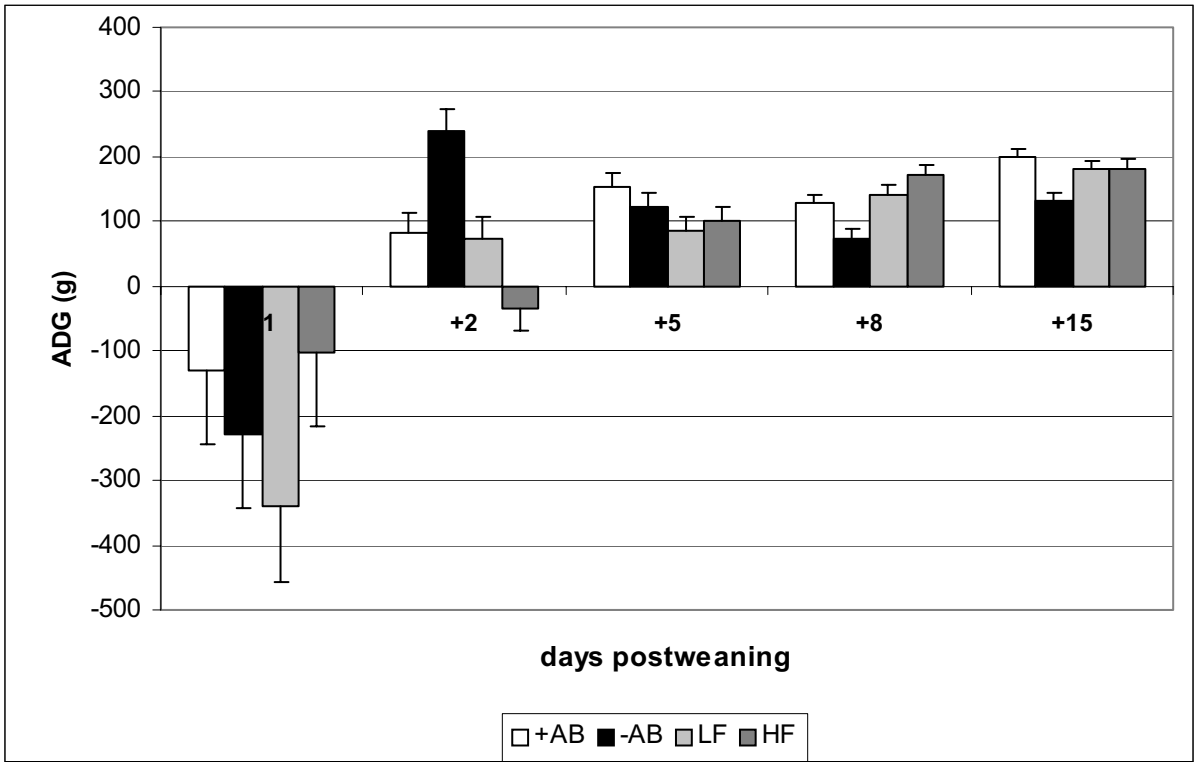
4 Results

4.1 Slaughter trial

4.1.1 Animal parameters

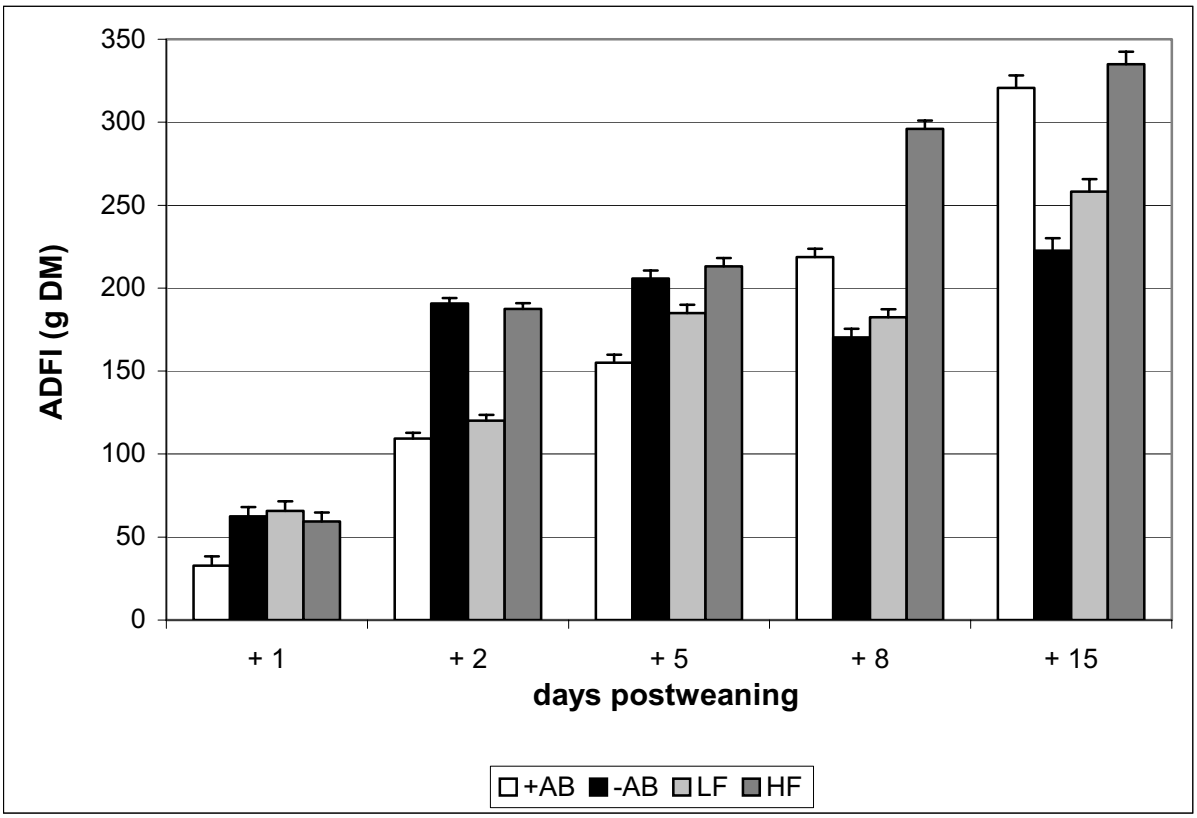
Animals were weight at birth, weaning and slaughter day. Average daily weight gain (ADG) was calculated and plotted in a graph (Figure 10), with preweaning values of 241.89 ± 8.04 g being significantly higher than postweaning values. Statistical significant differences between the starter diets were observed on days +2, +8 and +15 postweaning: At day +2 the ADG of animals fed –AB was significantly higher than for all other diets, furthermore ADG for pigs fed +AB was significantly higher than those fed HF. On day +8 we noted the contrary situation with the lowest ADG in piglets fed –AB, which proofed statistically significant for the home-produced diets. This appeared alike at +15, with significant lower ADG in animals receiving –AB compared to diets +AB and HF.

Figure 10. Average daily weight gain of piglets postweaning (LSM ± SE)



Furthermore we estimated average daily feed intake (ADFI) of the starter diets on a pen basis (Figure 11). Until day +5 we noticed the lowest feed intake for +AB, significantly lower than all other starters at +1 and at +2 and +5 lower than –AB and HF, respectively. The situation reversed at +8 and +15, for now the highest feed intake was observed for the reference diet +AB and the home-produced diet HF. Animals receiving diets –AB and LF had a significantly lower feed intake at +8 and +15 days postweaning.

Figure 11. Average daily feed intake of piglets postweaning (LSM ± SE)



4.1.2 Chemical Parameters of Ileal Digesta

4.1.2.1 pH and DM

Before weaning pH of ileal digesta was 6.68 ± 0.14 and postweaning 6.60 ± 0.07. There was no significant difference between periods before and after weaning. Comparison between starter diets postweaning revealed a significant difference between +AB and HF on day 8 (6.8 ± 0.11 vs. 6.17 ± 0.11).

DM content (%) of ileal digesta was 11.74 ± 1.02 in suckling pigs and for each starter diet as follows: 7.81 ± 0.85 in +AB, 7.77 ± 0.85 in –AB, 8.83 ± 0.85 in LF and in HF 7.85 ± 0.85. Except for the low fibre diet LF, each starter diet was significant lower than sow milk and with this a significant decrease in DM content of ileal digesta could be observed postweaning. There was no difference in DM content between starter diets.

4.1.2.2 Ammonia

Also for ammonia (mmol/L) we observed a marked difference between pre- and postweaning periods, with values being significantly higher in suckling pigs than in weaners. In ileal digesta of suckling piglets ammonia amounted to 16.95 ± 0.81 and in weaners to 7.08 ± 0.51, 8.89 ± 0.51, 6.67 ± 0.51 and 6.85 ± 0.51 for +AB, -AB, LF and HF, respectively. Additionally we've found differences between starter diets on day 1 and 5 postweaning. On day 1 diet LF was distinctly lower than diet HF (7.45 ± 0.94 vs. 13.42 ±0.94) and day 5 HF was lower than –AB (2.76 ± 0.78 vs. 9.59 ± 0.78).

4.1.2.3 Volatile Fatty Acids (VFA)

We also determined total VFA and individual fatty acids (mmol/L), as mentioned earlier. Total VFA before weaning was 10.11 ± 1.72 and in weaning piglets fed solid diets 6.81 ± 1.49, 6.89 ± 1.49, 5.01 ± 1.49 and 7.24 ± 1.49 for +AB, -AB, LF and HF, respectively. Despite a noted decrease in values after weaning we could not proof any significant difference. For individual fatty acids the situation appeared alike Table 14.

Table 14. Individual short-chain fatty acids in ileal digesta of piglets pre- and postweaning

SCFA	SM	+AB	-AB	LF	HF
Acetic acid	9.70 ± 1.68	6.53 ± 1.32	6.16 ± 1.32	4.73 ± 1.32	6.69 ± 1.32
Propionic acid	0.28 ± 0.18	0.16 ± 0.18	0.49 ± 0.18	0.05 ± 0.18	0.26 ± 0.18
Butyric acid	0.02 ± 0.09	0.13 ± 0.21	0.24 ± 0.41	0.23 ± 0.36	0.29 ± 0.58
Valeric acid	nd	nd	nd	nd	nd
i-valeric acid	nd	nd	nd	nd	nd
i-butyric acid	nd	nd	nd	nd	nd

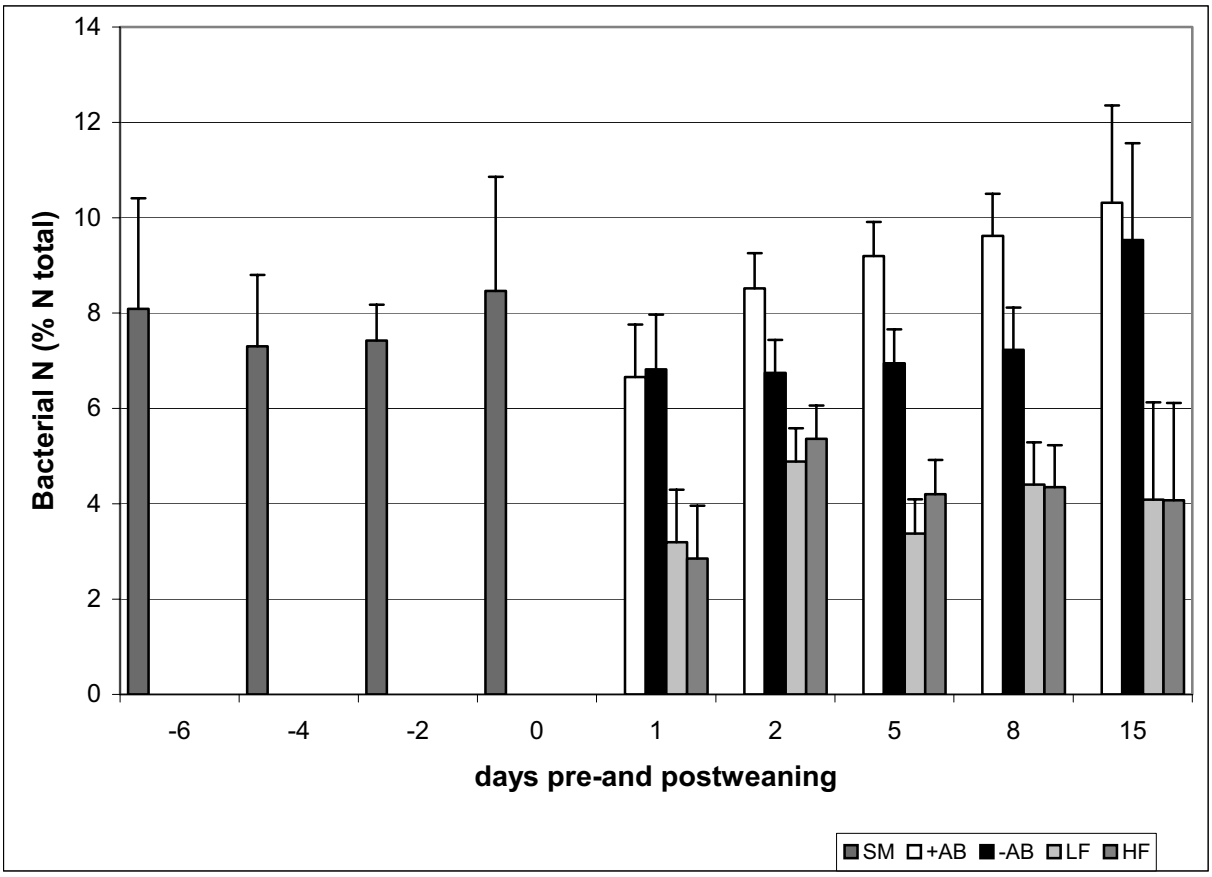
4.1.2.4 Lactic Acid (LA)

Lactic acid, another microbial metabolite in addition to VFA, showed a tendency to increase after weaning, but values did not reach statistical significance. In suckling piglets we determined 18.80 ± 2.55 mmol/L and in weaners 23.60 ± 2.44.

4.1.2.5 Bacterial Nitrogen

D-alanine, serving as a marker for microbial nitrogen, enabled us to calculate the bacterial nitrogen in ileal digesta of piglets before and after weaning (Figure 12). We observed almost equal bacterial nitrogen content (% of total N) in suckling pigs and those fed with reference diets +AB and –AB (7.82 ± 0.73 vs. 8.86 ± 0.52, 7.48 ± 0.52), but significantly lower values in weaners fed home-produced diets LF and HF (3.99 ± 0.52 and 4.17 ± 0.52). Comparing the individual slaughter days postweaning, we could see constant higher values in animals fed the reference diets. At day +5 and +8 the higher bacterial nitrogen in +AB reached statistical significance in respect to the home-produced diets LF and HF. Furthermore the bacterial N increases in time for the reference diets, although the differences between days postweaning did not reach statistical significance. In the home-produced diets we did not observe such an increase, values remained almost constant in time.

Figure 12. Bacterial nitrogen content in ileal digesta of piglets pre- and postweaning (% of total N)



4.1.2.6 Amines

The biogenic amines putrescine, histamine, cadaverine, spermine and spermidine were also measured and values are given in Table 15. Statistical analysis revealed, that spermine and histamine were significantly higher in suckling piglets compared to animals fed the starter diets, whereas preweaning spermidine values were only significantly higher than starters –AB and LF. Although there was a marked difference for putrescin values between pre- and postweaning periods, we couldn't proof statistical significance, likely due to the high standard deviation.

Table 15. Biogenic amines in ileal digesta pre-and postweaning (mg/kg ADM), over all days

Amines	SM	+AB	-AB	LF	HF
Putrescine	90.96 ± 19.21	45.84 ± 12.27	41.31 ± 11.93	48.00 ± 11.93	65.93 ± 11.93
Histamine	221.05 ± 50.13 [a]	60.29 ± 3.78 [b]	45.06 ± 7.20 [b]	75.12 ± 7.20 [b]	64.58 ± 7.89 [b]
Cadaverine	48.10 ± 22.24	29.38 ± 14.65	31.65 ± 14.05	44.03 ± 14.05	69.36 ± 14.05
Spermidine	44.38 ± 7.26 [a]	27.92 ± 6.11 [ab]	14.71 ± 5.92 [b]	14.44 ± 6.28 [b]	22.39 ± 5.74 [ab]
Spermine	97.66 ± 15.46 [a]	35.47 ± 6.27 [b]	32.65 ± 6.00 [b]	42.35 ± 6.00 [b]	40.61 ± 6.00 [b]

Mean values with unlike superscripts in one line are significantly different (Tukey-test, P<0.05)

4.1.3 Classical Microbiology

We cultivated four microbial groups to investigate the microbial flora in ileal content, *Enterobacteriaceae*, *Enterococcus spp.*, *Lactobacillus spp.* and yeast. Colony-forming units were counted, given as log cfu/g and statistically processed, comparing the obtained data in regard to diets. We observed differences in *Lactobacillus spp.* and yeast counts before and after weaning. *Lactobacillus spp.* decreased in weaned piglets and yeast increased (Table 16).

Table 16. Microbial counts in ileal digesta pre-and postweaning (log cfu/g)

Microorganism	SM	+AB	-AB	LF	HF
Enterobacteriaceae	7.21 ± 0.29	7.30 ± 0.24	7.36 ± 0.24	7.68 ± 0.24	7.96 ± 0.24
Enterococcus spp.	5.43 ± 0.71	3.56 ± 0.44	5.90 ± 0.44	5.30 ± 0.44	5.18 ± 0.44
Lactobacillus spp.	8.81 ± 0.22 [a]	7.89 ± 0.22 [b]	8.26 ± 0.22 [ab]	8.30 ± 0.22 [ab]	7.93 ± 0.22 [b]
Yeast	1.49 ± 0.48 [a]	2.43 ± 0.36 [ab]	3.59 ± 0.36 [b]	2.76 ± 0.40 [ab]	2.55 ± 0.36 [ab]

Mean values with unlike superscripts in one line are significantly different (Tukey-test, P<0.05)

Additionally we observed significant differences when individual slaughter days were compared with respect to diet. At day 5 postweaning *Enterococcus spp.* counts of piglets fed +AB were significantly lower than of animals fed –AB (2.52 ± 0.94 vs. 8.38 ± 0.94 log cfu/g). At the same day yeast counts differed as well between diets, with –AB and LF being significantly higher than +AB and HF (4.13 ± 0.39, 2.83 ± 0.39 vs. 0.50 ± 0.39, 0.60 ± 0.39 log cfu/g). To obtain a deeper insight into the relations between the cultivated microorganisms, the Pearson Correlation Coefficient (Table 17) was calculated. We did

not observe a significant correlation between the microbial groups except between *Lactobacillus spp.* and yeast. For this we determined a significant negative correlation.

Table 17. Pearson Correlation Coefficient r, calculated between cultivated microorganisms of ileal digesta

log cfu / g	*Entero-bacteriaceae*	*Entero-coccus spp.*	*Lactobacillus spp.*	Yeast	Parameter
Enterobacteriaceae	1.00	0.18 0.07	0.05 0.66	-0.05 0.66	r p
Enterococcus spp.	0.18 0.07	1.00	- 0.07 0.52	0.18 0.08	r p
Lactobacillus spp.	0.05 0.64	- 0.07 0.52	1.00	- 0.24 0.02	r p
Yeast	- 0.05 0.66	0.18 0.08	- 0.24 0.02	1.00	r p

Another Pearson correlation was calculated between the microbiota and biogenic amines. Here we noticed a positive correlation between *Lactobacillus spp.* and the biogenic amines, which we were unable to detect for *Enterobacteriaceae, Enterococcus spp.* and yeast. Pearson Correlation Coefficients are displayed in Table 18.

Table 18. Pearson Correlation Coefficients r between amines (mg/kg ADM) and cultivated microorganisms (log cfu/g)

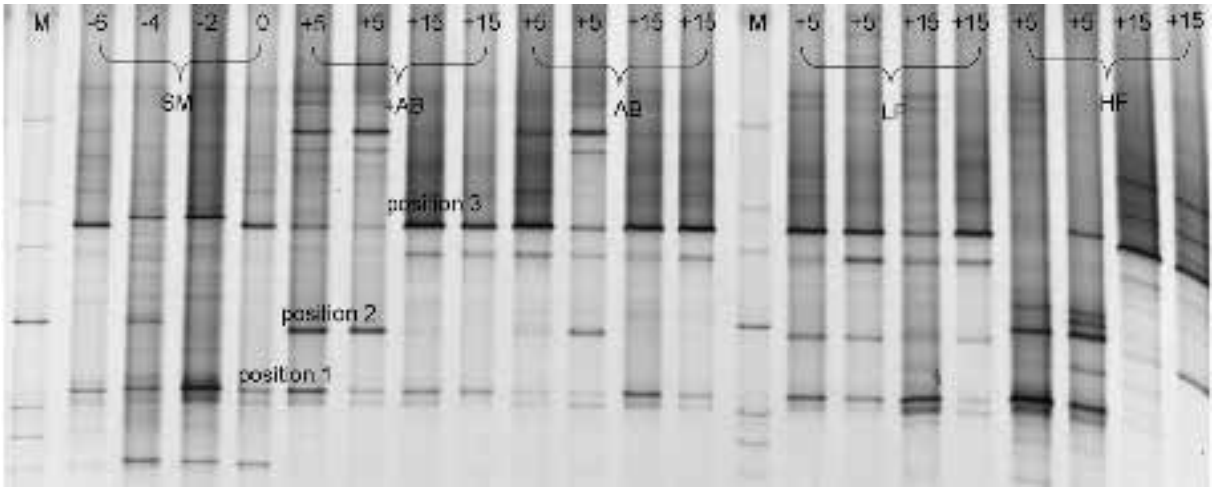
	Putres-cine	Hist-amine	Cada-verine	Spermi-dine	Spermine	Para-meter
Yeast	- 0.13 0.23	- 0.19 0.07	- 0.06 0.58	- 0.22 0.04	- 0.17 0.11	r p
Enterococcus spp.	0.08 0.46	- 0.11 0.30	-0.08 0.43	0.04 0.70	0.22 0.04	r p
Enterobacteriaceae	0.19 0.07	- 0.03 0.79	0.12 0.27	0.10 0.33	0.05 0.65	r p
Lactobacillus spp.	0.41 < .0001	0.36 0.0005	0.35 0.0006	0.23 0.03	0.28 0.007	r p

4.1.4 Molecular Microbiology

4.1.4.1 Denaturing Gradient Gel Electrophoresis (DGGE)

To perform DGGE a range of samples was chosen from the entire sample set. To cover the preweaning period samples from day –6, -4, -2 and 0 were taken and postweaning days 5 and 15 for each starter diet. Figure 13 displays the DGGE profile, i.e. the "genetic fingerprint" of the ileal microbial flora of piglets around weaning. The entire profile appears simple with just three dominant bands: a lower band (position 1) in each sample, a middle band (position 2) in the postweaning samples only and an upper band (position 3) in the major part of all samples. Samples before and after weaning differed in the appearance of a middle band postweaning, but apart from this the entire sample set showed a high similarity. For cluster analysis the Dice-coefficient of similarity was calculated and a dendogramm generated.

Figure 13. Universal DGGE profile of ileal digesta in piglets pre-and postweaning, selected timepoints



Furthermore a universal DGGE profile for all time-points taken postweaning was prepared and its dendogram was generated using the Bionumerics software (Figures 14, 15). We observed a clustering rather to age than dietary treatment, with days 5, 8 and 15 postweaning (+AB, -AB, LF) clustering closest together (similarity > 74%). To assess the diversity of the microbiota the Shannon-index of general diversity was calculated and plotted in a graph (Figure 16). It can be seen from the graph that there was no marked difference in diversity at day 1 postweaning, but already at day 2 a considerable difference between the dietary treatments was observed. Animals fed with the antibiotic-

supplemented diet showed a lower diversity in their microbiota, whereas in those fed the other diets we noticed even an increase in bacterial diversity. Nevertheless at 15 days after weaning the diversity of the four starters appears to equal each other and the Shannon-index is 0.76 ± 0.46, 0.76 ± 0.39, 0.90 ± 0.29 and 0.73 ± 0.33 for +AB, -AB, LF and HF, respectively.

Figure 14. Universal DGGE profile of ileal digesta in piglets, all timepoints postweaning

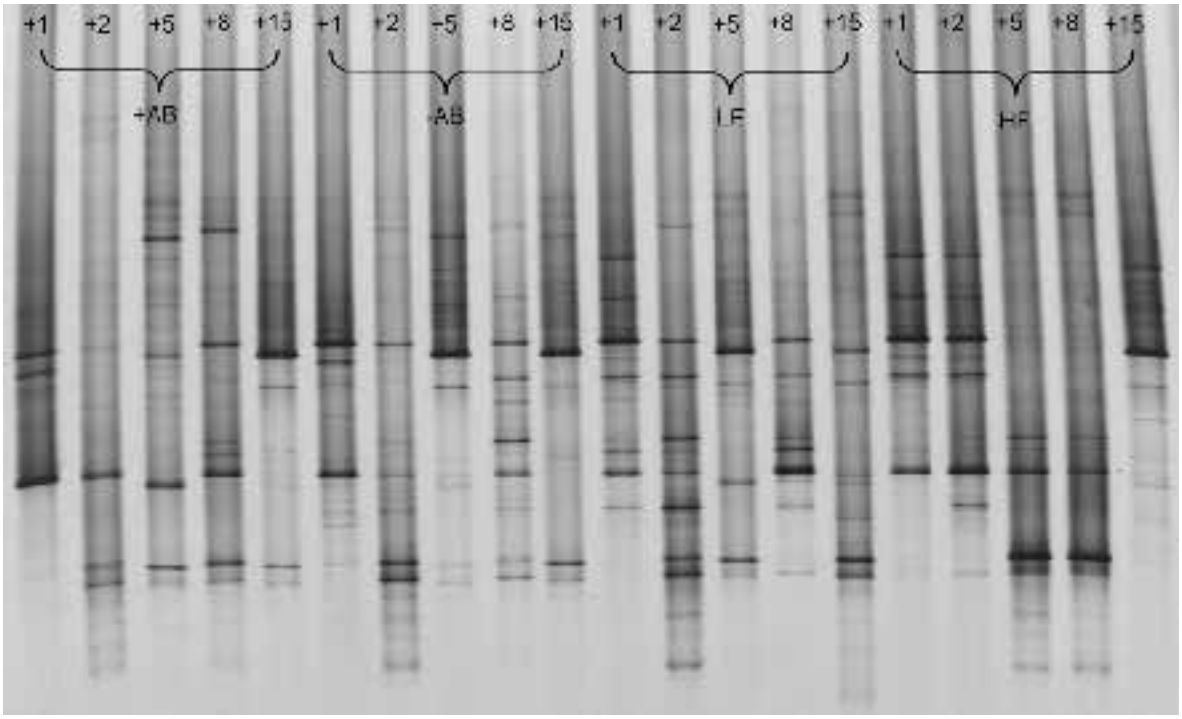


Figure 15. Dendogramm of universal DGGE profile of ileal digesta in piglets postweaning, all timepoints

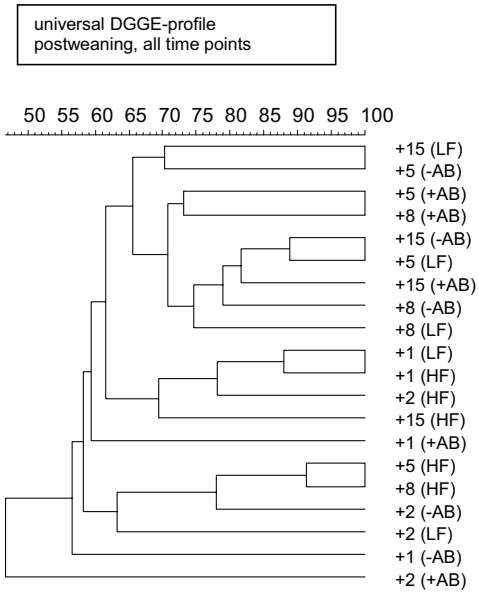
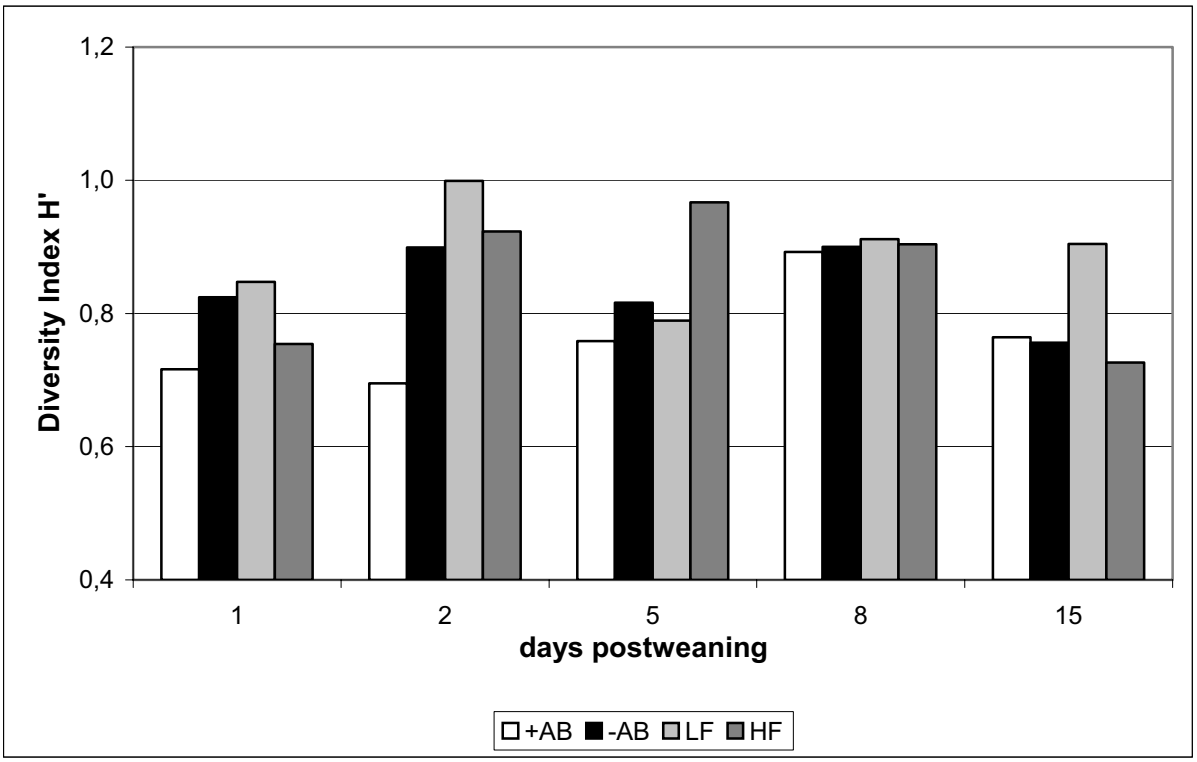
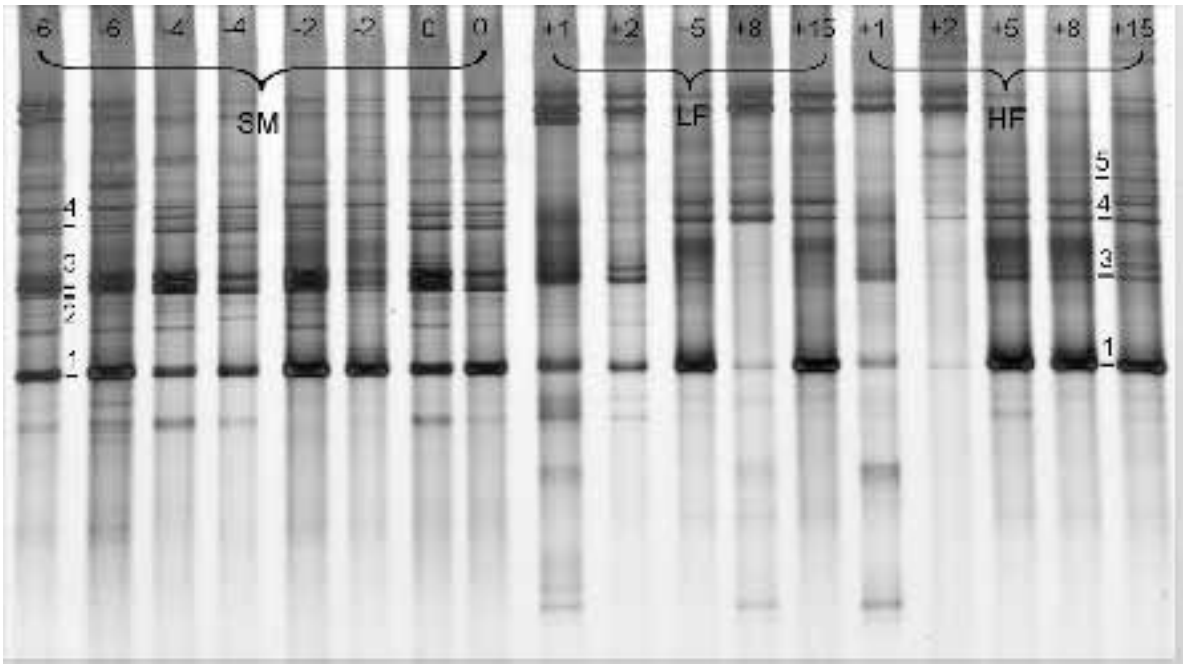


Figure 16. Shannon-index of general diversity, all timepoints postweaning



To achieve a better insight into the *Lactobacillus*-group, DGGE profiles specific for *Lactobacillus* were prepared. Samples of all time points per dietary regime were incorporated into the gels. Loading a sample selection and *Lactobacillus spp.*-clones of defined origin on a DGGE gel we were able to compare the clones with bands appearing in the samples. With this we could identify a few *Lactobacillus spp.* in the profile (Figure 17, 18).

Figure 17. Lactobacillus-specific DGGE profile of porcine ileal digesta pre-and postweaning



1 – L. amylovorus (type strain DSMZ20531)

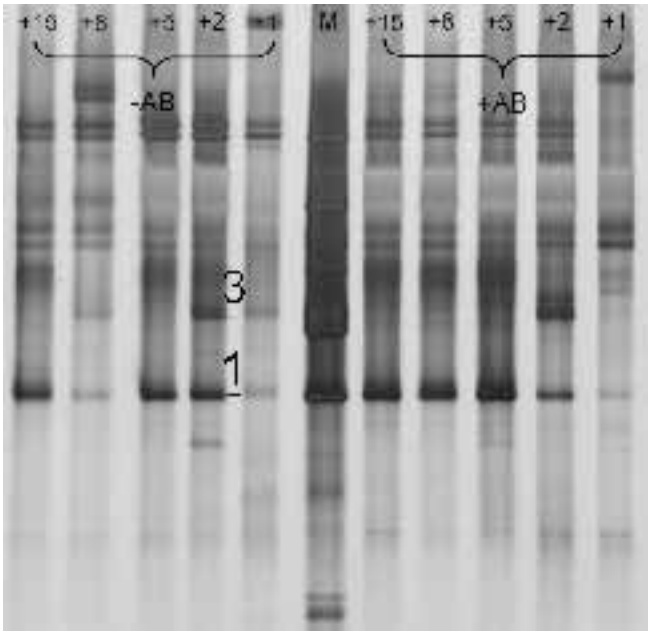
2 – L. vaginalis

3 – L. panis

4 – L. acidophilus

5 – L. delbrueckii

Figure 18. Lactobacillus-specific DGGE profile of porcine ileal digesta postweaning



1 – L. amylovorus (type strain DSMZ20531)

3 – L. panis

Both profiles were analysed with the Bionumerics software and a combined dendogram was generated (Figure 19). Number of bands and the Shannon-index of general diversity were calculated (Figure 20, Figure 21). There are two main clusters visible in the dendogram: one including all samples from pigs fed the reference diets +AB and –AB (similarity > 62%) and one including the home-produced diets LF, HF and the preweaning period SM (similarity > 57%). In between the latter samples of the suckling animals are clustered closer together (similarity > 76%) than for the diets LF and HF (similarity > 71 %), with the exception of +1d.

The highest *Lactobacillus spp.* diversity we observed in suckling piglets, which decreased markedly 1 day after weaning. Subsequently an increase in diversity was noticed until day 15 postweaning, although values did not reach their preweaning counterparts. 15 days after weaning a clear distinction was established between reference diets and home-produced diets. Animals that received the home-produced diets showed a higher diversity of *Lactobacillus spp.* compared to those fed with both the reference diets. Further repetition of this approach is needed to reach statistically significance.

Figure 19. Dendogramm of combined Lactobacillus-specific DGGE profiles of ileal digesta in piglets pre-and postweaning, all timepoints

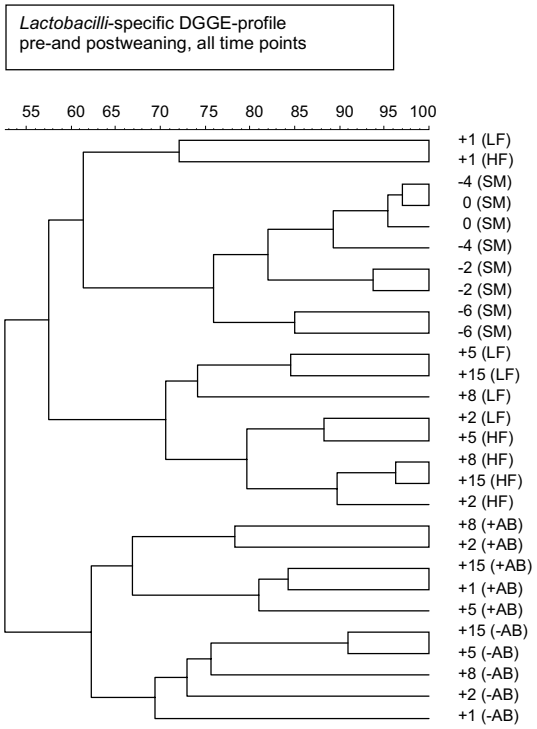


Figure 20. Number of bands of combined Lactobacillus-specific DGGE profiles of ileal digesta in piglets pre-and postweaning, all timepoints

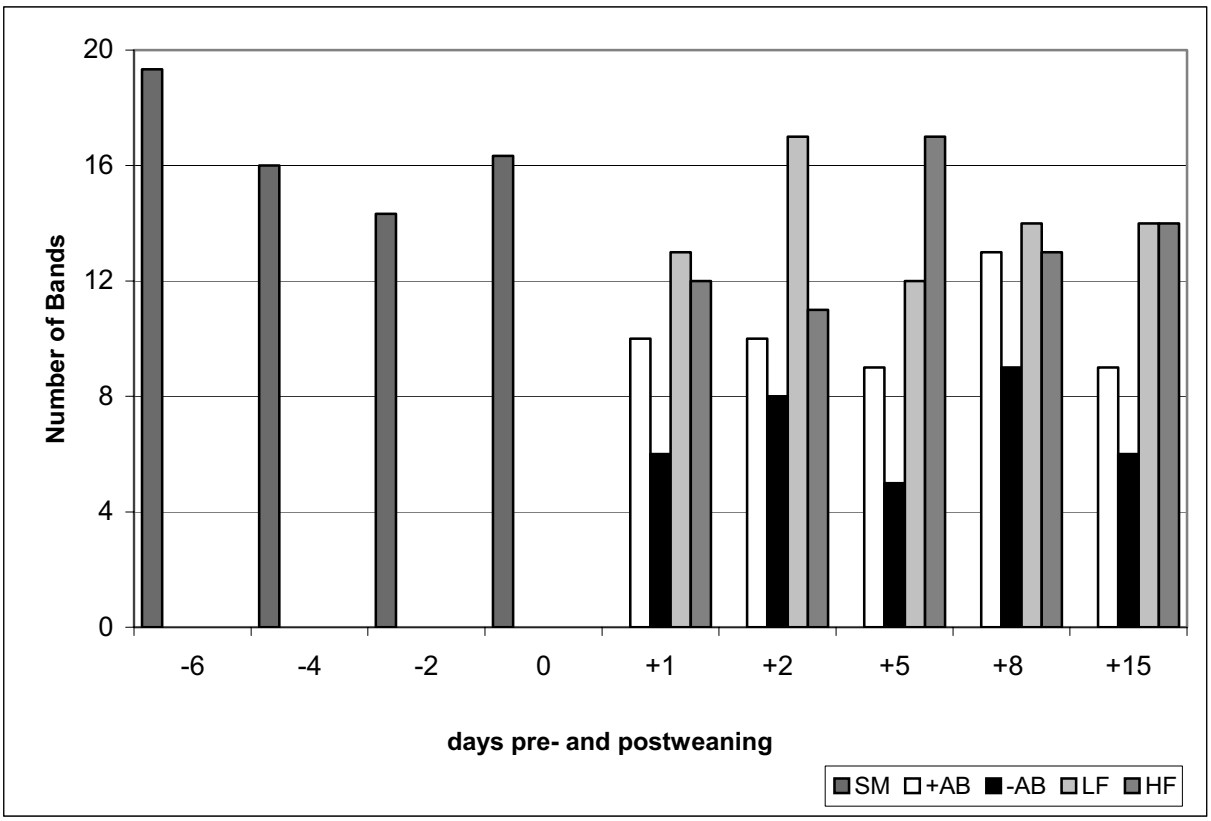
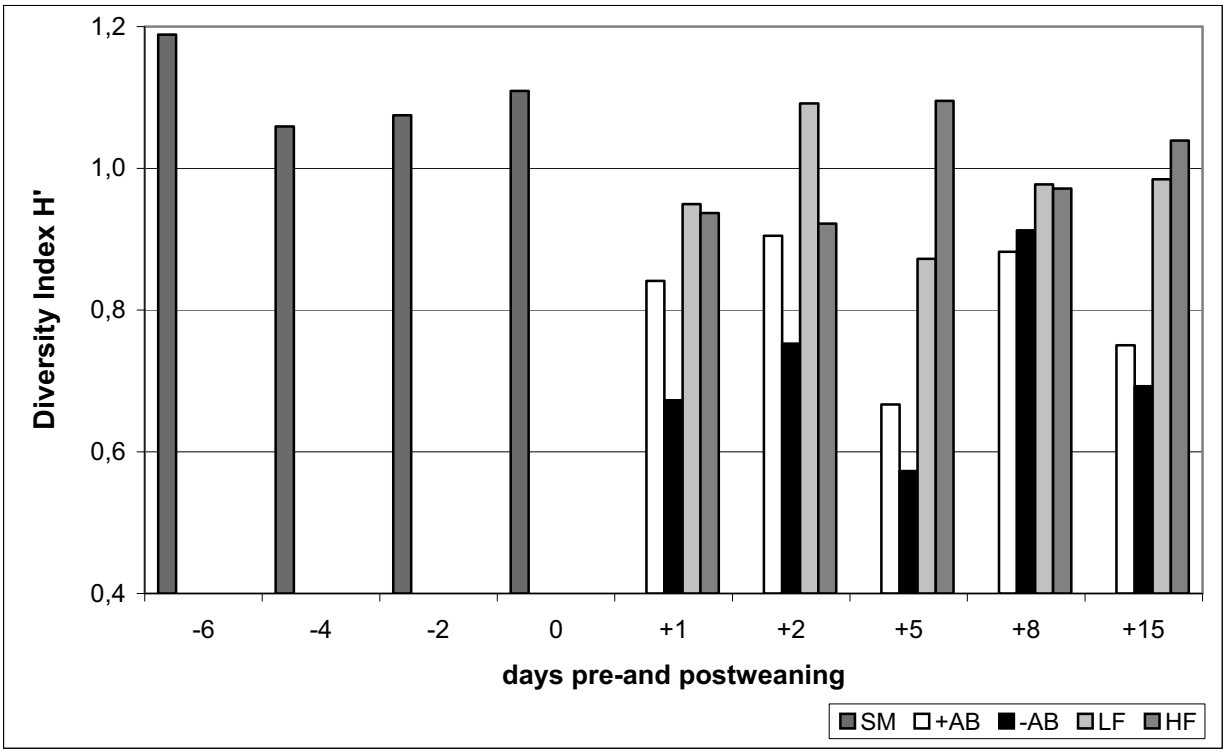


Figure 21. Shannon-index of combined Lactobacillus-specific DGGE profiles of ileal digesta in piglets pre-and postweaning, all timepoints



4.1.4.2 Sequence analysis

To identify the three dominant band in the positions mentioned above a sequence analysis was performed. Obtained sequences were compared to sequences available in public databases by using BLAST analysis of sequences from the Ribosomal Database Project. By means of this we were able to identify bands in position 1 and 2: position 1 represented uncultured bacterium clones p-3301-23G2, p-37-a5 and p-3443-SwA2 belonging to the cluster of *L.amylovorus* (Leser, 2002) and position 2 the uncultured clone S4D belonging to *E.coli* (Favier, 2002). Unfortunately our attempts to identify the upper band (position 3) failed up to now.

4.1.4.3 Fluorescence *in situ* Hybridisation (FISH)

For FISH we applied various oligonucleotide probes: for *Eubacteria* EUB-0338, for *E.coli* EC 1531 and for *Lactobacillus spp.* LAB-0158 and LAB-0722. Representative samples - preweaning -6 days, postweaning +5 days of reference diet +AB and +5 and +15 days of home-produced diet HF - were chosen from the whole set to quantify these bacteria. The selected probes have worked properly, except for LAB-0158. Despite identically accomplished hybridisation procedures for both *Lactobacillus spp.* probes – samples and probes were applied on the same slide – only with LAB-0722 we were able to detect *Lactobacillus spp.* under the epifluorescence microscope. As the two probes are labelled with different dyes one can apply both on the same sample and view under the microscope with different filters. Cells labelled with LAB-0722 appears orange and with LAB-0158 green (Figure 22). Subsequently we used LAB-0722 only for the quantification of *Lactobacillus spp. spp.* (Figure 23). We observed a definite decline of general bacterial counts from 1.0×10^{11} bacteria/g six days preweaning to 1.8×10^{9} bacteria/g and 1.9×10^{9} bacteria/g five and fifteen days postweaning, respectively (Figure 24). The number of *Lactobacillus spp.* was estimated as 97 % and 77 % of general bacteria at six days preweaning and five days postweaning, respectively. We couldn't detect any *E.coli* before weaning (Figure 25), but already 1.5×10^{9} bacteria/g at five days and 9.4×10^{8} bacteria/g at fifteen days postweaning, respectively (Figure 26).

Figure 22. Lactobacilli in ileal digesta of unweaned pig (-6d), labelled with LAB-0722 and LAB-0158

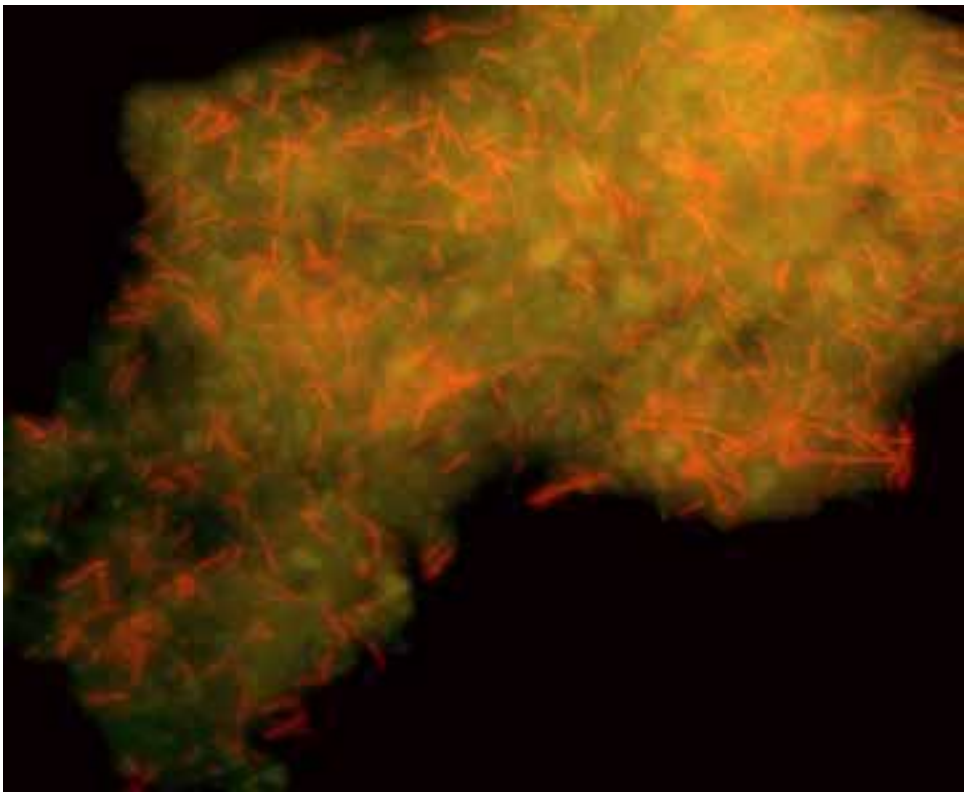


Figure 23. Lactobacilli in ileal digesta of unweaned pigs (-6d), labelled with LAB-0722

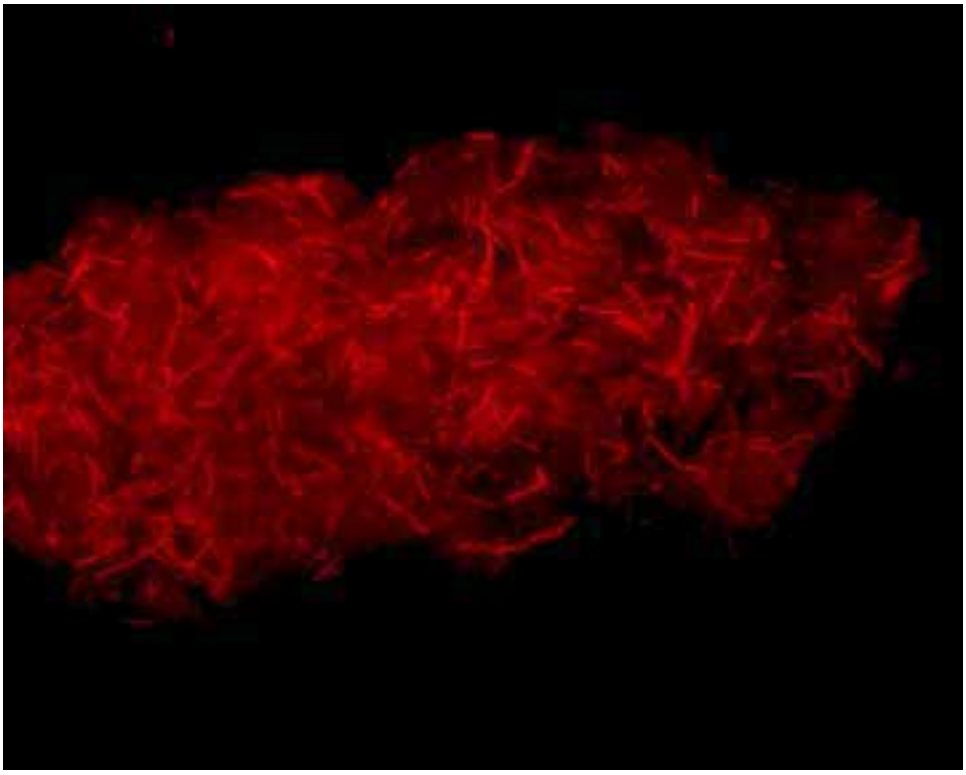


Figure 24. Lactobacilli in ileal digesta of weaned pigs (+5d), labelled with LAB-0722

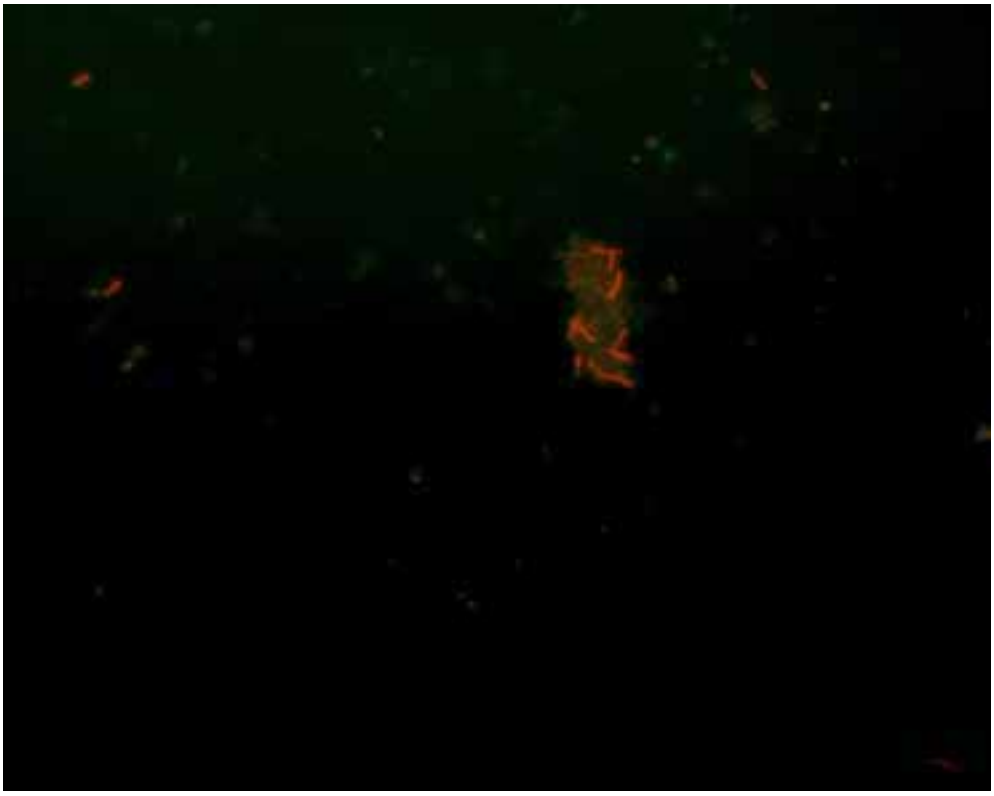


Figure 25. Enterobacteriaceae in ileal digesta of unweaned pigs (-6d), labelled with EC-1531

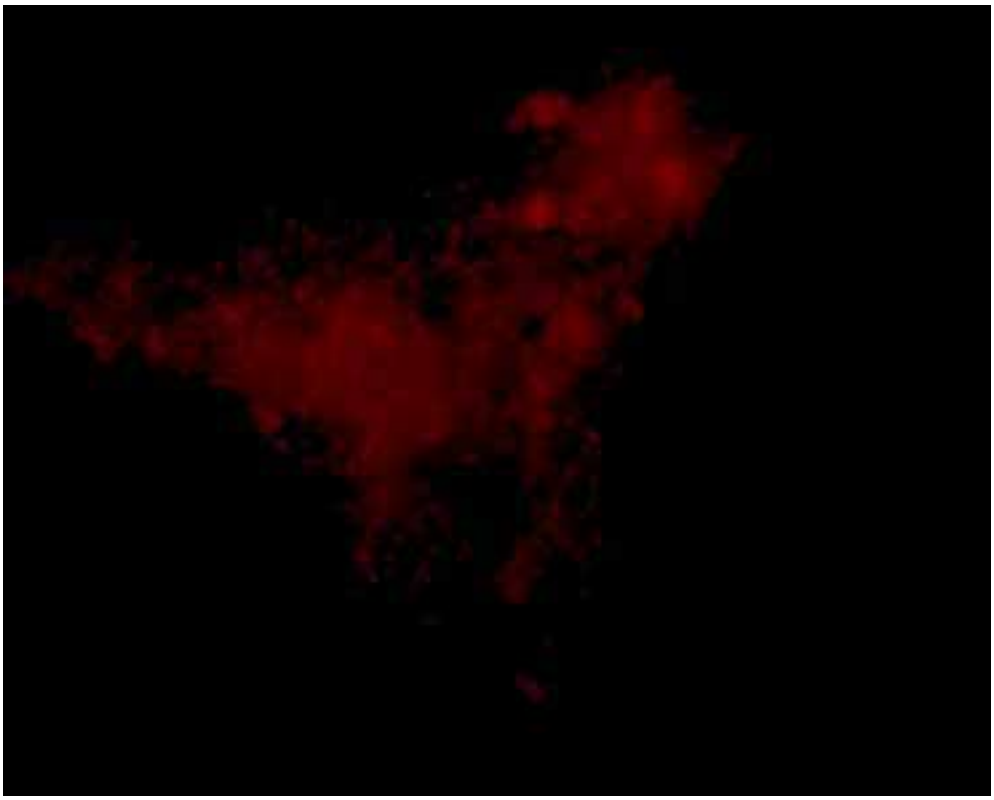


Figure 26. Enterobacteriaceae in ileal digesta of weaned piglets (+5d), labelled with EC 1531