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Novel *ex vivo* screening assay to preselect farm specific pre- and probiotics in pigs

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RESEARCH ARTICLE

Abstract

A novel rapid *ex vivo* assay was developed as part of a concept to determine potential tailor-made combinations of pre- and probiotics for individual farms. Sow faecal slurries from 20 German pig farms were anaerobically incubated with pre- and probiotics or their combinations together with pathogenic strains that are of interest in pig production. Aliquots of these slurries were then incubated with media containing antibiotic mixtures allowing only growth of the specific pathogen. Growth was monitored and lag time was used to determine the residual fitness of the pathogenic strains. The background growth could be inhibited for an *Escherichia coli*- and a *Clostridium difficile*- but not for a *Clostridium perfringens* strain. The prebiotic fructo-oligosaccharides (FOS) and its combination with probiotics reduced the residual fitness of the *E. coli* strain in some farms. However, notable exceptions occurred in other farms where FOS increased the fitness of the *E. coli* strain. Generally, combinations of pre- and probiotics did not show additive effects on fitness for *E. coli* but displayed farm dependent differences. The effects of pre- and probiotics on the residual fitness of the *C. difficile* strain were less pronounced, but distinct differences between single application of prebiotics and their combination with probiotics were observed. It was concluded that the initial composition of the microbiota in the samples was more determinative for incubations with the *C. difficile* strain than for incubations with the *E. coli* strain, as the presumed fermentation of prebiotic products showed less influence on the fitness of the *C. difficile* strain. Farm dependent differences were pronounced for both pathogenic strains and therefore, this novel screening method offers a promising approach for pre-selecting pre- and probiotics for individual farms. However, evaluation of farm metadata (husbandry, feed, management) will be crucial in future studies to determine a tailor-made solution for combinations of pre- and probiotics for individual farms. Also, refinement of the *ex vivo* assay in terms of on-farm processing of samples and validation of unambiguous growth for pathogenic strains from individual farms should be addressed.

Keywords: colonic microbiota, pigs, *Clostridium* spp., enterotoxigenic *E. coli* (ETEC), fructo-oligosaccharides (FOS), probiotics

1. Introduction

Conventional pig farming in Europe faced a major challenge in the last decades as antibiotic growth promoters were banned in the EU (Anadón, 2006). Before, in-feed antibiotics have been used regularly to enhance the growth, performance and health of the animals. With the ban came a demand for suitable alternatives that could compensate for losses in performance. Especially during the critical weaning period, new therapies and preventive methods are still an urgent need in pig production and the search for better feeding- and management strategies without antibiotics are still ongoing (Dlamini *et al.*, 2017; Li *et al.*, 2017).

In this respect, pre- and probiotics are considered promising candidates. Probiotics are used in many ways in all stages of production in modern pig farming (Barba-Vidal *et al.*, 2019). The popularity of probiotics in pig production has increased considerably and their beneficial effects have been demonstrated in many studies (Azad *et al.*, 2018; Liao and Nyachoti, 2017; Ohashi and Ushida, 2009). For instance, the use of probiotics can reduce digestive problems that may consequently increase production parameters (Anand *et al.*,

2018; Joysowal *et al.*, 2018). However, there are also studies, which could not demonstrate significant benefits from the use of probiotics (Kreuzer *et al.*, 2012; Walsh *et al.*, 2012). In a recent review, the authors suggest that probiotics should not be seen as an equivalent replacement for antibiotics, as their effects are per se not comparable (Barba-Vidal *et al.*, 2018). Instead, the use of probiotics should be combined with feed and management strategies to achieve the best possible results (Barba-Vidal *et al.*, 2018). Although there have been many studies on this subject, their results are difficult to compare. It becomes clear that not only the probiotic strain, but also dosage, host-specific factors, genetic aspects and especially environmental conditions like housing, hygiene management and feed play a major role for the success of a probiotic application (Patil *et al.*, 2019). This impedes the use of uniform strategies for probiotics in conventional pig farming and it may not be possible to use a 'one-size-fits-all' approach.

Prebiotic feed additives also became popular due to their effect on performance and health as well as easy preparation and storage stability (Duan *et al.*, 2016; Samolinska *et al.*, 2018). Per definition, prebiotics are meant to be fermented by non-pathogenic intestinal bacteria, which thus gain a colonisation advantage and consequently control growth of pathogenic bacteria (Oyarzabal and Conner, 1995). Secondary effects include beneficial effects on the hosts immune system (San Andres *et al.*, 2019) or specific effects on pathogenic bacteria (Halas and Nochta, 2012). The modification of the intestinal microbiota may lead to healthier animals with better performance (Sivieri *et al.*, 2014), however, prebiotics are substrates for intestinal microbiota, which varies in composition and activity. Therefore, similar to probiotics, different responses in individual animals or -farms lead to varying degrees of success and thus inconsistent results are obtained with prebiotics (Bielecka *et al.*, 2002; Kadlec and Jakubec, 2014).

The response of the intestinal microbiota to pre- and probiotics has been examined in many studies (Quigley, 2010), however most of these studies were done in scientific institutions that do not mirror practical conditions of pig production. Furthermore, screening different feed additives under different housing, feed-, or management conditions in scientific feeding trials is prohibitively expensive. An alternative to screen the influence of different feed additives or their combinations on the animal's microbiota are *ex vivo* assays with digesta content (Ren *et al.*, 2019; Starke *et al.*, 2013; Vahjen *et al.*, 2012). *Ex vivo* assays are the middle ground between cost intensive feeding trials and unreliable *in vitro* tests. Due to the high number of samples or combinations of feed additives that can be tested, *ex vivo* assays are a promising method to screen the effect of feed additives on the intestinal microbiota. The read-out of these assays depends on the scientific question. Consequently, the bacterial response to an inhibiting feed additive requires monitoring the growth of the intestinal microbiota (Starke *et al.*, 2013), while the search for a probiotic that is specifically active against a bacterial pathogen requires monitoring the growth of that pathogen in the presence of the probiotic or its culture supernatant (Ren *et al.*, 2019).

Finally, a unique relationship between mother and offspring is present in pig production. The transfer of the maternal microbiota to their piglets has been recognised as an important factor that shapes the early development of the piglet microbiota (Thum *et al.*, 2012). Furthermore, it is also acknowledged that the early microbial programming in mammals has an impact on health later in life (Nowland *et al.*, 2019). Therefore, it seems reasonable to modify the maternal microbiota via feed additives to induce beneficial changes in the microbiota of their offspring.

As described above, inconsistencies in studies with pre- and probiotics in pigs are a result of many different factors that influence the intestinal microbiota, even before these feed additives are administered. Consequently, the success of a given feed additive is also dependent on the external factors that should not be underestimated. As many different management systems, animal genetics and feeding regimes exist in the pig industry, the search for individual solutions in the application of pre- and probiotics seem more successful than the 'one-size-fits-all' approach. With this in mind, we aimed to develop a suitable screening strategy for the selection of pro- and prebiotics in conventional pig farming within the larger project 'Optibiom'. In combination with other data (microbiome analysis, farm metadata on husbandry, feed, management) the presented *ex vivo* assay may help to decide, which feed additive is most beneficial for a specific farm.

We propose that individual farm conditions lead to individual responses to the presence of pre- and probiotics. To monitor these responses, an *ex vivo* assay was developed that tested the efficacy of pre- and probiotics to inhibit the growth of three bacterial pathogens with importance in pig production.

2. Material and methods

Experimental design

Sow faecal slurries were anaerobically incubated with 15 different combinations of pre- and probiotics and three bacterial pathogens in a first 24 h incubation. Aliquots thereof were transferred in an antibiotic medium that allowed only growth of the given pathogen. Turbidity was followed overnight and growth parameters were computed from growth curves and compared to controls without addition of pre- and probiotics to monitor the residual fitness of the pathogen.

Strains, media and antibiotics

Throughout the study pathogenic strains of an *Escherichia coli* 'Abbotstown' isolate (IMT203/7, serotype O149:K91, haemolytic), a *Clostridium perfringens* type strain (DSM 756) and a *Clostridium difficile* isolate (serotype 078) were used as model strains. The *E. coli* and the *C. perfringens* strains were cultured throughout the study in brain heart infusion broth (BHI). The *C. difficile* strain was cultured in BHI broth supplemented with 0.5 g/100 ml yeast extract, 0.1 g/100 ml L-cysteine-hydrochloride and 0.1 g/100 ml taurocholic acid sodium salt hydrate (BHIS).

The antibiotic resistance of the pathogenic strains was tested with an agar plate antibiogram and consequently, suitable antibiotics used for the growth assay were selected based on their resistance profile (Supplementary Table S1). Different antibiotic mixtures were tested that (a) allowed growth of the pathogen and (b) inhibited background growth in sow faecal samples. In order to prepare specific antibiotic mixtures for each pathogenic strain, stock solutions were diluted immediately before use with sterile demineralised water according to the desired concentration (see SupplementaryTable S1).

Prebiotic- and probiotic products

All prebiotic products are commercially available and are commonly used in sows and piglets. Fructo-oligosaccharides (FOS) produced by partial enzymatic hydrolysis from chicory inulin (93-97% β-(2,1)-linkage oligofructose; partial hydrolysis from chicory inulin; DP 2-8), mannan-oligosaccharide (MOS) produced from *Saccharomyces cerevisiae* yeast cell walls (min. 24% mannans; 25% beta-glucans, 25% protein) and inulin derived from chicory (90% inulin from chicory; DP 2-60) were diluted in phosphate buffered saline with 0,5 mg/ml L-cysteine (PBS, pH 7.0) and filter-sterilised (0.2 µm cellulose acetate; VWR international, Radnor, PA, USA) under anaerobic conditions. MOS and inulin were diluted to a final concentration of 20 mg/ml, while FOS was adjusted to 10 mg/ml final concentration.

All probiotic products are licensed in the EU as feed additives in sows and piglets. An *Enterococcus faecium* (1×10^{10} cfu/g), a *Saccharomyces cerevisiae* (2.0×10^{10} cfu/g) and a multistrain product containing *Bacillus licheniformis* and *Bacillus subtilis* (3.25×10^{9} cfu/g) were diluted anaerobically from original powders in PBS. The final concentration for all probiotics was 10^{7} cfu/ml in each *ex vivo* assay. Viable cell number per g product was determined before use.

Selection of samples and sampling

A total of 60 freshly voided faecal samples were obtained from sows two weeks ante partum in 20 different German pig farms (three sows per farm) during February to August 2019. After collection, samples were shock-frozen in liquid nitrogen and shipped at -20 °C. After reception at the institute, samples were stored at -80 °C. Before the assay, 1 g of frozen faeces was transferred into 15 ml tubes (Cellstar Tubes, Greiner Bio-One GmbH, Frickenhausen, Germany) and transferred into an anaerobic chamber.

Determination of suitable antibiotic combinations for pathogenic strains and test of antibiotic combinations

The broth micro-dilution method in 96-well microplates was used to determine individual minimum inhibitory concentration (MIC) of suitable antibiotics as determined via plate antibiogram (see Supplementary Table S1). OD_{690} measurements of cultures incubated with two-fold serial dilutions of antibiotics were taken every 5 min for 24 h in a microplate reader (Tecan Infinite® 200Pro, Maennedorf, Switzerland). Different combinations and concentrations of antibiotics were tested for each pathogen. Resulting growth curves were visually analysed and suitable antibiotic mixtures and concentrations were tested on sow faecal samples and probiotic products to ensure absence of growth from antibiotic-resistant bacteria.

Ex vivo assay

All work with faecal suspensions, inocula and pre- and probiotic products was carried out in an anaerobic chamber. For each sample, faecal slurries were prepared inside the anaerobic chamber by mixing 1 g of faeces with 9 ml of PBS. After mixing, the slurries were sedimented for 5 min and 160 µl supernatant was transferred to a microplate. In the next step, prepared suspensions (20 µl) of each pre- and probiotic product were inoculated into the faecal slurry. Each of the three pre- and probiotic products was inoculated singly or in combination, yielding 15 different combinations. Suspensions without pre- and probiotic inoculation (use of PBS instead) served as control. Finally, the pathogenic strain was inoculated to all wells in a concentration of 10^{6} cfu/ml. The microplate was then incubated anaerobically at 37 °C for 24 h. After the incubation, suspensions were homogenised by pipetting and 15 µl was taken anaerobically from each well and transferred to a new microplate containing strain dependend BHI or BHIS medium with the respective specific antibiotic mixture for each pathogenic strain. To ensure anaerobic conditions in the next step, microplates were sealed airtight with a cover foil (Viewseal Sealer clear, Greiner Bio-One GmbH).

For the second incubation, the microplate was transferred into a microplate reader and growth was measured at OD_{690} every 5 min for 24 h at 37 °C. The microplate was shaken for 10 s prior to each measurement. After the incubation, growth parameters (lag time, specific growth, maximum growth) were calculated from growth data with

the nonlinear 3-parameter logistic growth model. After visual inspection, only data from 0-12 h were considered, as these curves showed the typical appearances of bacterial growth curves. Furthermore, differences to positive controls (samples inoculated with pathogenic strain only) were expressed as percentage difference to compare the impact of pre- and probiotic combinations on the residual fitness of the pathogenic strains.

DNA extraction, 16S rDNA sequencing and 16S rDNA analysis

Total DNA was extracted from 0.25 g faeces with a commercial extraction kit (QIAamp PowerFecal Pro DNA Kit, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions with an additional lysis step at 65 °C. DNA extracts were subjected to amplicon sequencing using an Illumina NextSeq500 sequencer (LGC, Berlin, Germany) with 150 bp-paired reads using 16S rDNA primers 341f and 785r. Demultiplexing was achieved with Illumina bcl2fastq (v. 2.17.1.14); combination of paired reads was done with BBMerge (v. 34.48). The resulting 16S-rDNA sequences were analysed using the QIIME2 pipeline (Bolyen *et al.*, 2019) and the SILVA SSU database (Yilmaz *et al.*, 2014). Quality control and determination of sequence counts were performed using the DADA2 (Callahan *et al.*, 2016). Sequence variants with less than five counts were excluded from further analysis to increase confidence of sequence reads and reduce bias by possible sequencing errors (Huse *et al.*, 2007). Normalisation of sequence reads was done by rarefaction with an equal representation of 10,000 sequences per sample (Weiss *et al.*, 2017).

Statistical analysis

All data is presented as means and standard error of the means for each farm and all pre- and probiotic products. Percentages of lag time relative to pathogen-inoculated controls were calculated to study the impact of pre- and probiotics on pathogenic strains. An ANOVA procedure with Tukey-HSD as post-hoc test was done with the software SPSS 24 (IBM SPSS 24; Armonk, NY, USA) and served as indicator for statistical significance at $P \leq 0.05$. Spearman correlation analysis was used to correlate individual growth parameters. Similarity analysis and hierarchical clustering of lag times for individual farms and pre- and probiotics was calculated by the Ward method and Euclidian distance method.

3. Results

Microbiome composition of samples

A characterisation of the initial state of the microbiome in the samples was done via 16S-rDNA sequencing. Figure 1 shows a similarity dendrogram (Ward D Method, complete clustering) that was constructed with data of the project 'Optibiom' (Luehrmann *et al.*, unpublished data). Most individual farms showed a high 16S-rDNA sequence similarity in their samples that set them apart from other farms. However, some farms (F, O, P, R and T) showed low similarity within the data set.

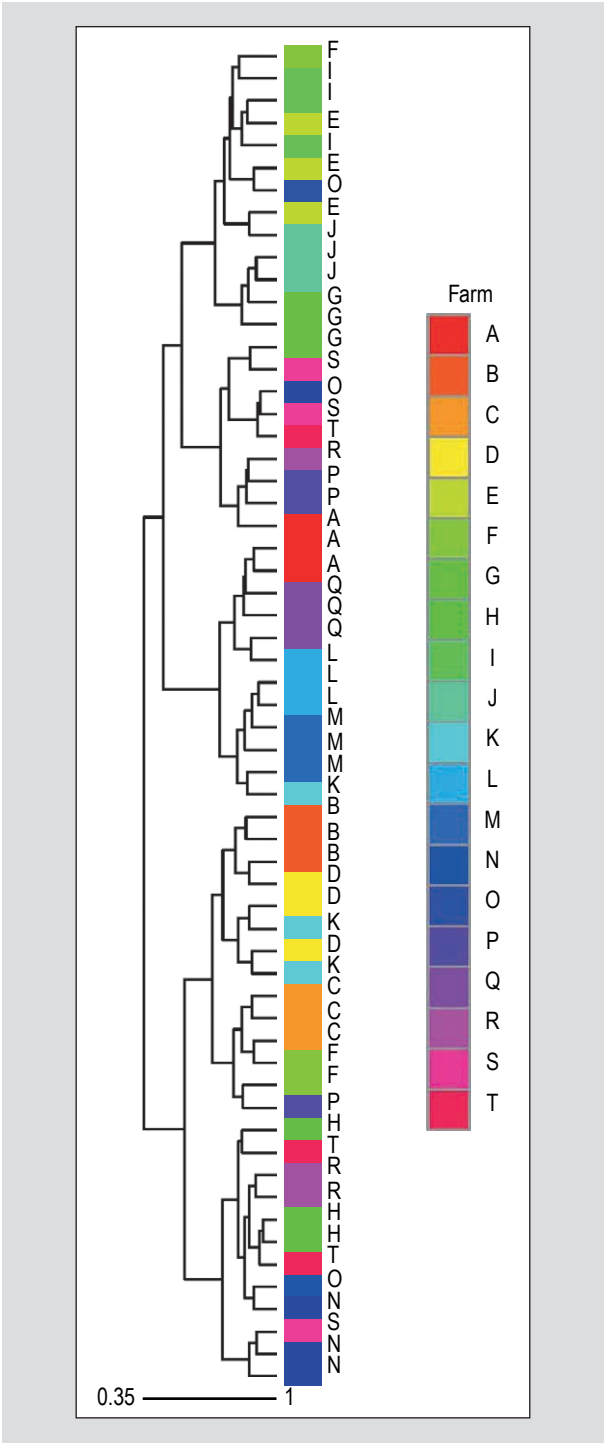

Figure 1. Similarity dendrogram of the faecal microbiota (16S-sequences) of 20 different German pig farms (n=3 per farm).

Ex vivo assay method

No growth occurred in non-inoculated controls for antibiotic mixtures used for the *E. coli*- and the *C. difficile* strain, but some samples showed spurious growth in the late incubation period (>18 h), which was not part of the analysis of 0-12 h. However, under the conditions used (BHI & antibiotic mixture), it was not possible to inhibit growth in controls with different antibiotic combinations for the *C. perfringens* strain. Even at extremely high concentrations and four different antibiotics (see Supplementary Table S1), substantial background growth in non-inoculated samples was still observed. Therefore, we chose to exclude the *C. perfringens* strain from further analysis in this study. Incubated samples were subcultured on BHI and resulting colonies were taxonomically assigned *E. coli* by MALDI-TOF analysis (data not shown).

Positive controls (no addition of pre- or probiotics) for samples of all 20 farms displayed a high variation after an initial growth phase (Supplementary Figure S1). The *E. coli* strain generally showed shorter lag times than the *C. difficile* strain.

A typical growth curve (Farm B) for the residual fitness of the *E. coli* strain after incubation with different prebiotics is shown in Figure 2. Relative to the positive controls, FOS showed a prolonged time before reaching the exponential growth phase, while MOS seemed to yield an even better growth than the control in samples from farm B.

Most often, the specific growth during the exponential phase was not different between the different feed additives in these incubations. Although in this case the maximum OD showed highest values for the control, most other experiments did not yield reliable data for this parameter. Indeed, correlation of lag time and specific growth showed highly significant correlation coefficients (0.857 for *E. coli*; 0.730 for *C. difficile*), but correlation of these parameters to maximum OD only yielded significant, but much lower coefficients (0.267 and 0.189 for *E. coli*; 0.252 and 0.111 for *C. difficile* for lag time and specific growth, respectively). Therefore, we decided to focus on lag time as the primary parameter for pathogen residual fitness.

The complete data set for the *E. coli*- and the *C. difficile* strain is given in Supplementary Tables S2 and S3, respectively. Due to the high number of variables (20 farms, 15 combinations, 960 assays per strain) it is only possible to show exemplary data as follows.

Residual fitness of the pathogenic *E. coli* strain after incubation with pre- and probiotics

Table 1 shows the post-hoc multiple comparison test for lag time data to estimate the fitness of the *E. coli* strain after incubation in pre- and probiotic supplemented faecal slurries. The combined data for all 20 farms shows shortest lag times after incubations with the prebiotic MOS, while the longest lag times occurred after incubation with the prebiotic FOS. Although overall differences for lag times

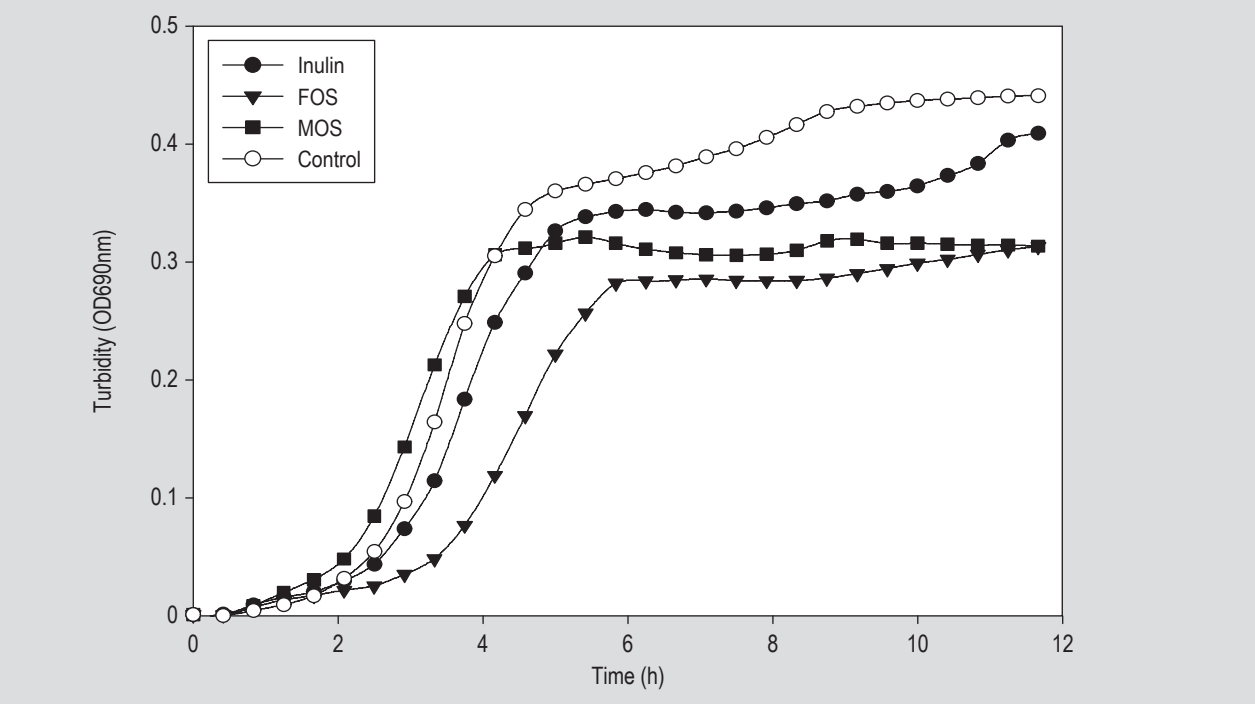

Figure 2. Typical growth curves for the residual fitness of an *E. coli* strain after incubation with different prebiotics in faecal slurries (Farm B). FOS = fructo-oligosaccharides; MOS = mannan-oligosaccharide.

were minor (2.74-3.31 h), the prebiotics MOS and inulin and their combinations with probiotic products consistently yielded shorter lag times than control incubations. On the other hand, the *Bacillus*- and *Enterococcus* probiotic extended overall lag times in a similar fashion as the prebiotic FOS.

Regarding the residual fitness of the *E. coli* strain in faecal slurries from individual farms, the addition of pre- and probiotic products showed farm dependent differences (see also Supplementary Table S2). As an example, the fitness of the *E. coli* strain relative to the controls after incubation of faecal slurries and FOS is shown in Figure 3. Faecal slurries from many farms showed no significant impact on *E. coli* fitness compared to controls for any feed additive. However, farms G, L and T yielded in part drastically reduced lag times, i.e. increased residual fitness of the strain. On the other hand, strongly increased lag times were noted in farm J and especially in farm P. Neither inulin nor MOS showed a significant impact on the residual fitness of the *E. coli* strain between farms. However, strong numeric differences in lag time were observed for inulin, ranging from 80 to 232%, while differences for MOS (74 to 104%) were much lower (data not shown).

Table 1. Overall post-hoc multiple comparison test of lag time for *Escherichia coli* after incubation in sow faecal slurries supplemented with pre- and probiotics [h].1

	Subsets[2]		
	a	b	c
MOS	2.74		
MOS & *Bacillus*	2.79		
MOS & *Enterococcus*	2.82		
MOS & yeast	2.86	2.86	
Inulin & yeast	2.96	2.96	2.96
Yeast	3.01	3.01	3.01
Inulin & *Bacillus*	3.02	3.02	3.02
Inulin & *Enterococcus*	3.05	3.05	3.05
Inulin	3.06	3.06	3.06
Control	3.07	3.07	3.07
FOS & yeast	3.11	3.11	3.11
Enterococcus	3.11	3.11	3.11
FOS & *Bacillus*		3.21	3.21
FOS & *Enterococcus*			3.23
Bacillus			3.24
FOS			3.31
Significance	0.059	0.113	0.093

[1] FOS = fructo-oligosaccharides; MOS = mannan-oligosaccharide.
[2] ANOVA Tukey-HSD Post-hoc test at $P<0.05$; n=960).

Figure 4 depicts the similarity clustering and heat map of the lag time for all feed additives and all farms. Clear cluster formations were visible for all feed additives, as FOS, probiotics, MOS and inulin each formed different clusters. Individual farms showed less clear clustering, however the residual fitness of the *E. coli* strain after incubations in faecal slurries from farms P, J and E seemed to be negatively influenced by more feed additives than farms L, G and I.

In general, farm specific responses were observed for the residual fitness of the *E. coli* strain after incubation with feed additives. From the data, it can be concluded that the presence of FOS alone or in combination with probiotics showed the highest degree of influence, followed by inulin and MOS. However, notable exceptions occurred in some farms, where FOS increased the fitness of the pathogenic *E. coli* strain.

Residual fitness of the pathogenic C. difficile strain after incubation with pre- and probiotics

The overall impact of pre- and probiotics on lag time was not significantly different between feed additives according to an ANOVA Post-Hoc multiple comparison test (Supplementary Table S4). Overall lag time differences between feed additives were more pronounced (7.73-9.00 h) than for the *E. coli* strain.

Contrary to results for the *E. coli* strain, significant differences for individual farms were scarce with the *C. difficile* strain for all feed additives. In general, lag time data showed higher standard deviations than for incubations with the *E. coli* strain. Compared to control incubations, the single application of prebiotics did not show pronounced numeric differences in lag time for FOS, but increased lag time in more farms for inulin and MOS (Supplementary Table S3). Similarly, no obvious changes in lag time were generally observed for the single application of probiotics. However, in combination with FOS or inulin, the *Bacillus* probiotic showed numerically increased lag times in eight of twenty farms compared to only four farms for the other probiotic products. Interestingly, there was no exact match of farms that showed an increased lag time for both FOS and inulin (see Farm A, C, E and F). In combination with MOS, the addition of the *Enterococcus* probiotic led to an increased lag time in seven farms, but also displayed reduced lag times in seven other farms. Drastically reduced lag times were also observed in nine farms for the combination of MOS and the yeast probiotic.

Although higher numeric differences for lag time were visible between farms, significant differences relative to control incubations were infrequent. Significant differences relative to the control were only observed for combinations of FOS with the *Bacillus*- or the yeast probiotic (Figure 5). Residual fitness in samples from farms E, J and P was

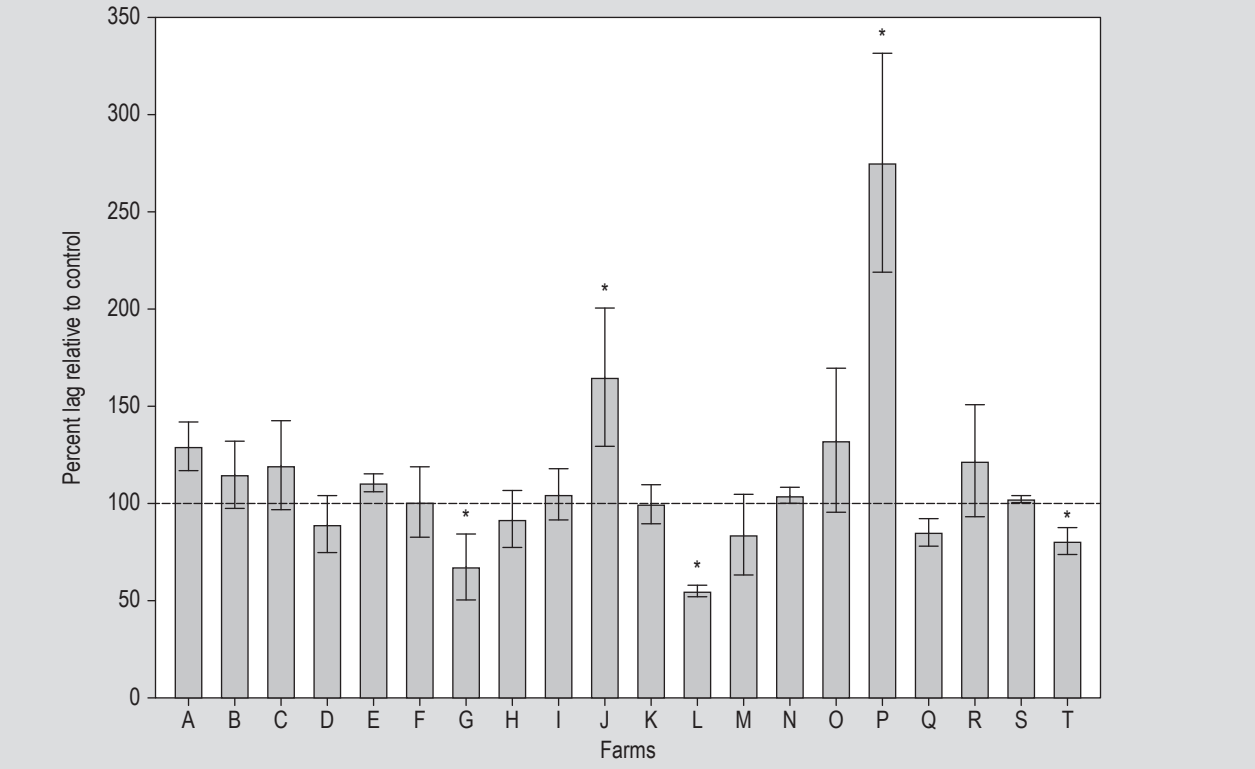

Figure 3. Lag time of the *Escherichia coli* strain relative to controls for all farms after incubation with fructo-oligosaccharides in sow faecal slurries [%].

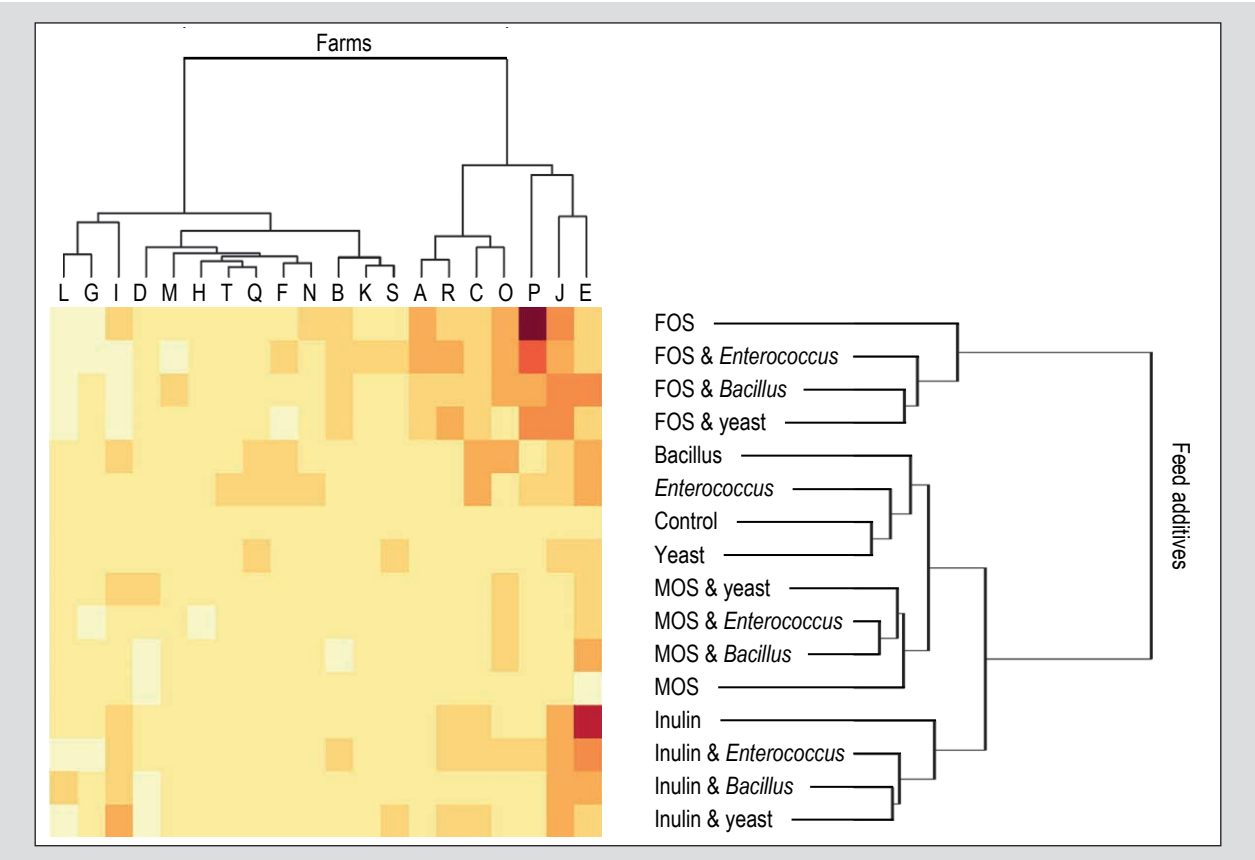

Figure 4. Heatmap and hierarchical clustering of *Escherichia coli* lag data after incubation pre- and probiotic products. FOS = fructo-oligosaccharides; MOS = mannan-oligosaccharide.

significantly decreased relative to the control but, as was observed for the *E. coli* strain, significantly better fitness relative to the control were also observed for the *C. difficile* strain in some farms (D, L). Overall, feed additives had no significant effect the residual fitness of the *C. difficile* strain compared to control incubations.

Figure 6 shows the similarity cluster analysis for the *C. difficile* strain. Contrary to the clear cluster formations for the *E. coli* strain, no clear clustering according to feed additives was observed. However, six of nine combinations of pre- and probiotics formed one of the two main clusters. The prebiotics FOS and inulin together with the yeast probiotic and the combination of inulin and *Bacillus* formed a subcluster with the control, while the probiotics *Enterococcus* and *Bacillus* formed a subcluster together with the combinations MOS & *Bacillus* and inulin & yeast. Regarding the similarity of individual farms, only farm A seemed to be especially responsive to many combinations of pre- and probiotics, while the remaining farms formed clusters with nine and ten farms, respectively.

Overall, farm specific responses were less pronounced for the *C. difficile* strain. However, similar to the *E. coli* strain, individual farms showed not only increased lag times for some feed additives, but also reduced lag times, i.e. increased residual fitness.

4. Discussion

Ex vivo assay method

This study used a novel *ex vivo* assay to screen the impact of three pre- and three probiotic products and their combinations on the residual fitness of pathogenic *E. coli* and *C. difficile* strains as part of the larger project 'Optibiom' to determine tailor-made solutions for pre- and probiotic supplementation in individual German sow farms. Thus, the present study is a proof-of-principle that the novel *ex vivo* assay is a rapid, inexpensive method to screen for most suitable combinations of pre- and probiotic products for individual farms.

Based on the hypothesis that sow faeces are an important transfer vector for the development of the piglet microbiota and could compromise general farm hygienic conditions, sow faeces were chosen as a suitable matrix. The importance of sow faeces as inoculant for bacterial development on their offspring (McCormack *et al.*, 2019), known differences in the microbiota composition between individual animals as well as in humans (Aluthge *et al.*, 2019; Kolodziejczyk *et al.*, 2019) as well as differences in microbiota composition in different farms (Yang *et al.*, 2018) formed the scientific rationale behind the employed assay.

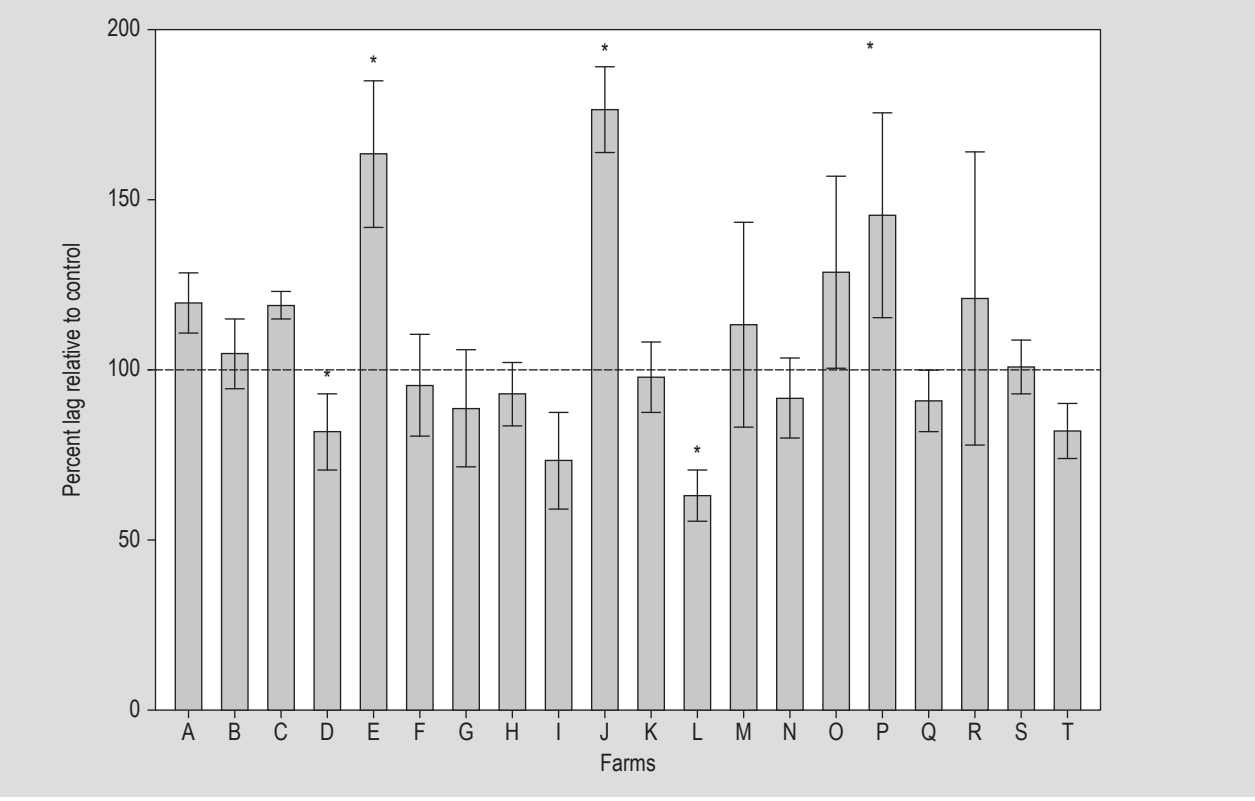

Figure 5. Lag time of the *Clostridium difficile* strain relative to controls for all farms after incubation with fructo-oligosaccharides in sow faecal slurries [%].

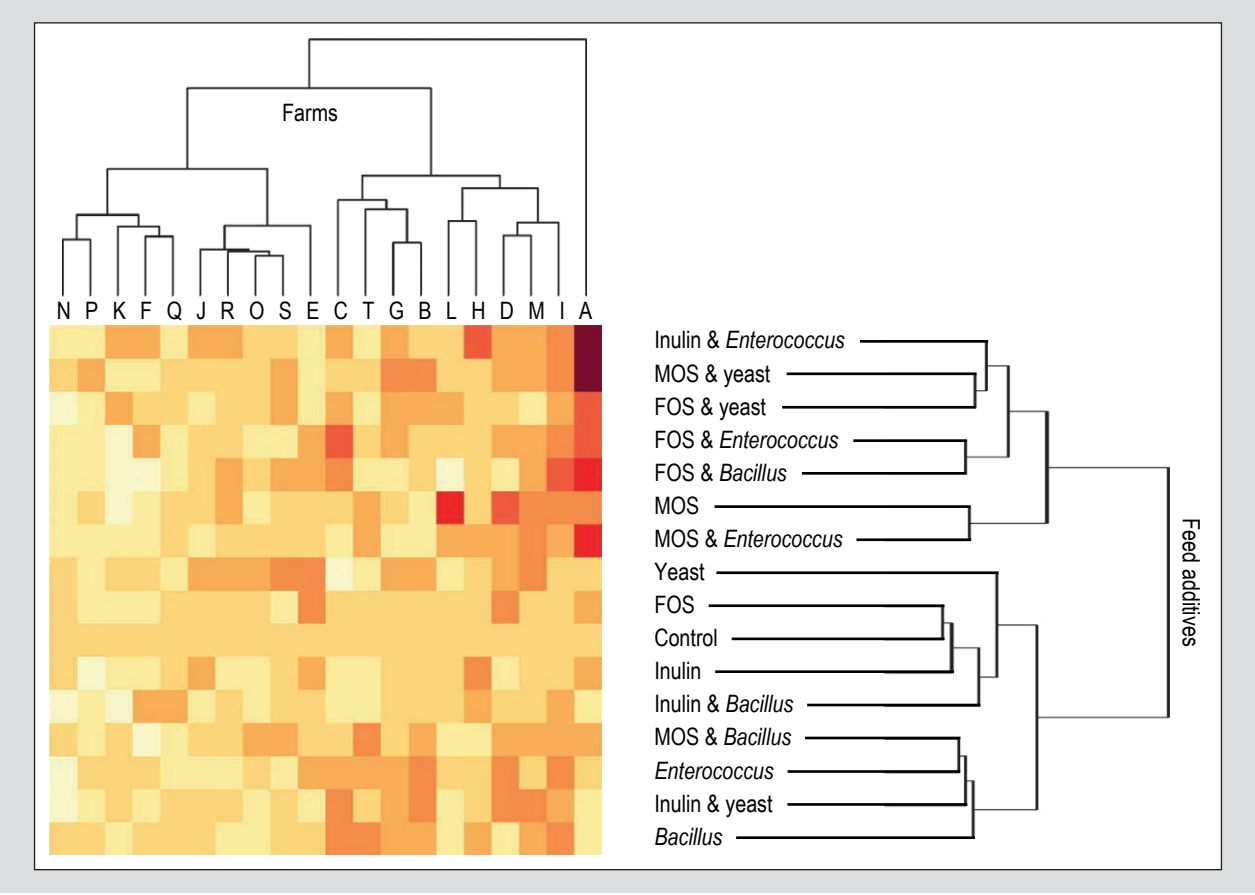

Figure 6. Heatmap and hierarchical clustering of *Clostridium difficile* lag data after incubation pre- and probiotic products. FOS = fructo-oligosaccharides; MOS = mannan-oligosaccharide.

The first incubation of the pathogenic strains in faecal slurries together with pre- and probiotics simulated the presence of these feed additives in the hindgut. The response of the microbiota to the presence of the feed additives could then create an adverse environment for the fitness of the inoculated pathogenic strains. The second incubation allowed only growth of these strains via the use of specific antibiotic mixtures in the medium. This enabled us to specifically monitor the residual fitness of the pathogenic strains and thus make predictions on the effectiveness of pre- and probiotics. This principle and similar assays have been used in our working group for some time (Ren *et al.*, 2019; Starke *et al.*, 2013). It is acknowledged that the use of antibiotic mixtures for the specific detection of the pathogenic strains introduces additional stress for the surviving pathogenic cells after incubation with the indigenous microbiota and the impact of pre- and probiotics. Nevertheless, this stress applied to all incubations.

The exclusive growth of the pathogenic strains after the first incubation is critical for the success of the assay. While it was possible to generate antibiotic mixtures for the *E. coli*- and the *C. difficile* strain according to their antibiogram profile, it was not possible to create a suitable antibiotic mixture for the *C. perfringens* stain, which could stop background growth from antibiotic resistant bacteria in samples. Due to shortage of time in the project, we did not pursue other means to ensure growth selectivity of the *C. perfringens* strain. However, as the antibiotic resistant bacteria were identified as *E. coli*, one could envision the use of media that inhibit Gram-negative bacteria in addition of antibiotic mixtures. In future projects, combinations of antibiotics, growth inhibition with specific media as well as the use of specific carbon sources may alleviate the encountered problems.

Lag time was chosen to monitor residual fitness of the pathogenic strains. Lag time defines the time point, before growth enters the exponential phase of a bacterial culture (Monod, 1949). Lag time not only correlates with initial cell number, but depends also on the physiological state of individual cells (Baranyi, 1998). Thus, for the purpose of this study, the use of growth curves represents a rapid and inexpensive means to estimate the effect of different feed additives on the residual fitness of pathogenic strains.

Specific growth and maximum OD that can also be gained from the equation of the 3-parameter logistic growth model, revealed no useful information in this study. This was not surprising, as specific growth relates to differences in growth during the exponential phase and is often used to compare the effectiveness of carbon sources but may be ineffective to describe bacterial fitness (Concepcion-Acevedo *et al.*, 2015). In this study, maximum OD did not show a strong correlation to lag time or specific growth and was therefore not used to investigate the residual fitness of the pathogenic strains.

On the other hand, faecal matter only poorly reflects bacterial conditions in the intestine. The bacterial composition of the small intestine is completely different from the hindgut microbiota (Quan *et al.*, 2020), which is more comparable to the faecal microbiota. For instance, fermentation of FOS may occur in the ileum (Conway, 1994) and generally, fermentable carbohydrates are already attacked in the small intestine (Jensen and Jorgensen, 1994). However, fermentation is far from complete in the small intestine and increased bacterial metabolite concentrations in the hindgut of weaned or suckling piglets that were given FOS via feed (Correa-Matos *et al.*, 2003) or orally (Ayuso *et al.*, 2020). This indicates that a considerable amount of FOS reaches the hindgut. Thus, fermentation of considerable amounts of FOS in the hindgut can be presumed and therefore, faecal matter is considered as an appropriate as well as convenient matrix to study the fitness of pathogenic bacteria to prebiotics.

Finally, it is always preferred for studies on microbial activity to use fresh and non-frozen samples due to loss of bacterial viability after freezing. In this study, this was not possible due to the screening process of 20 different farms. In future studies that focus on a specific farm, on-site incubation of fresh samples for the first incubation with feed additives is conceivable in portable temperature-controlled units.

Impact of feed additives on the residual fitness of the pathogenic *Escherichia coli* strain

E. coli induced diarrhoea is a significant factor in pig farming, especially for post-weaning piglets (Fairbrother *et al.*, 2005). As sows are in intimate contact with their offspring during the suckling period, the transfer of pathogenic *E. coli* strains via sow faeces is very probable (Bettelheim *et al.*, 1974). Thus, reducing pathogenic *E. coli* in sow faeces would reduce the bacterial burden for piglets. In our study, we used an *E. coli* strain that is used as challenge model in post-weaning piglets (Schroeder *et al.*, 2006; Spitzer *et al.*, 2014).

According to hierarchical clustering and comparison of lag times in different farms, the prebiotic FOS seemed to be the most consistent additive in different farms, either as single additive or in combination with all three probiotics. On the other hand, MOS as well as inulin showed less overall influence singly or in combination. Prebiotics are thought to improve growth and activity of beneficial bacteria, thereby inhibiting pathogenic bacteria via metabolite production or competition (Gibson and Roberfroid, 1995). Fermentation of prebiotics may indeed reduce pH-values and lead to lower fitness of bacteria that are not adapted to a low pH. It is established that MOS interferes with the attachment of enterobacterial fimbriae to epithelial tissues, but much less is known about its fermentation by hindgut bacteria (Halas and Nochta, 2012). In this study, MOS may not have been able to modify the faecal microbiota during time of incubation and may therefore not be an effective substrate for bacterial fermentation. However, it is also known that inulin can modify the composition of the hindgut microbiota in pigs (Sattler *et al.*, 2015). Interestingly, in respective studies bacterial metabolites often seem not to increase, but sometimes even decrease when inulin is supplemented in diets for sows (Passlack *et al.*, 2015), suckling piglets (Wang *et al.*, 2019) or growing pigs (Branner *et al.*, 2004; Eberhard *et al.*, 2007; Loh *et al.*, 2006). Thus, if one follows the proposed inhibition of the pathogenic *E. coli* via bacterial metabolites, inulin would not be a suitable candidate. Changes in bacterial composition may not have taken place during the 24 h incubation period of the pathogenic *E. coli* strain with pre- and probiotics, but rather changes in bacterial activity. Finally, regarding inulin, a meta study also found that the initial concentration of bifidobacteria is important for the often quoted 'bifidogenic' effect of inulin (Rao, 1999). Regarding prebiotics, it may be concluded that the assay cannot detect any beneficial long-term effects of prebiotic supplementation that are related to microbiota changes, but rather identifies direct effects via fermentation. Nevertheless, the assay is very suitable for any prebiotic that is created to decidedly interfere with bacterial fermentation in the hindgut.

There were notable exceptions for the residual fitness of *E. coli* in samples from different farms. Thus, while samples from Farm P drastically reduced *E. coli* fitness, other farms like Farm G and L even showed an increased fitness. This was evident also for combinations of FOS with probiotic products for several farms. These results are most probably due to different farm-specific bacterial composition and -activity in the samples. Not all farms may possess sows with a microbiota that is able to induce such effects. Therefore, if one would use FOS on such farms as feed additive in sows feed with the intent to reduce the pathogenic *E. coli* load, there may be negative consequences. Results from this assay in combination with in-depth analysis of metadata as well as bacterial composition would give valuable information for specific farms, on which type of prebiotic may be more suitable. To that end, we examined the data on the faecal microbiota of the samples used and found a high in-farm similarity for most farms, but lower similarity between

farms. This has also been found in other studies (Yang *et al.*, 2018) and emphasises that microbiota data should be included in the search for tailor-made pre- and probiotics.

The single application of probiotic products did not show significant differences for *E. coli* residual fitness compared to controls, the only exception being the *Bacillus* probiotic in farm I. Probiotics have different modes of action (Fuller, 1989) and some probiotics may primarily act more on host response than directly on the microbiota or specific pathogenic bacteria (Chaucheyras-Durand and Durand, 2010). However, probiotic bacteria have to compete with the indigenous microbiota as any other exogenous bacterium. Therefore, the 'fitness' of the three probiotic products during the incubation may be different depending on the existing microbiota in the samples. It is unknown and not central to this study, if the probiotic bacteria were able to adapt to the environment. Apparently, their influence in the residual fitness of the pathogenic *E. coli* were marginal.

Impact of feed additives on the residual fitness of the pathogenic *Clostridium difficile* strain

C. difficile infections are of serious concern to neonatal piglets (Songer *et al.*, 2000). *C. difficile* does not affect adult pigs or post-weaning piglets but has a window of opportunity for infection in the hindgut of two-to-ten-day old suckling piglets (Hopman *et al.*, 2011; Weese *et al.*, 2010). Interestingly, detection of *C. difficile* in sows is often difficult, but high cell numbers can be found in their offspring (Grzeskowiak *et al.*, 2019). Thus, rapid multiplication of this pathogen occurs in the suckling piglets and the actual microbiota composition and -activity may be the decisive factor for *C. difficile* colonisation and outbreak of disease (Grzeskowiak *et al.*, 2019). Furthermore, the success of faecal transfer from human donors on *C. difficile* remission also indicates that bacteria are strongly involved in the control of *C. difficile* (Konturek *et al.*, 2017). A modification of the sow microbiota may therefore be an attractive way to reduce *C. difficile* infections, as the transfer of the maternal faecal microbiota to their offspring is of importance.

In our study, we used a *C. difficile* strain with the ribotype 078 as a model strain, which is not only of interest in the pig industry. This ribotype is also found in human patients suffering from *C. difficile*-associated diarrhoea (Goorhuis *et al.*, 2008) and thus, this strain is of interest within the concept of the 'One-Health'-approach. Although there are some studies in human medicine concerning direct beneficial effects of probiotics on *C. difficile* infections (Dendukuri *et al.*, 2005; McFarland, 2006), knowledge about promising strategies is still limited in swine nutrition.

Compared to the *E. coli* strain, incubations with the *C. difficile* strain suffered from high standard deviations and thus, fewer significant differences were observed. This may be related to individual differences in growth conditions after the incubation with pre- and probiotics, as control incubations consistently showed much lower standard deviations (data not shown). Therefore, the effect of the pre- and probiotics on the residual fitness of the *C. difficile* strain may have depended much more on the initial bacterial composition of each individual faecal slurry before incubation than it was the case for the *E. coli* strain. Here, additional in-depth analysis of the microbiota composition and metadata is especially recommended to elucidate farm-specific differences.

Neither the single application of pre- nor probiotic products yielded decisive changes on *C. difficile* residual fitness, although inulin tended to decrease fitness in more farms than FOS and MOS. In combination with FOS and inulin, the *Bacillus* product tended to decrease fitness in eight farms, while the *Enterococcus* probiotic in combination with MOS decreased fitness in seven farms. However, some farms displayed even an increased residual fitness for *C. difficile* with the mentioned combinations. In addition, the overall similarity analysis for all farms shows that FOS again showed a strong impact on the *C. difficile* strain, but only in combinations with probiotics. From these observations, several conclusions can be drawn. Firstly, single application of the probiotic products did not show any noticeable effect on both pathogenic strains. This result may question their activity during the incubation period. However, the quite diverse changes observed for the *C. difficile* strain in combination with prebiotics indicate that the probiotics indeed modified the activity or composition of the faecal microbiota during the incubation period. Thus, different modes of action regarding the residual fitness of the two pathogenic strains can be presumed for the probiotic products. Secondly, the *E. coli* strain seemed to be much more vulnerable to the addition of the easily fermentable FOS. Therefore, a subsequent reduction of pH due to release of short chain fatty acid metabolites may have a direct effect on *E. coli*, but not on *C. difficile*. Subsequently, resilience of the *C. difficile* strain against an increased fermentation rate may be higher. Finally, certain combinations of pre- and probiotics (e.g. *Bacillus* and FOS/inulin; *Enterococcus* and MOS) may work on many farms, but not on all farms. It is therefore very likely that the fitness of the *C. difficile* strain was mainly based on the composition of the indigenous microbiota in each individual sample.

Farm dependent differences and farm specific solutions

The data shows that the residual fitness of the pathogenic strains after incubation with pre- and probiotics in sow faecal slurries are farm dependent. Although some combinations of the feed additives were more successful overall, these combinations sometimes even led to

an increased fitness in other farms. For instance, the combination of the *Bacillus* probiotic with FOS decreased residual fitness of *C. difficile* in samples from Farm L, but the same combination drastically increased fitness in Farm A. Furthermore, this combination was not able to decrease fitness for the *E. coli* strain.

Therefore, a 'one-size-fits-all' approach for pre- and probiotics as well as for different pathogenic bacteria is questionable. The different outcomes also imply that the composition of the sow faecal microbiota is a primary factor for the success of these feed additives in inhibiting the colonisation of the tested strains. It is thus likely that individual differences in different farms determine the success of pre- and probiotic additives. The analysis of these differences will be part of further studies within the project 'Optibiom' that focus on bacterial composition and a wide range of acquired metadata.

The presented data in this study show that it is possible to pre-screen many different combinations of potentially beneficial feed additives that target the intestinal microbiota. Information on the bacterial response to these feed additives is a valuable parameter to guide the user in the choice of feed supplements to reduce the transfer of specific pathogenic bacteria from the sow to their offspring. Similar to tests for antibiotic resistance to decide on the use of appropriate antibiotic on a farm, this assay is at its minimum able to exclude pre- or probiotics that show increased fitness of a given pathogenic strain. At its best, the assay can point to feed additives that are capable to inhibit a specific pathogen on an individual farm. Such a tailor-made approach would replace a trial-and-error method that is not only cost intensive, but also negatively impacts long-term animal welfare and performance.

5. Conclusions

The present study demonstrated that pre- and probiotics and their combinations led to specific responses of a pathogenic *E. coli*- and a pathogenic *C. difficile* model strain after incubation in sow faecal slurries. Using lag time as parameter for residual fitness, farm specific responses to pre- and probiotics were demonstrated. Combined with microbiome analysis and evaluation of farm metadata on husbandry, feed and management, this novel screening method offers a promising approach for the pre-selection of pre- and probiotics for individual pig farms. However, more studies are needed to further refine the use of the *ex vivo* assay in terms of on-farm processing of samples and validation of farm specific pathogenic strains.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2020.0226.

Figure S1. Growth of the *Escherichia coli* and the *Clostridium difficile* strain after incubation in sow faecal slurries.

Table S1. Antibiotics used in this study.

Table S2. Impact of pre- and probiotic products on the lag time of a pathogenic *Escherichia coli* strain after incubation in sow faecal slurries.

Table S3. Impact of pre- and probiotic products on the lag time of a pathogenic *Clostridium difficile* strain after incubation in sow faecal slurries.

Table S4. Overall multiple comparison test of lag time for *Clostridium difficile* lag times after incubation in sow fecal slurries supplemented with pre- and probiotics.

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Conflict of interest

The authors declare no conflict of interest.

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