence in Feed Efficiency on the Intestinal Microbiota and the Intestinal Immune Response in Both Unchallenged and Lipopolysaccharide Challenged Ileal and Colonic Explants. PLoS One, 2016. **11**(2): p. e0148145.
8. Zierer, J., et al., The fecal metabolome as a functional readout of the gut microbiome. Nat Genet, 2018. **50**(6): p. 790-795.
9. Schirmer, M., et al., Linking the Human Gut Microbiome to Inflammatory Cytokine Production capacity. Cell, 2016. **167**(7): p. 1897.
10. Salonen, A., et al., Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. ISME J, 2014. **8**(11): p. 2218-30.
11. Jackson, M.A., et al., Gut microbiota associations with common diseases and prescription medications in a population-based cohort. Nat Commun, 2018. **9**(1): p. 2655.
12. Ottosson, F., et al., Connection Between BMI-Related Plasma Metabolite Profile and Gut Microbiota. J Clin Endocrinol Metab, 2018. **103**(4): p. 1491-1501.
13. Chevalier, C., et al., Gut Microbiota Orchestrates Energy Homeostasis during Cold. Cell, 2015. **163**(6): p. 1360-74.
14. Scheithauer, T.P., et al., Causality of small and large intestinal microbiota in weight regulation and insulin resistance. Mol Metab, 2016. **5**(9): p. 759-70.
15. Inman, C.F., et al., Rearing environment affects development of the immune system in neonates. Clin Exp Immunol, 2010. **160**(3): p. 431-9.
16. Ley, R.E., et al., Microbial ecology: human gut microbes associated with obesity. Nature, 2006. **444**(7122): p. 1022-3.
17. Mulder, I.E., et al., Restricting microbial exposure in early life negates the immune benefits associated with gut colonization in environments of high microbial diversity. PLoS One, 2011. **6**(12): p. e28279.
18. Mulder, I.E., et al., Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. BMC biology, 2009. **7**: p. 79.
19. Penders, J., et al., Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut, 2007. **56**(5): p. 661-7.
20. Penders, J., et al., Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics, 2006. **118**(2): p. 511-21.
21. Schmidt, B., et al., Establishment of normal gut microbiota is compromised under excessive hygiene conditions. PLoS One, 2011. **6**(12): p. e28284.
22. Rossi-Tamisier, M., et al., Cautionary tale of using 16S rRNA gene sequence similarity values in identification of human-associated bacterial species. Int J Syst Evol Microbiol, 2015. **65**(Pt 6): p. 1929-34.

A. de Greeff, A., et al., Effect of maternal antibiotic intervention in sows on gut development and microbiota in offspring. 2015.

B. Jansman, A.J.M., et al., Effects of a high level of dietary zinc over different post-weaning periods on intestinal microbiota and mucosal gene expression in piglets. 2016.
C. Jansman, A.J.M., et al., Effects of dietary zinc concentration on development of immune competence in post-weaning piglets. 2017.
D. de Greeff, A., et al., Effect of neonatal and maternal dietary interventions on gut health of piglets. 2017.

Appendix 1 Overlap of all bacterial genera in jejunal digesta associated with RBWG between studies

Bacterial genera	Study			
[Paraprevotellaceae];	VDI12.W-W3*			
[Prevotella]	VDI12.W-W3			
[Weeksellaceae];	VDI12.W-W3			
Acinetobacter	VDI5.1.W-W14			
Actinobacillus	VDI5.1.W14-W23			
Actinobacteria.Other.Other	VDI5.1.W-W14			
Actinomycetaceae.	VDI5.1.W14-W23			
Alcaligenaceae.	VDI5.2.W-W14			
Alcaligenaceae.Other	VDI2.W-W4			
Anaerococcus	VDI5.1.W23-W35			
Anaerovibrio	VDI12.W-W3			
Bacilli.Other.Other	VDI2.W4-W28	VDI5.1.W14-W23		
Bacteroidia.	VDI2.W4-W28			
Bacteroidia.Other.Other	VDI2.W4-W28	VDI5.1.W23-W35	VDI2.0-7	VDI2.0-W
Bacteroidia;f__;	VDI2.0-W			
Blautia	VDI5.1.W-W14			
Butyrivibrio	VDI2.W4-W28			
Catenibacterium	VDI2.W4-W28	VDI2.W-W4		
Chlamydia	VDI2.0-W	VDI2.W-W4		
Clostridia.Other.Other	VDI2.W4-W28	VDI5.1.W-W14		
Clostridium	VDI2.0-W			
Coriobacteriaceae.Other	VDI5.1.W23-W35	VDI5.1.W-W14		
Deltaproteobacteria;	VDI12.W-W3			
Desulfovibrio	VDI12.W-W3	VDI2.0-W		
Desulfovibrionaceae;	VDI12.W-W3			
Enterobacteriaceae.Other	VDI2.0-7	VDI2.W4-W28		
Enterococcaceae.Other	VDI5.1.W14-W23	VDI5.1.W23-W35		
Enterococcus	VDI2.0-7			
Epulopiscium	VDI2.0-7			
Erysipelotrichaceae;Other	VDI2.0-W			
Escherichia	VDI2.0-7	VDI2.W4-W28	VDI2.W-W4	
Facklamia	VDI12.W-W3			
Firmicutes.Other.Other.Other	VDI5.1.W23-W35	VDI5.1.W-W14		
Gammaproteobacteria.Other.Other	VDI2.0-7			
Gemella	VDI2.0-W	VDI5.2.W-W14		
Ignatzschineria	VDI5.1.W23-W35			
k__Bacteria.Other.Other.Other.Other	VDI2.W4-W28			
Lachnospiraceae.Other	VDI2.W4-W28	VDI2.0-W		
Lactobacillaceae;	VDI2.0-W			
Lactobacillus	VDI5.2.W-W14	VDI2.W4-W28		
Listeria	VDI12.W-W3			
Microbacteriaceae.Other	VDI5.1.W23-W35			
Mitsuokella	VDI2.0-7			
Mogibacterium	VDI5.1.W-W14			
Mollicutes.	VDI2.W4-W28			

Bacterial genera	Study			
Mollicutes;f__;	VDI2.0-W			
Moraxella	VDI5.2.W-W14			
Moraxellaceae.Other	VDI5.1.W14-W23			
Moraxellaceae;	VDI12.W-W3			
Neisseria	VDI5.2.W-W14			
Neisseriaceae.	VDI5.2.W-W14			
p-75-a5	VDI12.W-W3			
Parabacteroides	VDI2.0-W			
Peptococcus	VDI2.0-7	VDI2.W4-W28		
Phascolarctobacterium	VDI2.0-W			
Porphyromonas	VDI12.W-W3			
Prevotella	VDI2.0-W	VDI5.1.W23-W35	VDI2.0-7	
Proteobacteria.Other.Other.Other	VDI2.W-W4			
Pseudomonas	VDI5.2.W-W14			
Ruminococcaceae.Other	VDI2.W4-W28	VDI5.1.W-W14		
Ruminococcus	VDI5.1.W-W14			
Sarcina	VDI5.1.W-W14			
Sharpea	VDI2.0-7	VDI2.W-W4		
Sphaerochaeta	VDI2.0-W			
Staphylococcus	VDI5.1.W14-W23	VDI5.1.W23-W35		
Streptococcaceae.Other	VDI2.W-W4	VDI2.0-7		
Streptococcus	VDI2.W4-W28	VDI5.1.W14-W23	VDI5.1.W23-W35	VDI12.W-W3
Succinivibrio	VDI12.W-W3			
Sutterella	VDI12.W-W3			
Treponema	VDI2.W4-W28			
Unassigned;Other;Other;Other;Other	VDI12.W-W3			
Ureibacillus	VDI5.1.W23-W35			
Veillonella	VDI5.1.W23-W35			
Veillonellaceae.Other	VDI5.1.W23-W35	VDI2.0-W		
Weissella	VDI5.1.W14-W23	VDI5.1.W23-W35		
X.Eubacterium.	VDI2.W-W4			
Xanthomonadaceae.	VDI5.2.W-W14			
YRC22	VDI2.0-W			

* This represents the study and time-period, e.g. VDI2.0-W is the VDI2 study and the period 0 (birth) to W (weaning)

To explore the potential of nature to improve the quality of life

Wageningen Livestock Research
P.O. Box 338
6700 AH Wageningen
The Netherlands
T +31 (0)317 48 39 53
E info.livestockresearch@wur.nl
www.wur.nl/livestock-research

Wageningen Livestock Research creates science based solutions for a sustainable and profitable livestock sector. Together with our clients, we integrate scientific knowledge and practical experience to develop livestock concepts for future generations.

Wageningen Livestock Research is part of Wageningen University & Research. Together we work on the mission: 'To explore the potential of nature to improve the quality of life'. A staff of 6,500 and 10,000 students from over 100 countries are working worldwide in the domain of healthy food and living environment for governments and the business community-at-large. The strength of Wageningen University & Research lies in its ability to join the forces of specialised research institutes and the university. It also lies in the combined efforts of the various fields of natural and social sciences. This union of expertise leads to scientific breakthroughs that can quickly be put into practice and be incorporated into education. This is the Wageningen Approach.