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Research Paper

The measurement of volatile organic compounds in faeces of piglets as a tool to assess gastrointestinal functionality

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There is an increasing interest in developing innovative means to monitor animal health through precision farming. As part of this drive, we have targeted digestive health and in particular the microbiota. In this study, we investigated the effect of different dietary interventions in piglets, feeding these piglets with one of two different feeds (high protein and low protein). We then evaluated its effects by measuring the volatile organic compounds (VOC) that emanated from these faecal samples using various forms of Ion Mobility Spectrometry. Piglets were monitored for 19 days, with faecal samples collected on days 6, 12 and 19, providing a total of 69 samples. The statistical analysis attempted to separate the samples using either dietary intervention or faecal score. First, the faecal score was investigated using a value based on a visual faecal scoring system, from 0 to 3, with 0 being normal and 3 having diarrhoea. Then the VOCs were analysed in regard to dietary intervention (high vs low protein). Results indicated that our approach was able to separate the dietary intervention (area under the curve (AUC) 0.81) using VOC data. Furthermore, we were able to separate samples based on faecal score (AUC between 0.71 and 1, with six different comparisons undertaken). We believe that faeces volatiles hold potential as a future means to monitor animal health.

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1. Introduction

At present, there is a strong drive to develop innovative means to monitor animal health within the livestock sector. This is normally referred to as Precision Livestock Farming (PLF) and is designed to bring more sophisticated and precise animal management to both increase productivity and improve animal health (Berckmans, 2014, 2017). Effective functionality of

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the gastrointestinal system and its health are important factors in determining livestock performance and their welfare (Celi et al., 2017). The understanding of biomarkers of gastrointestinal functionality is crucial in deepening our understanding of the complex mechanisms involved in the regulation of the several patho-physiological events that occur within the gastrointestinal tract. This has the potential to aid in livestock management through the monitoring of gastrointestinal functionality. For example, nutritional imbalances, management practices, environmental challenges and diseases can often result in diarrhoea, especially in young piglets (Jayaraman & Nyachoti, 2017). If this could be detected earlier, farmers would be able to make a rapid intervention, which would result in improved animal health, welfare and production performances (average daily gain, feed efficiency). Current advances have yet to be taken up in a farm setting, potentially due to the various challenges, including the large numbers of animals involved, their short production cycle, and the lack of a specific biomarker that can capture the complexity of the gastrointestinal tract and its functionality (Celi, Verlhac, Pérez Calvo, Schmeisser, & Kluenter, 2019).

After weaning, piglets suffer marked changes in gastrointestinal physiology, microbiology and immunology characterised by a high incidence of intestinal disturbances with diarrhoea and depression of growth performance (Pluske, Hampson, & Williams, 1997). In addition, the weaned piglet has to cope with the sudden withdrawal of sow milk and adapt to less digestible, plant-based dry diets containing proteins and carbohydrates including various anti-nutritional factors (Lallès, Bosi, Smidt, & Stokes, 2007). Both leguminous plant proteins and high levels of dietary protein can influence negatively the gastrointestinal functionality in weaned piglets (Pluske, Pethick, Hopwood, & Hampson, 2002). There is evidence that feeding a high protein diet immediately after weaning could cause protein maldigestion (Högberg & Lindberg, 2004) and consequently, increasing amounts of undigested crude protein materials in the large intestine. This contributes to an unbalanced growth of proteolytic (protein digesting) vs. saccharolytic (carbohydrate digesting) microbiota, encouraging growth and fermentation of nitrogen-utilising bacteria (Piva, Panciroli, Meola, & Formigoni, 1996). In this regard, microbial fermentation of the undigested dietary protein can provoke post weaning diarrhoea (PWD) by contributing to an increased production of toxic by-products such as branched-chain fatty acids, indole, phenols, ammonia and biogenic amines in the gastrointestinal tract (Pluske, Turpin, & Kim, 2018). On the contrary, feeding a low-protein diet in the post-weaning period reduces protein fermentation in the gastrointestinal tract and improves faecal consistency (Nyachoti, Omogbenigun, Rademacher, & Blank, 2006) but also inflammatory responses may be reduced (Opapeju, Rademacher, Payne, Krause, & Nyachoti, 2010).

Currently, the diagnosis of gastrointestinal diseases is performed by means of a series of tests that require animals to be restrained, therefore it would be desirable to develop non-invasive or minimally invasive biomarkers of gastrointestinal functionality. The visual faecal consistency score is routinely used to quantify the severity of diarrhoea in pig farms. However, the faecal consistency score system is subjective and although substantial agreement has been reported in detecting diarrhoea and faecal consistency (Pedersen, Holyoake, Stege, & Nielsen, 2011), more objective measures than faecal scoring systems may still be required. A potential solution is to monitor the inorganic gases and volatile chemicals in animal waste for biomarkers of disease and health. There has previously been a small number of papers that have used gases and volatiles targeted towards animal health. As examples, there has been work on monitoring odours coming from livestock farms (Pan & Yang, 2009) and in detecting bacterial infections in cattle (Ellis, Stahl, Nol, Waters, & Palmer, 2014) and badgers (Fend et al., 2005), but more work is needed in the field to understand its potential in a farming environment. In the wider field, gases and volatiles have been used for undertaking breath analysis in cattle and pigs (Traxler et al., 2018; Turner et al., 2012). In addition, Ciganek & Neca (2008) have looked at the chemical composition in the air around different locations within a farm.

The purpose of this study was to investigate if it was possible to distinguish pigs that were fed a high or a low protein diet, based on faecal volatiles. A secondary aim was to investigate if we could relate faecal volatiles to the faecal scoring system, which is used as a marker of gastrointestinal functionality in early weaned piglets.

2. Materials and methods

2.1. *Piglets, experimental diets, and housing*

Ethical approval was received from the Comité d'étique du CRNA-DSM 123 in accordance with French Legislation. The animal trial was performed from May 18th to June 8th 2017 at the Research Centre for Animal Nutrition (DSM Nutritional Products France, Village-Neuf, France) according to the official French guidelines for experiments with live animals. Initially a total of 64 castrated crossbred male (Large-White x Redon) weaned piglets at 21 days of age (body mass 6.1 ± 0.6 kg, mean ± SD) were weighed and allocated to one of two treatment groups using a randomised block design with initial weight as the blocking factor. The two treatments consisted of feeding the animals with two different experimental diets: 1) Low Protein (LP; n = 32); 2) High Protein (HP; n = 32). The experimental diets used in the current study were based on corn, barley and soybean meal. The diets (Table 1) differed in crude protein (CP) content but contained the same amount of metabolisable energy (ME). Diet LP (Low Protein) contained 16.7% CP, and diet HP (High Protein) contained 21.0% CP supplemented with crystalline amino acids (AA). All other nutrients were supplied in amounts meeting or exceeding NRC (2012) nutrient standards for pigs weighing 6–10 kg. Diets were offered to pigs as mash. On day 12 of the study, 16 animals per treatment were selected randomly and euthanised to analyse other parameters not relevant for this manuscript. The remaining animals, 16 animals per treatment, continued the trial until day 19. Even though our dietary intervention generated diarrhoea in some of the animals, overall the animals remained healthy and their growth performance at the end of the study was as expected. Therefore, there were no other differences between the two groups.

Table 1 – Composition of the experimental diets, as feed basis.

Feed Ingredient	LP Content (%)	HP Content (%)
Corn	56.60	32.60
Barley	10.00	20.00
Soybean meal 48%	23.00	35.00
Soy concentrate	–	3.00
Soybean oil	3.00	4.00
Dicalcium phosphate	2.00	1.80
Calcium Carbonate	0.50	0.50
Salt	0.50	0.50
Vitamin-mineral Premix[a]	3.00	3.00
L-Met	0.30	0.20
L-Thr	0.30	0.10
L-Lys HCl	0.80	0.30
Analysed composition		
Dry Matter	89.40	89.41
Crude Protein	16.68	21.01
Fat	6.46	5.81
Fibre	2.23	2.69
Metabolisable Energy (MJ/kg)	13.45	13.68

[a] Vitamin-mineral premix provided per kilogram of diet: Vitamin A: 15,000 I.U.; Vitamin E: 100 mg; Vitamin K: 20.0 mg; Vitamin C: 100 mg; Vitamin B1: 3.00 mg; Vitamin B2: 10.00 mg; Vitamin B6: 6.00 mg; Vitamin B12: 0.04 mg; Pantothenic acid: 25.0 mg; Folic acid: 1.50 mg; Biotin 0.2 mg; Choline: 326 mg; Mn: 60.0 mg; Fe: 200 mg; Cu: 160 mg; Zn: 100 mg; I: 2.0 mg; Se: 0.4 mg; Lys: 2848 mg; Met: 513 mg; Thr: 1354 mg; Trp: 296 mg; L-Val 196 mg.

Pigs had unlimited access to feed and water throughout the experimental period. The body mass of the animals was monitored as an indicator of the growth performance of the animals. Room temperature was maintained at 31 °C during week 1 and reduced weekly by about 2 °C for the remaining period. The relative humidity was 50%. A 16 hour lighting system was also maintained in the room.

2.2. *Faecal sample collection*

Faeces from each pig were collected after 6, 12 and 19 days of nutritional intervention. Not all animals gave a sample at each time point. Each fresh sample was given a faecal score (FS) according to the consistency and appearance using the faecal consistency score system (Fig. 1) including 4°; where 0 = normal faeces, 1 = soft faeces, 2 = mild diarrhoea, and 3 = severe diarrhoea.

Samples were collected directly from the anus in a plastic container, kept on ice and immediately separated into different aliquots. The sampling was undertaken over a maximum duration of 2 hours, early in the morning. If an animal did not defecate during this time period, this sampling point was considered as 'missing'. One aliquot consisting of 1 g of faecal matter was heated at 105 °C for 24 h to measure the dry matter (DM) content of the faeces. The remaining aliquots were frozen and stored at −80 °C until analysis. The numbers of samples collected by dietary treatment per day are given in the Results section.

2.3. *Faecal volatile measurement*

In this study two different IMS (Ion Mobility Spectrometers) instruments were used. Specifically a G.A.S. FlavourSpec (Dortmund Germany), which uses GC-IMS principles (Gas Chromatography-Ion Mobility Spectrometry) and an Owlstone Lonestar (Cambridge, UK), which uses the principles of FAIMS (Field Asymmetric Ion Mobility Spectrometry). The FlavourSpec is equipped with a SE-54 mid-polarity column (CS Chromatographie, Germany) for gas chromatographic separation, based on chemical interactions with the column. This stage is followed by a drift tube IMS detector, whereby analytes are ionised and injected into a drift tube. The ions drift against a buffer gas under influence of a uniform electric field, where the ions achieve different velocities, inversely related to their size, mass and charge. The ions are then collected on a Faraday plate to produce a time-dependent signal that corresponds with ion mobility. This technique can measure substances in the low parts-per-billion (ppb) range and delivers measurement results in less than 10 minutes (Arasaradnam et al., 2018). In comparison, the Owlstone Lonestar instrument operates by measuring the movement of ions in high electric fields. In FAIMS, ions are pushed between two plates, onto these plates a high-electric field is applied that either attracts, repels or has no effect on the ions. The applied electric field is asymmetrical, such that a short positive high-electric field is followed by a longer smaller negative electric field, but with the time x electric field strength the same. Thus, ions that move more in the high electric field will drift towards one of the plates. If an ion touches a plate, it loses its charge. A compensation voltage is added to remove the effect of this drift and thus, by stepping through compensation voltage values, a range of ion movements can be measured. Furthermore, ion movement is not linear with electric field, thus the magnitude of the electric field is also stepped through a range of values (Lewis et al., 2017). A slightly different pre-analysis methodology was applied due to the two laboratories working independently at this stage and using their own internal methods.

For the analysis with the G.A.S. FlavourSpec, 500 ± 20 mg of each sample was weighed into a 20 mL glass headspace vial and suspended with 1 mL of a saturated sodium chloride solution by vortexing. The vials were closed with a magnetic screw cap and placed into the sample tray of the autosampler. After 20 min of incubation at 60 °C, 300 μL of sample headspace was automatically injected by a heated syringe. For GC separation, the following carrier gas flow gradient was applied: 2–40 mL min^{-1} (1–10 min), 40–80 mL min^{-1} (10–11 min), 80 mL min^{-1} (11–15 min). The IMS drift gas flow rate was kept constant at 150 mL min^{-1}.

The method for sample analysis using the Owlstone unit Lonestar unit required the samples to be placed in 10 mL glass headspace vials, with 20% by weight of ultra-pure water (UPW) added and then sealed with appropriate crimp vial caps containing a septum. The instrument was attached to a bespoke auto sampler setup (MPS, Gerstel, Germany). The samples were placed in a cooling tray set to 4 °C to reduce chemical degradation during sample wait time. When analysed, the samples are moved by the auto sampler into a heated agitator, where they were heated to 40 °C and agitated for 10 minutes. Once complete, the samples were purged into the Lonestar unit with a flow rate at 300 mL min^{-1} and a makeup flow rate of 1700 mL min^{-1}. Samples were analysed for 10 min using continuous flow over the sample, the dispersion field was

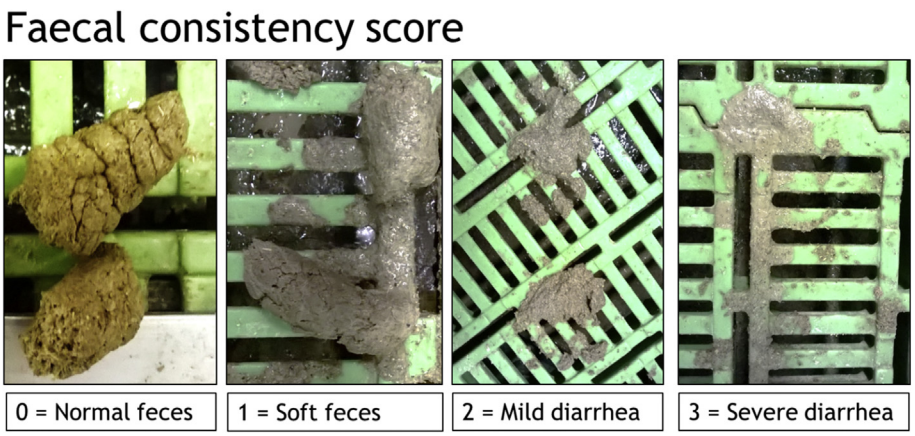

Fig. 1 – Faecal consistency score system.

stepped from 0 to 99% in 51 steps, compensation voltage +6 V to −6 V in 512 steps. Once a sample had been analysed, an air blank was run to reduce sample carry over. When sample analysis was complete the data files were exported using Owlstone's file export program (ver 4.6, Owlstone, UK).

2.4. *Statistical analyses*

Faecal score and faecal DM data were subjected to one-factorial analysis of variance with the animal as the experimental unit, using Newman–Keuls test in the StatGraphics Centurion XVI statistical software package (Manugistics, Rockwille, MD). The X^2 test was used to analyse frequency of diarrhoea between the 2 treatments. Statistical significance was considered at $p < 0.05$.

For VOC analysis, the instrument data were processed using our well-established pipeline, which has been developed specifically for these types of studies (Arasaradnam et al., 2018; Lewis et al., 2017). For FAIMS analysis, a pre-processing step is first applied to each sample in the form of a 2D wavelet transform (using Daubechies D4 wavelets). This performs two tasks, first as a data compression step and secondly, it can aid in the selection of chemical species by extracting 'peaks', which results in concentrating the chemical information into a small number of wavelet coefficients. This has the effect of improving and simplifying subsequent analysis steps. A threshold is then applied to remove data with little or no discriminatory power (background noise). For GC-IMS, the data are first cropped to leave only the areas of chemical interest and then a threshold is applied to remove background noise. In both cases, this is followed by a 10-fold cross-validation, with the data split into a training set and a test set. Within each fold, important features were identified using a Wilcoxon rank sum test from the training set and then applied to the test set using different classifiers. A total of four different classifiers were applied, specifically sparse logistic regression, random forest, support vector machines and neural network. From this, ROC (receiver operator curve) and area under the curve (AUC), specifically, sensitivity and specificity were calculated. The analysis was performed using R version 3.3.3.

3. Results and discussion

Faecal score, DM (%) and frequency of diarrhoea of weaned pigs are given in Table 2. Each sample is classified based on both dietary intervention and on faecal score. Pigs fed with the HP treatment had higher faecal consistency score and frequency of diarrhoea (considering faecal score 2 and 3) during the trial, in particular after 12 or 19 days of feeding. Consequently, lower faecal DM compared to LP treatment was obtained at every day of measurement (Table 2). A high negative correlation ($r = -0.86$) was found between both methods of diarrhoea evaluation.

Figure 2 shows example outputs from a G.A.S. FlavourSpec and Owlstone Lonestar. On the G.A.S. FlavourSpec data, each "spot" represents either a single chemical entity, or potentially

Table 2 – Effect of dietary intervention on the faecal score, faecal DM (%) and frequency of diarrhoea of weaned pigs.

Item	Treatment						P value
	LP			HP			
	n	Mean	SD	n	Mean	SD	
Faecal score							
D6	16	1.2[b]	1.05	8	2.0[a]	1.07	0.089
D12	21	1.3[b]	0.86	14	2.2[a]	0.89	0.006
D19	12	0.9[b]	0.67	8	2.3[a]	0.89	0.001
DM (%)							
D6	16	25.5[a]	8.80	8	17.4[b]	7.9	0.039
D12	21	29.3[a]	9.83	14	20.9[b]	8.8	0.015
D19	12	26.1[a]	3.92	8	19.1[b]	5.1	0.003
Frequency of diarrhoea							
	n	Frequency (%)		n	Frequency (%)		
D6-D19	54	25.90		25	80.00		0.0007

n = number of observations; Frequency = number of animals with diarrhoea (scores 2 or 3); LP low protein diet; HP high protein diet; D6, D12, D19 sampling days; DM dry matter.
[a,b] Means within a row that do not have a common superscript differ ($P < 0.05$).

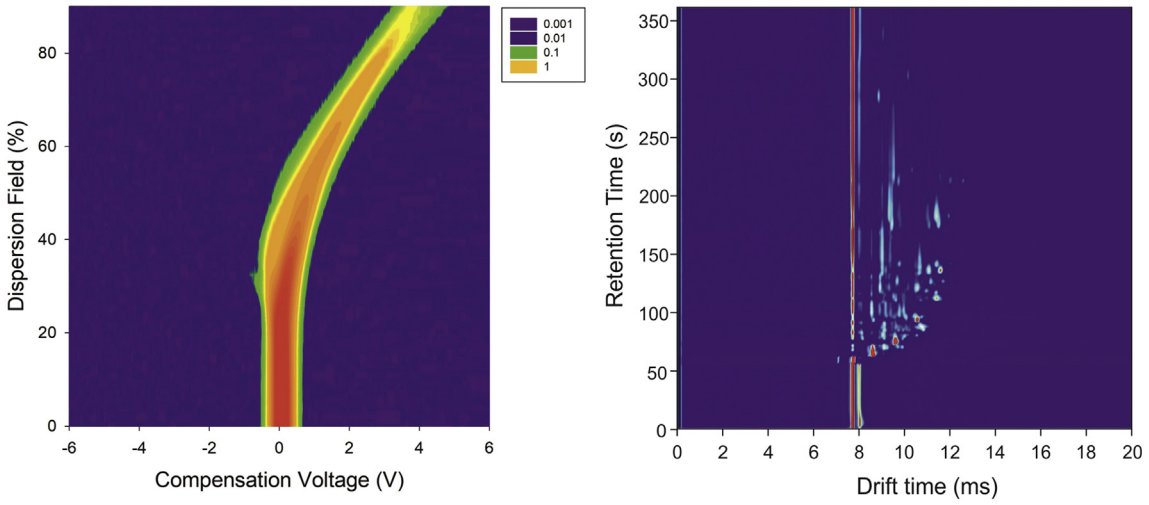

Fig. 2 – Typical data output from (a) Owlstone Lonestar and (b) G.A.S. FlavourSpec to a faecal sample.

more than one chemical with similar retention and drift time. For the Lonestar, as the electric fields are increased, the volatiles produce a plume-like data structure, with molecules with similar mobilities overlapping. Data from both instruments shows that the samples contain a significant amount of chemical information.

For the data analysis steps, the high-protein diet versus low protein diet was analysed. Figure 3 gives the ROC for this analysis and Table 3 provides a comparison between the results obtained with the two different technologies used in this study. It indicates that both instruments, to some extent, show that differentiation between high and low protein diet could be achieved, with the G.A.S. FlavourSpec providing the most notable separation.

Following this, each combination of faecal score class was evaluated (0 vs 1, 0 vs 2, 0 vs 3, 1 vs 2, 1 vs 3 and 2 vs 3). Table 4 shows the different scores attained for this analysis and the technology that produced the highest separation. In addition, Fig. 4 gives the ROC for the different analyses.

The aim of this paper was to investigate if faecal samples can be used as a means to identify pigs that were on different dietary interventions. Analysis of the faecal VOCs indicate that indeed this is possible, with a good sensitivity and specificity. The results also indicate that the two different measurement technologies were comparable (as seen with the complete analysis in the e-component). Faecal score differentiation of samples with consecutive scores was challenging, whilst scores with larger differences (for example group 0 vs 2), could more reliably be separated. This is not unexpected as significant differences are easier to both visually score and the

Table 3 – A comparison between the two analytical techniques in separation animals on a low and high protein diet. (results from all the classifiers are provided as an e-component linked to this paper). Numbers in brackets are 95% confidence intervals and are between 0 and 1).

	HP vs LP	HP vs LP
Instrument	G.A.S. FlavourSpec	Owlstone Lonestar
AUC	0.85 (0.75–0.95)	0.78 (0.66–0.90)
Sensitivity	0.90 (0.78–0.97)	0.61 (0.43–0.76)
Specificity	0.77 (0.59–0.90)	0.91 (0.71–0.99)
p-value	$1.05\ 10^{-9}$	0.0001

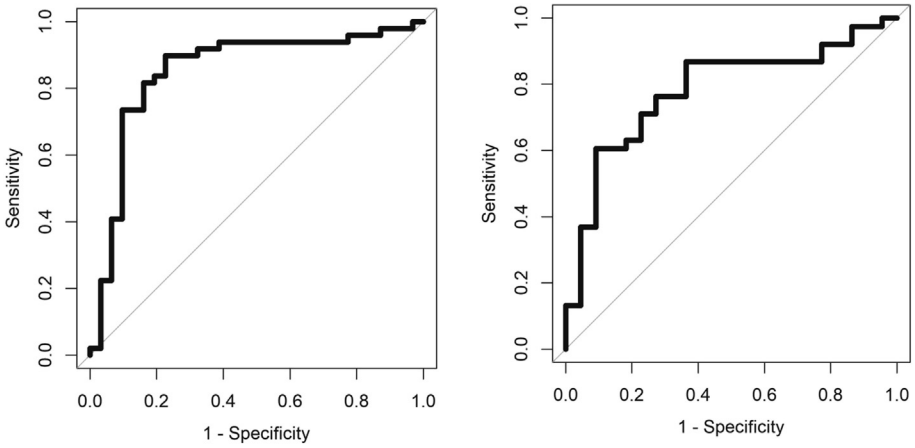

Fig. 3 – ROC for diet for LP vs HP, (a) G.A.S. FlavourSpec and (b) Owlstone Lonestar.

Table 4 – Technology by which the best separation of faecal scores could be achieved (full results are in Appendix S1).

Best	0 vs 1	0 vs 2	0 vs 3	1 vs 2	1 vs 3	2 vs 3
Instrument	FlavourSpec	FlavourSpec	FlavourSpec	Lonestar	FlavourSpec	FlavourSpec
AUC	0.76 (0.61–0.92)	0.82 (0.67–0.96)	1 (1–1)	0.86 (0.73–0.98)	0.97 (0.95–1)	0.89 (0.78–0.99)
Sensitivity	0.56 (0.30–0.80)	0.75 (0.48–0.93)	1 (0.79–1)	0.91 (0.76–0.98)	0.90 (0.74–0.98)	0.84 (0.60–0.97)
Specificity	0.90 (0.74–0.98)	0.84 (0.60–0.97)	1 (0.81–1)	0.67 (0.3–0.93)	1 (0.81–1	0.83 (0.59–0.96)
p-value	0.0014	0.0005	4.54E-10	0.0003	2.35E-11	7.49E-6

samples will have a more diverse chemical composition. The approach here was to use pattern recognition techniques to find features with discriminatory power. Therefore, at this stage, we are unable to state what the chemical differences are, and this is something we are currently pursuing. For a farm setting, FAIMS is closer to real-time whilst GC-IMS has a slightly longer analysis time. Both techniques are portable and can be used in the field, though not specifically the models as used here, and they have a similar unit cost. IMS has the potential to be used in a farm setting to analyse samples in a short period of time. Thus, the experimental model adopted in this study (high protein diet in early weaned piglets) was successful in challenging the gastrointestinal functionality of young piglets as reflected in the observed increase in diarrhoea in the piglets that were fed the HP diet. Previous investigations have shown the possible associations between the incidences of diarrhoea of piglets and dietary CP sources as well as levels (Heo et al., 2008; Kim, Heo, Mullan, & Pluske, 2011).

Though, in this study, we were unable to identify specific VOCs, we know that VOCs are a large and highly diverse group of compounds, which include hydrocarbons, alcohols, aldehydes, ketones, esters and organic acids. Furthermore, we know that a faecal sample represents the end-product of diet, digestive and excretory processes, and gastrointestinal microbial metabolism. Fluctuations in VOCs may be ascribed to changes in cellular (host) and microbial metabolism, which may be indicative of a diseased state (Pluske et al., 2018). In piglets affected by PWD, the composition of the intestinal microbiota is disrupted (Pluske et al., 2002). It is quite plausible that these changes affect the proportions of VOCs in the faecal samples. This is consistent with our results, where FAIMS and GC-IMS were able to discriminate samples collected from piglets fed diets with different protein content and faecal samples of different consistency.

We acknowledge that our study has some limitations and that the data gathered so far present the opportunity to improve the design of future experiments. While the number of samples was sufficient to allow piglets fed a HP or LP diet to be distinguished and to relate faecal VOCs to the faecal scoring system, a higher number of samples would be desirable to increase the sensitivity to discriminate consecutive faecal scores (especially 0 from 1) using the different instruments evaluated in this study. Also, as faecal VOCs

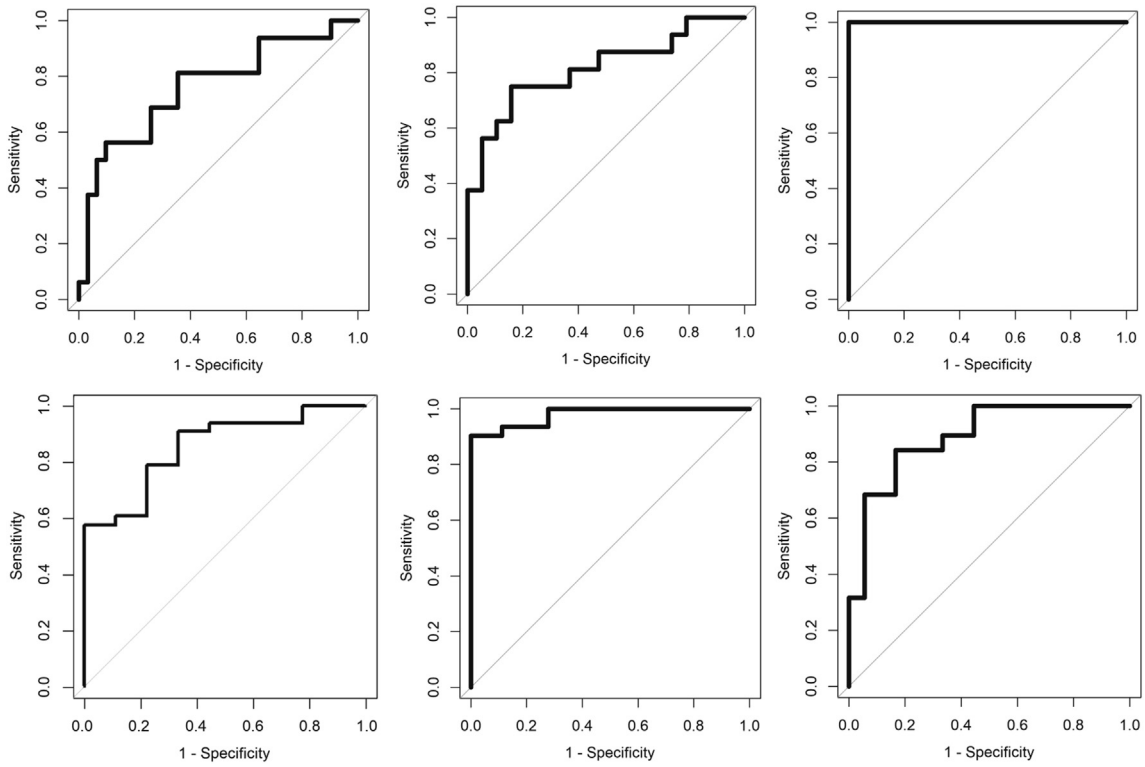

Fig. 4 – ROC for faecal score. Top row (left to right) scores 0 vs 1; 0 vs 2; 0 vs 3; Bottom row (left to right) 1 vs 2; 1 vs 3; 2 vs 3.

provide us with valuable information about the gastrointestinal microbiota composition and metabolic activity, further studies should also include the characterisation of the microbiota. This approach would also allow the identification of individual VOCs that might be specific for certain bacteria (commensal or pathogenic). Finally, further studies should consider longitudinal changes in faecal VOCs as temporal changes might be more indicative than absolute levels. Therefore, the characterisation of VOCs in faecal samples may facilitate the development of a rapid non-invasive tool to monitor gastrointestinal functionality (Celi et al., 2019).

4. Conclusions

In this paper, we investigated volatiles in faecal samples as a means to monitor gastrointestinal health expressed as different grades of diarrhoea. Piglets were fed with two different dietary interventions and faeces samples were collected over a period of 19 days. The volatiles emanating from samples were later analysed using different IMS instruments. The analysis of the data indicated that volatiles from faeces provide a reasonable means to separating piglets on different dietary interventions. Furthermore, they could also provide a means of replacing faecal score in diagnosis of diarrhoea, independent of dietary intervention. Overall, the data gathered in this study suggest that the detection of VOCs from faecal samples holds great potential to develop into a non-invasive tool to monitor gastrointestinal functionality in piglets.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biosystemseng.2019.06.005.

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