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Development of piglet gut microbiota at the time of weaning influences development of postweaning diarrhea – A field study

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ABSTRACT

Postweaning diarrhea is a common issue in pig production which is currently controlled by feed supplementation with zinc oxide. However, new alternatives are being sought due to an expected ban on zinc oxide in feed supplementation from 2022 in the EU. One possible alternative is to use novel types of probiotics consisting of microbiota characteristic for healthy weaned piglets. In this study, we therefore collected rectal swabs of piglets 3 days before weaning and 4 days after weaning in a commercial farm considering all risks of field trial like the use of antibiotics, classified the piglets as predisposed, healthy or sick and using 16S rRNA sequencing, we determined and compared the microbiota composition. Increased Actinobacteria before weaning was a marker of piglets predisposed for diarrhea. Increased *Chlamydia* or *Helicobacter* before weaning was surprisingly a marker of healthy and resistant piglets after weaning. After weaning, unclassified Clostridiales, Deltaproteobacteria, Selenomonadales, *Fusobacterium*, *Akkermansia* or *Anaerovibrio* increased in microbiota of piglets with post-weaning diarrhea while an increase in *Prevotella* and *Faecalibacterium* was characteristic for healthy, weaned piglets. Both changes in individual microbiota members and also correct timing of microbiota reshaping around weaning and the increase of mainly *Prevotella* species just after weaning are equally important for resistance to postweaning diarrhea in piglets under field conditions.

1. Introduction

Similar to other animal species, pig gut microbiota is subjected to age-dependent development with birth and weaning being the most critical periods of life. Colonisation of the pig intestinal tract begins with birth and selection of appropriate microbiota members in newborn piglets is highly determined by feed composition, i.e. by milk diet. The first coloniser is ubiquitously present *E. coli* but soon after both Gram-positive Firmicutes and Actinobacteria as well as Gram-negative representatives of phyla Fusobacteria and Bacteroidetes appear (Frese et al., 2015; Kubasova et al., 2017; Slifierz et al., 2015). Despite intimate contact of piglets with its sow, the microbiota of sows and piglets differ considerably due to the different diet of sows and nursed piglets under lactation (Kubasova et al., 2017; Motta et al., 2019). One of the striking differences is the dominance of *Prevotellaceae* over *Bacteroidaceae* in the microbiota of adult pigs, including sows, and the dominance of *Bacteroidaceae* over *Prevotellaceae* in the microbiota of nursed piglets (Kubasova et al., 2018; Motta et al., 2019). Soon after weaning, piglets reshape their gut microbiota as they respond to the change in diet and one week after weaning, piglet microbiota is nearly of the same composition as in adult pigs (Gerzova et al., 2015; Kubasova et al., 2018).

Weaning is sometimes associated with the development of diarrhea. Postweaning diarrhea is a multifactorial disease in piglets which is affected by change in diet, piglet genetics or the presence of particular pathogens, such as enterotoxigenic *E. coli* (Alexa et al., 1997; Cox et al., 1991; Li et al., 2019; Massacci et al., 2020; Sun and Kim, 2017). Despite this, it develops only in some of weaned piglets. Since faecal microbiota transplantation prevents the development of post-weaning diarrhea (Hu et al., 2018), we speculated that gut microbiota might develop differently around weaning in resistant and susceptible piglets. Aim of this study therefore was to determine microbiota members positively or negatively associated with postweaning diarrhea. At a commercial farm, we therefore determined the composition of rectal microbiota in piglets

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3 days before and 4 days after weaning. When sampling after weaning, piglets were diagnosed as with or without diarrhea and samples collected before and after weaning from the same piglet were matched. This allowed to determine microbiota composition before and after weaning in resistant and predisposed piglets. Consequently, individual microbiota members as well as general trends of microbiota composition characteristic for sensitive and resistant piglets could be defined.

2. Materials and methods

2.1. Animals

Piglets of DanBred line were born on August 31 to September 3, 2019 and irrespective of the day of birth, thereafter all the piglets were collectively treated always at a single time point. Any antibiotic treatment, change in diet or weaning was therefore performed at a single time point which means that the age of piglets at the time of the treatment varied ±4 days. If a single piglet age is mentioned in this paper, it represents an average age and is used only to simplify the description. Teeth grinding and castration was performed in the piglets on day 3 of life, if appropriate.

2.2. Rearing conditions

Since the samples were collected at a commercial farm, the practice adopted at this farm had to be accepted. All the piglets were subcutaneously treated with a single dose of 0.1 ml ceftiofur at 100 mg/ml concentration and with a single dose of 1 ml toltrazuril at 50 mg/ml concentration on day 3 of life because of therapeutic reasons. From day 5 of life, the piglets were allowed access to creep feed consisting of wheat, barley, soybean meal, fish meal, dried acid whey and soybean vegetable fat. Creep feed consisted of 89.7% of dry matter, of which crude protein formed 19.60%, crude fibre 25.8%, crude fat 6.44%, calcium 0.65%, total phosphorus 0.52% and sodium 0.25%. The feed was supplemented with ZnO at 2500 mg/kg concentration. Piglets were weaned at 30 days of age and from the time of weaning, i.e. for the last 4 days of this study, the feed was supplemented with sulfamethoxazole and trimethoprim at 120 mg/kg and 600 mg/kg of feed, respectively. In both cases, the antibiotics were used for therapeutic purposes. Animal house microclimate was automatically controlled for temperature and humidity as recommended for particular age category. To increase temperature comfort, all the pens were equipped with heated floors.

2.3. Sample collection

Three days before weaning, rectal swabs were collected from more than 100 piglets. Increased number of piglets had to be sampled before weaning since we did not know which piglet would remain healthy (majority of piglets sampled before weaning) and which would develop postweaning diarrhea. Four days after weaning, i.e. one week after the preweaning sample collection, the number of collected samples was reduced to 17 rectal swabs from piglets with postweaning diarrhea and an additional 17 rectal swabs from healthy piglets. Piglet classification as healthy or diarrheic was done based on faeces composition. Piglets with solid faeces were classified as healthy and piglets with loose faeces were classified as diarrheic. All piglets were ear tagged and the same tag was used to identify samples collected before and after weaning. Next, samples collected after weaning were paired with the samples collected before weaning so that 68 individual samples were analysed for microbiota composition in total. This approach also allowed to select animals with extreme phenotype, i.e. clearly sick with loose faeces and clearly healthy with solid stools.

Rectal swabs were obtained by inserting a sterile cotton swab approx. 15 mm into the rectum and rotating the swab. Swabs were withdrawn and placed in sterile plastic bags marked with the same number as piglet tag. Rectal swabs were frozen at −20 °C within 20 min after sampling and DNA was purified from frozen rectal samples within 3 months. Collection of rectal swabs is a procedure which according to Act 166/1999 on veterinary care does not require approval of ethic committee in the Czech Republic.

2.4. Microbiota characterisation by sequencing of V3/V4 region of 16S rRNA genes

Rectal swab samples were homogenised in a MagNALyzer (Roche). Following homogenisation, the DNA was extracted using a QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen). The DNA concentration was determined spectrophotometrically and DNA samples diluted to 5 ng/ml were used as a template in PCR with forward primer 5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-MID-GT-CCTACGGGNGGCWGCAG-3′ and reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-MID-GT GAC-TACHVGGGTATCTAATCC-3′. MIDs represent different sequences 5, 6, 7, or 9 nucleotides in length which were used to identify individual samples after the whole sequencing run. PCR amplification was performed using a KAPA HiFi Hot Start Ready Mix kit (Kapa Biosystems) and the resulting PCR products were purified using Agencourt AMPure beads (Beckman Coulter). In the next step, the concentration of PCR products was determined spectrophotometrically, the DNA was diluted to 100 ng/μl and groups of 14 PCR products with different MID sequences were indexed with the same index from Nextera XT Index Kit following the manufacturer's instructions (Illumina). The next set of 14 PCR products with different MID sequences were indexed with the next index from Nextera XT Index kit etc. thus allowing us to increase the number of samples analysed in a single sequencing run. Prior to sequencing, the concentration of differently indexed samples was determined using a KAPA Library Quantification Complete kit (Kapa Biosystems). All indexed samples were diluted to 4 ng/μl and 20 pM phiX DNA was added to 5% final concentration (*v*/v). Sequencing was performed using MiSeq Reagent Kit v3 (600 cycle) and MiSeq apparatus according to the manufacturer's instructions (Illumina). Quality trimming of the raw reads was performed using TrimmomaticPE v0.32 with sliding window 4 bp and quality read score equal or higher than 20 (Bolger et al., 2014). Minimal read length must have been at least 150 bp. The fastq files generated after quality trimming were uploaded into QIIME software (Caporaso et al., 2010). Forward and reverse sequences were joined and chimeric sequences were predicted and excluded by the slayer algorithm. The resulting sequences were then classified by RDP Seqmatch with an OTU (operational taxonomic units) discrimination level set to 97%. Principal coordinate analysis (PCoA) implemented in QIIME was used for data visualisation. Multiple alignment was performed using Clustal Omega at https://www.ebi.ac.uk/Tools/msa/clustalo/ with default settings. Obtained phylogenetic tree file was finally edited in iTol (https://itol.embl.de/).

2.5. Statistics

Depending on taxonomic level, only phyla forming more than 3%, classes forming more than 1%, orders forming more than 0.33%, families forming more than 0.1%, genera forming more than 0.033% and OTUs (operational taxonomic units) forming more than 0.01% of total microbiota in at least one group of piglets were considered. Kruskal-Wallis test was used to identify differently abundant taxa and differences with $p < 0.05$ were considered as significant.

3. Results

3.1. Basic sequencing characteristics

All 68 samples were characterised by 3,874,446 reads in total, which corresponds to an average sample coverage of 56,977 reads. The sample with the highest coverage was characterised with 99,888 reads and the

sample with the lowest coverage was characterised with 29,480 reads.

3.2. Gross differences in microbiota composition in healthy, predisposed and sick piglets

Using PCoA clustering, samples collected from piglets 3 days before weaning formed a separate cluster from the samples collected from the same piglets 4 days after weaning indicating rapid microbiota remodelling in the week around weaning. However, PCoA analysis did not indicate extensive differences in the microbiota of healthy or predisposed piglets before weaning, or sick or healthy piglets after weaning (Fig. 1A).

3.3. Identification of differently abundant taxa in healthy, predisposed and sick piglets

Despite the absence of gross differences in microbiota composition in healthy, predisposed and sick piglets, we performed additional analyses to identify taxa which could be differently abundant without affecting the PCoA (Fig. 1B and C). First, we compared how many taxa at various taxonomic levels were differently abundant in healthy and sick piglets before and after weaning. Not a single phylum which formed more than 3% of total microbiota was differently abundant in predisposed or healthy piglets before weaning, or healthy and sick piglets after weaning. At the class level, four classes (Synergistia, Erysipelotrichia, Deltaproteobacteria and Negativicutes) were significantly more abundant in sick piglets after weaning compared to healthy weaned piglets. Performing the same comparison at the remaining taxonomic levels showed that a higher number of differentially abundant taxa was always recorded in piglets after weaning compared to before weaning and more taxa were significantly more abundant in sick than healthy piglets after weaning (Table S1 for full list). At OTU level, there were 91 differently abundant taxa between healthy and predisposed piglets before weaning and 130 differently abundant OTUs between healthy and sick piglets after weaning (Fig. 2).

Each of significantly different taxa in the Table S1 may contribute to the development of postweaning diarrhea. However, of these we highlight the following results. An increased abundance of opportunistic pathogens in gut microbiota before weaning resulted in a decrease in postweaning diarrhea. Indeed, a significantly increased abundance of genera *Chlamydia* or *Helicobacter* were characteristic for healthy piglets before weaning, which remained healthy also after weaning (Table S2). On the other hand, an increased abundance of Actinobacteria three days before weaning was a marker of predisposed piglets which developed post weaning diarrhea (Fig. 3).

Fusobacterium or *Akkermansia* exhibited increased abundance in

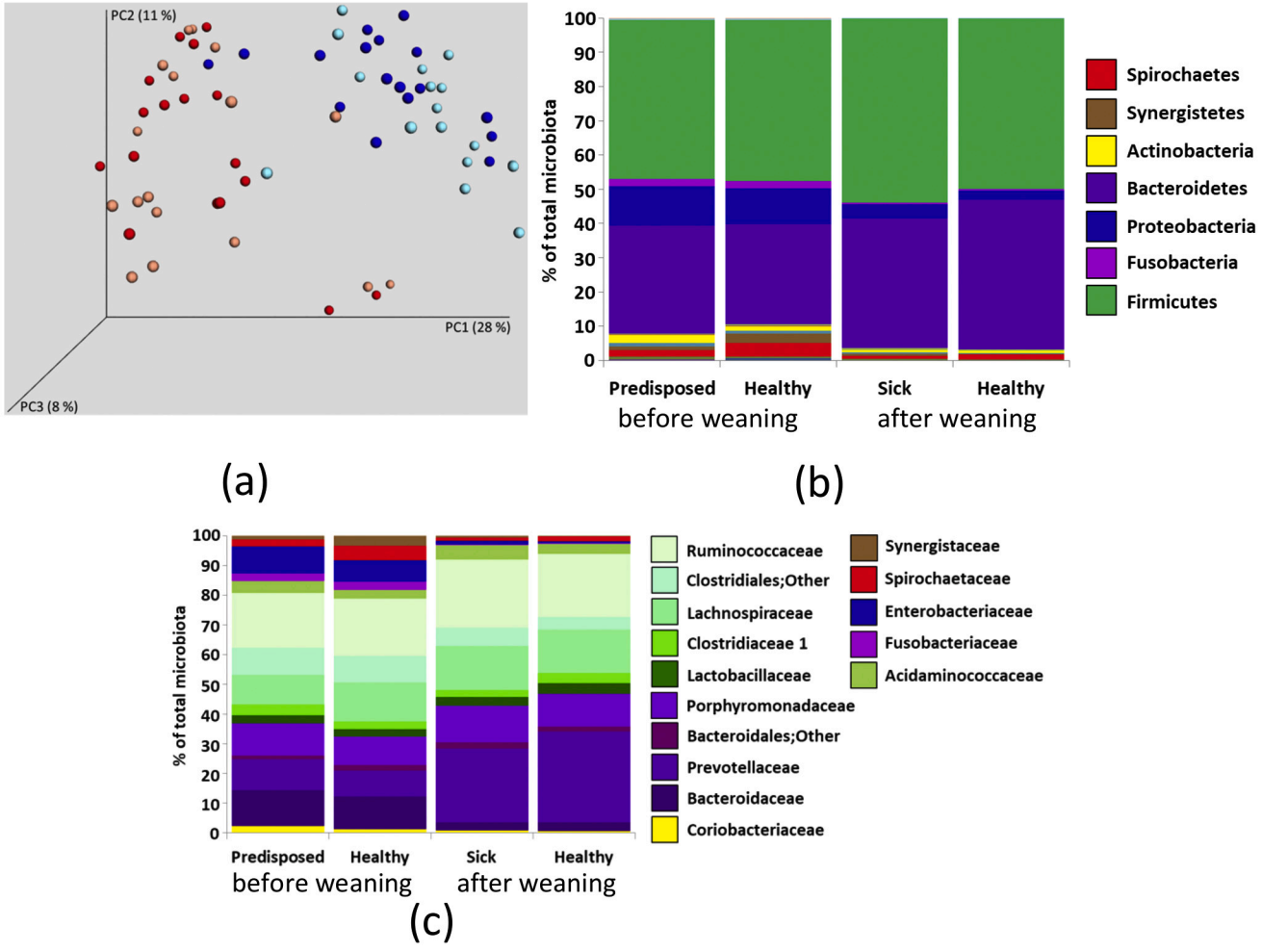

Fig. 1. Microbiota composition in 34 piglets tested before and after weaning. Panel (a) - Weighted PCoA of samples collected from piglets before and after weaning. Red dots, healthy piglets before weaning; pink dots, predisposed piglets before weaning; dark blue dots, healthy piglets after weaning; light blue dots, piglets which developed diarrhea after weaning. Panel (b), average microbiota composition of piglets before and after weaning at phylum level. Panel (c), average microbiota composition of piglets before and after weaning at family level. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

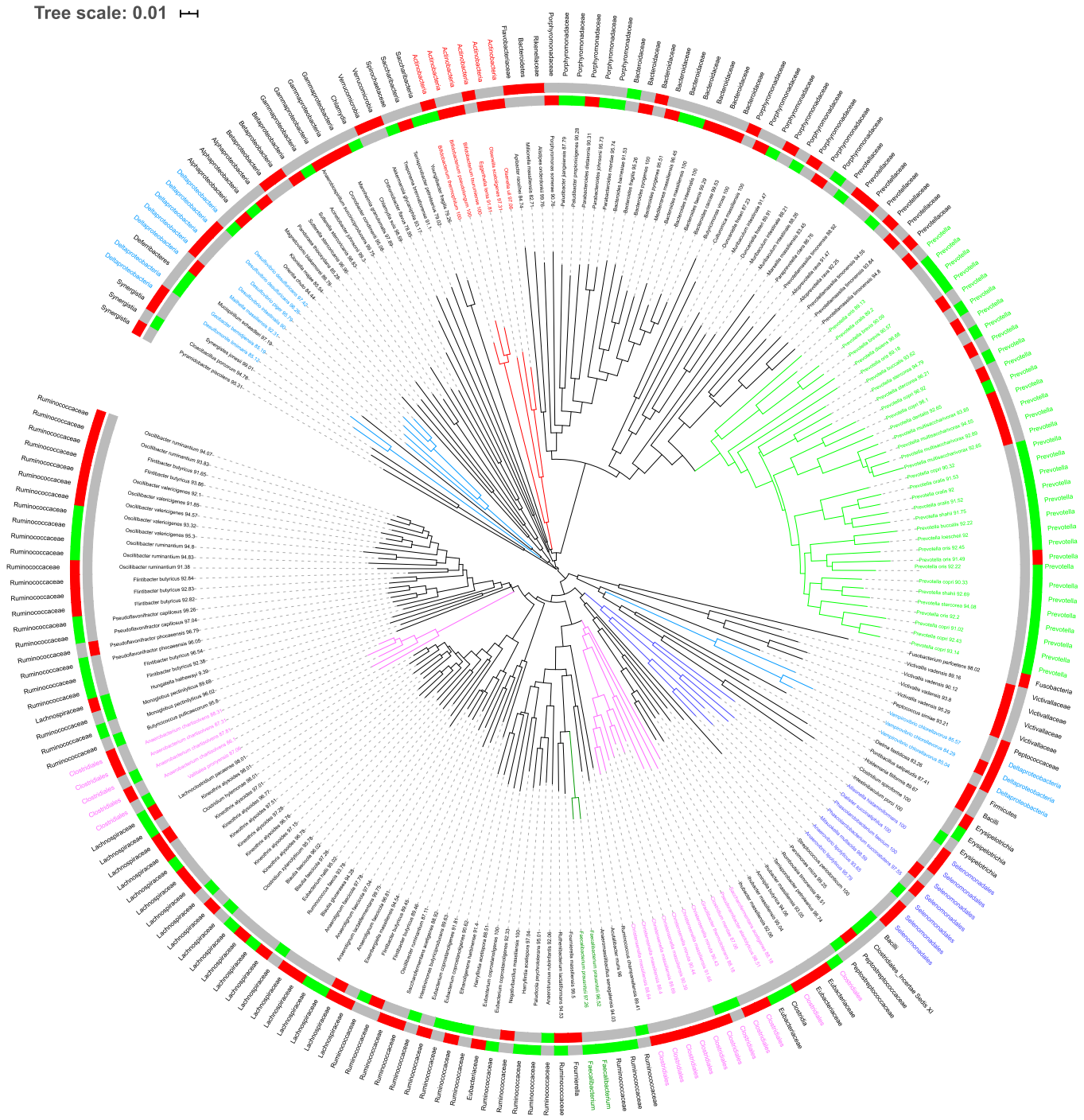

Fig. 2. Clustal alignment of partial 16S rRNA sequences (V3-V4 loop) of all differentially abundant OTUs. Colors of clades and of the taxonomic assignment with percentage similarity to the most related species in the GenBank OTUs highlights the taxa affecting the development of postweaning diarrhea the most. Inner circle, green color, significantly higher abundance of given OTU in healthy piglets in comparison to predisposed piglets before weaning; red color, significantly higher abundance of given OTU in predisposed piglets in comparison to healthy piglets before weaning; grey color, no difference in abundance of given OTU in predisposed piglets in comparison to healthy piglets before weaning. Outer circle, the same color coding as for the inner circle but for the comparison of healthy and sick piglets after weaning. To zoom in the figure, see Fig. S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

microbiota of piglets with postweaning diarrhea. Four different OTUs belonging to genera *Anaerovibrio*, *Allisonella*, unclassified Veillonellaceae and *Phascolarctobacterium*, all from order Selenomonadales, were more abundant in the microbiota of piglets with postweaning diarrhea. Seven OTUs from phylum Proteobacteria were also more abundant in the microbiota of sick piglets. Of these, six belonged to class Deltaproteobacteria (genera *Desulfovibrio*, *Bilophila*, *Vampirovibrio*). Finally, 12 different OTUs belonging to unclassified Clostridiales were also more abundant in the microbiota of piglets with postweaning diarrhea than in healthy ones (Fig. 3).

There was also a positive marker of resistant piglet microbiota. Two OTUs belonging to genus *Faecalibacterium* and 22 OTUs belonging to

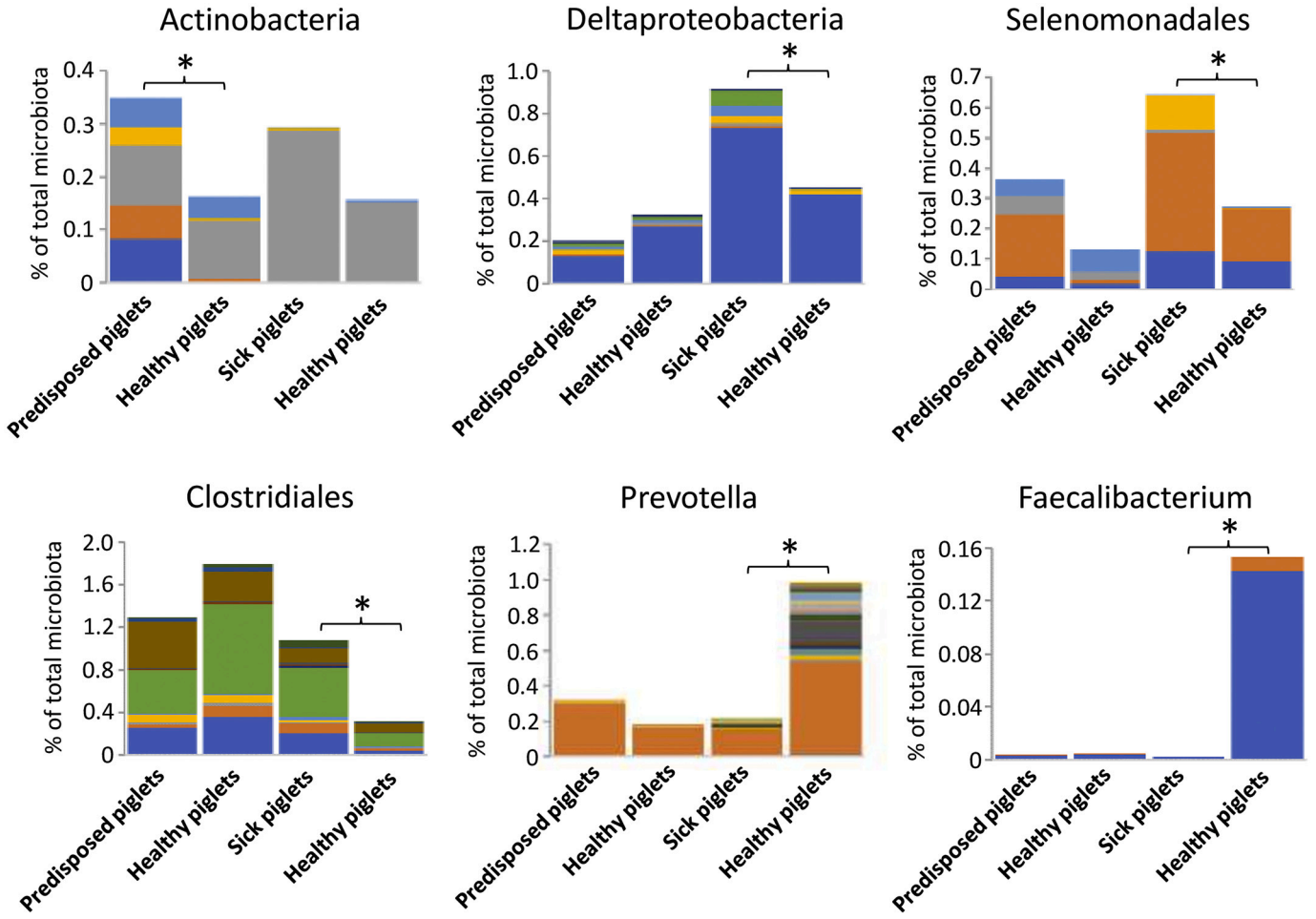

Fig. 3. Selected taxa differently abundant in microbiota of predisposed, healthy and diseased piglets. All differently abundant OTUs belonging to indicated taxon (class for Actinobacteria and Deltaproteobacteria, order for Selenomonadales and unclassified Clostridiales, and genus for *Prevotella* and *Faecalibacterium*) were superimposed one over another. Increased Actinobacteria abundance was a marker of predisposed piglets. Different OTUs belonging to Deltaproteobacteria, Selenomonadales and unclassified Clostridiales increased in microbiota of sick piglets. Microbiota of healthy piglets was enriched for OTUs belonging to genera *Prevotella* and *Faecalibacterium*. Asterisks indicate significantly different abundance by Kruskal-Wallis test ($p < 0.05$).

genus *Prevotella* were significantly more abundant in the microbiota of healthy piglets than in piglets which developed postweaning diarrhea (Fig. 3).

4. Discussion

In this study, we compared the microbiota of piglets before and after weaning with a special emphasis on microbiota in healthy and predisposed piglets before weaning, and healthy and sick piglets after weaning. We deliberately did not compare microbiota of piglets before and after weaning since such data have been reported repeatedly. Due to experimental design, we could identify the microbiota composition of predisposed piglets before weaning and those which were healthy before and remained so after weaning. The results of this study can be therefore used for the prediction of taxa indicative of correct gut microbiota development in healthy piglets and for the estimation of taxa which could be used as probiotics to prevent postweaning diarrhea. Conclusions of the study are limited by the fact that it was performed at a commercial farm and the practices used in this farm had to be accepted. This included a single dose cephalosporin administration on day 5 of life, zinc oxide supplementation, and trimethoprim and sulfamethoxazole feed supplementation after weaning. All of this could influence microbiota composition (Zeineldin et al., 2019). For example, taxa sensitive to trimethoprim (Crhanova et al., 2019) could be eliminated from piglet microbiota both in healthy and sick piglets and nothing can be said about their role in postweaning diarrhea. Due to all of this, our results should be considered as a field study. On the other hand, all the piglets were subjected to the same treatment which should equally affect microbiota composition in all piglets, and despite the use of antibiotics and zinc oxide, it was interesting to see that most of our conclusions on differential abundance of particular genera or OTUs were similar to the papers reporting no antibiotic treatment. Overall, this study therefore characterises microbiota composition in piglets under field conditions where antibiotics are sometimes used to decrease piglet mortality.

Colonisation of piglets with opportunistic pathogens like *Chlamydia* or *Helicobacter* before weaning made such piglets more resistant to postweaning diarrhea. We are aware that this is the first observation of this type which will have to be independently verified or excluded in a future. This therefore does not mean that such species should be considered as probiotic although opinion on *Helicobacter* is changing and developing (Malnick et al., 2014). However, we propose that these species activated innate immune response what made such piglets resistant to postweaning diarrhea. Similar protection was achieved in germ free piglets by their preinoculation with avirulent *Salmonella* Infantis or rough mutants of *S*. Typhimurium (Dlabac et al., 1997; Foster et al., 2003). Such preinoculation lead to the activation of innate immune response by infiltration of granulocytes and macrophages to the intestine and protection of piglets against lethal challenge with *S*. Typhimurium performed as early as 24 h after the preinoculation (Foster et al., 2003; Trebichavsky et al., 1997). The remaining taxa differently

abundant in healthy and sick piglets were reported also by other authors. *Fusobacterium* or *Akkermansia* were reported earlier as increased in piglets sensitive to postweaning diarrhea (Dou et al., 2017; Gilbert et al., 2019; Kubasova et al., 2018). Fusobacteria also increased in piglets with diarrhea before weaning (Hermann-Bank et al., 2015; Yang et al., 2019; Yang et al., 2017). An increased abundance of Actinobacteria was also reported as associated with neonatal diarrhea before weaning (Hermann-Bank et al., 2015). A lower abundance of *Prevotella* in piglets with postweaning diarrhea was also reported earlier (Dou et al., 2017; Sun et al., 2019). *Faecalibacterium* was described to increase in microbiota of healthy piglets after weaning (Kubasova et al., 2018). We recorded 4 different OTUs from order Selenomonadales as enriched in sick piglets with *Anaerovibrio* being 4 times more abundant in microbiota of sick piglets than in healthy ones. Since *Anaerovibrio* was earlier reported as increased in piglets with diarrhea (Yang et al., 2019), this species may represent a novel opportunistic pathogen. However, Selenomonadales (families *Veillonellaceae* and *Acidaminococcaceae*) are otherwise common microbiota members (Frese et al., 2015; Kubasova et al., 2018) resistant to bile salts (Crhanova et al., 2019). Our unpublished data show that *Megamonas funiformis* or *Megamonas hypermegale*, both from family *Veillonellaceae*, can rapidly multiply in vitro and overgrow different *Bacteroides* species. In the case of diarrhea with high electrolyte efflux, peristalsis and increased bile salt concentration due to their less efficient resorption, bacteria resistant to bile salts with a short generation time might be positively selected and may overgrow even without being the causative agents of infection.

Instead of overestimating the role of individual microbiota members as contributing to the development of postweaning diarrhea (see also Fig. 2, how many closely related OTUs exhibit opposite correlation with the development of postweaning diarrhea), we recommend to consider general principals of gut microbiota development during piglet weaning. Microbiota of piglets under lactation strongly differs from microbiota of sows (Kubasova et al., 2017; Motta et al., 2019) and is dominated by *Bacteroidaceae*, *Fusobacteriaceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Lactobacillaceae* and *Enterobacteriaceae* (Frese et al., 2015; Kubasova et al., 2017; Slifierz et al., 2015). Of these, *Fusobacteriaceae* and *Enterobacteriaceae* gradually decrease even before weaning (Kubasova et al., 2017; Slifierz et al., 2015) while additional reshaping is induced by weaning. Weaning results in a decrease of *Bacteroidaceae*, *Clostridiaceae* and *Enterobacteriaceae* and an increase of *Lactobacillaceae*, *Ruminococcaceae*, *Veillonellaceae* and *Prevotellaceae* (Adhikari et al., 2019; Frese et al., 2015; Kubasova et al., 2018). Our current results show that in addition to changes in individual microbiota members, precise timing is of utmost importance for the development of postweaning diarrhea. Both excessively rapid development, characterised by the presence of *Prevotella* in the gut microbiota 3 days before weaning, as well as delayed development and the absence of *Prevotella* in gut microbiota 4 days after weaning are characteristics of predisposed or sick piglets, respectively.

5. Conclusions

In this study, we have compared the microbiota composition of piglets before and after weaning in predisposed, healthy and sick animals in a field study at a commercial farm where antibiotics had to be used when forced by external factors. Increased Actinobacteria before weaning was a marker of piglets predisposed for diarrhea. After weaning, unclassified Clostridiales, Deltaproteobacteria or Selenomonadales increased in microbiota of piglets with postweaning diarrhea while an increase in *Prevotella* and *Faecalibacterium* was characteristic for healthy, weaned piglets. Although multiple additional taxa significantly changed in abundance in the microbiota of piglets around weaning, the exact timing and quick replacement of microbiota characteristic for piglets under lactation with adult-type microbiota is equally important for piglet resistance to postweaning diarrhea.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2020.12.022.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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