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Research Article

Common colonic community indicators of the suckling pig microbiota where diversity and abundance correlate with performance

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One sentence summary: Common colonic community indicators can be identified from suckling pigs in repeated trials without major significant differences in diversity, abundance, or microbiota composition.
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Abstract

The primary objective of this study was to investigate if common colonic community indicators could be identified from the microbiota of 22-day-old suckling pigs in repeated small-scale trials. A total of three separate trials were conducted at different times in the same year and facility with genetically similar animals. Colonic samples were collected from four pigs in each trial and the microbiome composition assessed by 16s rRNA gene sequencing. Pig weight, average daily gain (ADG), bacterial diversity, and abundance were not significantly different between repeated trials, except for a significant difference in Jaccard Similarity. At genus level, the most abundant taxa identified were *Porphyromonadaceae* unclassified (15.81%), *Ruminococcaceae* unclassified, (12.78%), *Prevotella* (7.26%), *Clostridiales* unclassified (6.99%), *Lactobacillus* (6.58%), *Phascolarctobacterium* (6.52%), and *Firmicutes* unclassified (5.69%). The secondary objective was to establish if pooled data in terms of microbial diversity and abundance of the colonic microbiota related to weight and ADG. Pig weight at day 22 and ADG positively correlated with α-diversity. Abundance of potential protein digesting and short-chain fatty acid producing operational taxonomic units ascribed to *Terrisporobacter*, *Ruminococcaceae* unclassified, *Intestinimonas*, and *Dorea* correlated with weight and ADG, suggesting a nutritional role for these common colonic community microbiota members in suckling pigs.

Keywords: microbiota, pigs, suckling, colonic, diversity, performance

Introduction

Suckling is a unique period in Porcine development, where the early-life environment significantly affects the development and composition of the adult microbiota. The gastrointestinal tract (GIT) microbiota, contribute to the developmental and metabolic needs of animals through vitamin synthesis, short-chain fatty acid (SCFA) production, complex carbohydrate digestion, and immune system regulation (Brestoff and Artis 2013; Kim and Isaacson 2015). In neonatal pigs, development of the intestinal microbiota is a gradual and sequential process (Inoue *et al.* 2015) in which the GIT is colonized by bacteria from maternal, and environmental sources (Katouli *et al.* 1997; Konstantinov *et al.* 2006; Thompson *et al.* 2008). During suckling, the formation of an increasingly differential, milk-oriented and protective *Lactobacillaceae* rich microbiota is favoured (Mulder *et al.* 2009; Petri *et al.* 2010; Frese *et al.* 2015; Bian *et al.* 2016). This is a unique period in porcine development, where acquisition of maternal immunity (Salmon *et al.* 2009) and the early-life environment heavily influences the development and composition of the adult microbiota and intestinal innate immune functions (Bauer *et al.* 2006; Mulder *et al.* 2009; Merrifield *et al.* 2016). In addition, GIT microbiota diversity may be predictive of the susceptibility of the animals to enteric disease postweaning (Dou *et al.* 2017). Indeed, diversity at weaning might not be an accurate predictor of diversity in later life, but earlier measures preweaning, may be more predictive (Lu *et al.* 2018). The abundance and diversity of the pig GIT microbiota increases with age (Niu *et al.* 2015; Chen *et al.* 2017), with operational taxonomic units (OTUs) ascribed to *Lachnospiraceae*, *Ruminococcaceae*, *Prevotella*, *Treponema*, and *Bacteroides* showing association with fatness in older pigs (He *et al.* 2016). It has also been shown that piglets with above average daily gain (ADG) had significantly higher abundances of *Lactobacillus*, unclassified *Ruminococcaceae*, and unclassified *Prevotella* (Gaukroger *et al.* 2020), and that microbial richness positively correlated with weight gain in preweaning pigs (Ding *et al.* 2019), thus indicating the link between microbiota composition and performance. Considering the profound influence of weaning weight on the lifetime growth and health performance (Collins *et al.* 2017), there is a lack of information on performance and the association between microbial diversity and abundance in the suckling pig. There are multifactorial influences on microbial diversity and composition, these being succession of bacterial populations, the age of the animal, the environment it inhabits, use of antimicrobial agents, dietary composition, stress, and genetics, to name but a few (Pluske *et al.* 2018).

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Given these influences and large variations between studies (Holman *et al*. 2017), there has been little discussion of the use of data between separate trials that have been repeated over time with the prospect of data pooling (Thompson *et al*. 2008; Pajarillo *et al*. 2015; Chen *et al*. 2017). The availability of controlled rearing environments with large animals and the incumbent costs to achieve reproducibility are important design considerations, which may dictate that small-scale trials are repeated to achieve statistical significance, in contrast to Murine models, which accommodate much larger study populations through ease of animal size and housing. Moreover, animal studies often pool data from repeated trials without due consideration of variance between trials and if they can be truly compared, especially for control data (Frommlet and Heinze 2021). In this study, we have attempted to reproduce three suckling pig trials in which multifactorial influences, as described above, on the microbiota were controlled for as far as possible. Not all variables can be controlled, e.g. succession of bacterial populations. However, examining animals of the same age, similar genetic traits, reproducing environmental and housing conditions, diets, and reducing physical contact between animals may allow studies to be compared. Indeed, long-term cohousing increases the similarity of pig faecal microbiota (Pajarillo *et al*. 2015) and there is a significant correlation between the intestinal microbiota of cohoused pigs particularly at 3–4 weeks postpartum (Thompson *et al*. 2008). We have attempted to reduce these pen effects in this study since samples relate to independently housed suckling pigs ($n = 12$ litters where "n" is the pen) rather than cohoused animals who might influence each other's microbiota as in other studies (Chen *et al*. 2017). In these respects, the objectives of this study were to investigate if common colonic community indicators could be identified from the microbiota of suckling pigs in repeated small-scale trials and if pooled data in terms of microbial diversity and abundance of the colonic microbiota related to animal performance in three controlled reproducible trials.

Materials and methods

Animals and trial design

This study was carried out under license and in accordance with UK Animals, (Scientific Procedures) Act 1986. All procedures were approved by the Local Ethics Committee of the University of Nottingham.

For all trials, A, B, and C, Landrace x Large white sows of parity seven were artificially inseminated with the same batch of Titan semen (JSR Genetics, Driffield, UK). Titan semen was pooled from three sibling boars bred from the same Piétran line and selected for similar breeding traits, as per industry standard by the supplier. On day 113 of gestation, sows were moved to individual 3.8 × 2.4 m farrowing pens with a 2.1 × 0.62 m farrowing crate (Figure S1, Supporting Information). Animals were housed in a single facility in separate pens. Pens were of solid concrete block construction with 1.5 m high walls. There were no apertures through which animals could physically contact one another through pens. Each pen was provided with its own colour coded tools for cleaning so as not to cross-contaminate pens. Pens had two secure lockable metal gates at opposite ends. One for allowing feeding of sows without technicians standing on bedding and one for removing contaminated bedding into a concrete corridor for disposal. This area was 60 cm lower than the base of the pen so that any "run off" could not contaminate other pens. The solid concrete construction of pens allowed no egress of contaminated bedding or fluid "run off" between pens. Technicians wore disposable gloves, facemasks, overshoes, and suits when cleaning pens and attending to animals. These were changed when attending to different pens. Trials were conducted during A; January–February, B; April–May, and C; October–November 2010 with four litters per trial kept under identical housing and environmental conditions. Pens were deep cleaned with Virkon between trials (VWR International Ltd, Lutterworth, UK). Pens were not used for any other experiments in between trials. Farrowing pens contained a 1 × 1 m piglet box heated with an industry standard heat lamp. Animals were bedded on a mixture of dust extracted straw and hemp bedding (Aubiose, Datesand Ltd., Stockport, UK) on concrete in farrowing pens and on straw, on plastic slats, and in weaning pens. Bedding was obtained from the same source for all three trials. Metal chain toys with plastic balls were provided in weaning pens as environmental enrichment. Temperature was kept at range 18–20°C for sows and 23–24°C for piglets with light periods from 7:30 a.m. to 7:00 p.m. Sows received a wheat-based lactation diet (BOCM Pauls ltd, Wherstead, UK) containing 16% protein, 4.5% oil, 5.5% crude fibre, 5.5% ash, 0.75% lysine, 1000 iu.kg^{-1} vitamin A, 2000 iu.kg^{-1} vitamin D3, 100 iu.kg^{-1} vitamin E, 0.40 mg.kg^{-1} selenium, and 25 mg.kg^{-1} copper, plus water *ad libitum*. For prevention of iron deficiency and coccidiosis, new-born pigs received a 1-ml intramuscular iron injection (Gleptosil, Alstoe Ltd, York, UK) 24 hours after birth, 0.7 ml of Baycox toltrazuril coccidiostat (Bayer, Newbury, UK) orally 3 days after birth and were ear tagged at day 5 for identification. Pigs did not receive any creep feed supplementation or any other prophylactic antibiotic treatment during the trials. Pigs were cross-fostered within 24 hours of birth to achieve homogenous litter size for welfare purposes and as per standard industry practice. However, cross-fostered pigs were excluded from euthanasia for collection of colonic samples. Not all pens had cross-fostered piglets. In Trial A, pen 2 had two cross-fostered pigs. Pens 1–4 contained 12, 13, 12, and 13 pigs, respectively. In Trial B, pen 4 had two cross-fostered pigs. Pens 1–4 contained 11, 12, 11, and 12 pigs, respectively. In Trial C, pen 1 had two cross-fostered pigs and pen 2, one cross-fostered pig. Pens 1–4 contained 10, 10, 10, and 12 pigs, respectively. From 24 hours of birth (post cross-fostering), to day 22 of sampling, there was no contact between litters and sows of different pens. Pigs were individually weighed at days 5, 12, 19, and 22 to determine ADG, with one pig per litter randomly selected at day 22 for euthanasia by intraperitoneal injection of Dolethal (1 ml kg^{-1} body weight; 20% w/v Pentobarbitone Sodium, Vétoquinol, Buckingham, UK).

Sample collection and DNA extraction

Samples of digesta from euthanized pigs were aseptically collected from the colonic lumen and held on ice for 5 minutes prior to transfer to the laboratory and storage at −80°C until bacterial DNA isolation. Bacterial DNA was isolated from ∼0.2 g colonic contents using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co. KG., GER) according to the manufacturer's instructions.

PCR amplification of 16S rRNA gene sequences

Using the isolated DNA as a template, the V4 region of the bacterial 16S rRNA genes were PCR amplified using primers 515f (5′ GTGCCAGCMGCCGCGGTAA 3′) and 806r (5′ GGACTACHVGG GTWTCTAAT 3′; Caporaso *et al*. 2011). Amplicons were sequenced on the Illumina MiSeq platform (Illumina, Inc., USA) using 2 × 250 bp cycles by LGC Genomics GmbH (GER). Sequence data were deposited in the NCBI database within the Bioproject PRJNA494528 under the SRA study SRP164374.

Microbiota diversity analysis

The 16S rRNA sequence analysis was performed using Mothur v. 1.39, using default settings (Schloss *et al*. 2009). Analysis was performed according to the MiSeq SOP (accessed online 09/11/2017; Kozich *et al*. 2013). The 16S rRNA gene sequences were aligned against a reference alignment based on the SILVA rRNA database (Pruesse *et al*. 2007) for use in Mothur (release 128; available at: https://www.mothur.org/wiki/Silva_reference files) and clustered into OTUs using the "opticlust" clustering algorithm (Westcott and Schloss 2017). The similarity cut off for OTUs was 0.03. The consensus taxonomy of the OTUs was generated using the "classify.otu" command in Mothur with reference data from the Ribosomal Database Project (version 14; Wang *et al*. 2007; Cole *et al*. 2014) adapted for use in Mothur (available at: https://www.mothur.org/wiki/RDP_reference_files).

Statistical analyses

Coverage and α-diversity expressed as Inverse Simpson diversity (Magurran 2004) and Chao Richness (Chao 1984) were calculated using the "summary.single" command in Mothur (Schloss *et al*. 2009). Quantile plots and Shapiro–Wilk tests (Shapiro and Wilk 1965) were used to determine normality for pig weights at days 5, 12, 19, and 22, ADG and α-diversity metrics. Significant differences were tested using ANOVA in R Studio (v4.1.1) with repeated measures for weight (R Core Team 2021). Estimates of β-diversity were calculated in Mothur as Yue and Clayton Dissimilarity (θ_{YC}; Yue and Clayton 2005), Bray–Curtis Dissimilarity (Bray and Curtis 1957) and Jaccard Similarity (Jaccard 1901). Homogeneity of variance for all three β-diversity metrics were analyzed by the Levene test (Levene 1960) using the "Car" package (v3.0-11) in R Studio. Analysis of molecular variance executed in Mothur (AMOVA) was used to test for differences in β-diversity between samples (Excoffier *et al*. 1992; Anderson 2001). Similarity Percentage (SIMPER) analysis was used to identify OTUs that most contributed to Bray–Curtis β-diversity measures (Clarke 1993) as performed in the "Vegan" Community Ecology Package (v2.4-3) in R Studio (Oksanen *et al*. 2017). Linear discriminant analysis effect size (LEfSe) was used to examine differential OTU abundances in Mothur (Segata *et al*. 2011). The abundance of phyla and OTUs at the genus level were analyzed by Kruskal–Wallis rank sum tests (Kruskal and Wallis 1952) to determine differences between trials. Correlations between pig weights, ADG, abundance of phyla, and OTUs at the genus level were analyzed by Kendall rank sum correlations with regression analysis performed using linear modelling in R as previously reported (Dill-McFarland *et al*. 2017). Pig weights at day 22, ADG, and α-diversity were correlated using Pearson's Product-Moment Correlation, with regression analysis performed using linear modelling in R Studio. Where appropriate, multiple comparisons (ANOVA and AMOVA) were adjusted for false discovery rates (FDR) by the Benjamini and Hochberg procedure (Benjamini and Hochberg 1995).

Results

Weight and ADG distribution

Pig weights at days 5, 12, 19, and 22 and ADG were normally distributed according to quantile plots, $R^2 = 0.96, 0.96, 0.95, 0.91$, and 0.94, respectively and Shapiro–Wilk normality tests, $P = .64, .78, .51, .27$, and 0.42, respectively. A total of four pigs each from separate litters in their own pens were analyzed for each trial, A, B, and C. Weights at days 5, 12, 19, and 22 and ADG were not significantly different between trials A, B, and C; $P = .92, .92, .78, .84$, and .79, respectively (ANOVA; Table 1). Weight significantly increased with time where D5–D12 $P = .002$, D12–D19 $P < .001$, and D19–D22 $P < .001$ (adjusted).

Colonic microbiota diversity

A total of 357 133 high quality V4 16S rRNA sequence reads were obtained from twelve suckling pig colonic microbiota samples, from which 8718 sequences per sample were subsampled to achieve a coverage of 97%–99%. Inverse Simpson diversity and Chao Richness were normally distributed according to quantile plots, $R^2 = 0.90$ and 0.97, respectively and Shapiro–Wilk normality tests, $P = .21$ and $P = .88$, respectively. Inverse Simpson diversity and Chao Richness were not significantly different between trials A, B, and C, $P = .70$ and $P = .10$, respectively (ANOVA; Table 1). Calculated β-diversity θ_{YC} and Bray–Curtis distances between trials A, B, and C were not significantly different, as determined by AMOVA, $P = .586$ and $P = .109$, respectively. Jaccard distances were significantly different for the overall comparison of trials A, B, and C, $P = .008$, but not for pairwise comparisons A–B, $P = .07$, A–C, $P = .09$, or B–C, $P = .09$ (Fig. 1). There were no significant differences in homogeneity of variance between trials for all three β-diversity metrics when analyzed by the Levene test, $P > .05$ in each case.

Colonic microbiota composition

Sequences were clustered into 4520 OTUs and classified into 18 phyla, 35 classes, 54 orders, 108 families, and 214 genera. Of these, 4132 OTUs occurred in colonic samples from all trials at the genus level. The remaining OTUs were exclusive to colonic samples from pigs in trials A (112), B (104), and C (64; Fig. 2). Relative abundances of bacterial taxa at the phylum and genus level for colonic samples from the three separate trials are shown in Fig. 3. The predominant phyla were *Firmicutes* (55.68%), *Bacteroidetes* (33.68%), *Proteobacteria* (1.64%), and *Spirochaetes* (1.37%). Unclassified bacteria accounted for 6.22% of the total sequences. There were no significant differences in phyla abundance between trials when analyzed by Kruskal–Wallis rank sum tests, $P > .05$. At the genus level, the most abundant taxa identified were *Porphyromonadaceae* unclassified (15.81%), *Ruminococcaceae* unclassified, (12.78%), *Prevotella* (7.26%), *Clostridiales* unclassified (6.99%), *Lactobacillus* (6.58%), *Phascolarctobacterium* (6.52%), and *Firmicutes* unclassified (5.69%). The top 30 OTUs accounted for 95.69% of total relative abundance, in contrast to the remaining 4490 OTUs, which accounted for the remaining 4.31% total relative abundance indicating the nonparametric and skewed distribution of OTUs identified.

OTUs contributing to variation in the Bray–Curtis dissimilarity indices were identified by analysis of similarity percentages (SIMPER). For trial comparison A–B, 49 OTUs contributed up to 70.08% of the variation, for comparison A–C, 46 OTUs contributed up to 70.27% of the variation, and for comparison B–C, 47 OTUs contributed up to 70.10% of the variation. Overall, 65 different OTUs representing 26 genera contributed up to 70% variation across all three trials (Figure S2, Supporting Information). In order of rank, the top 10 OTUs contributing the most variance between trials A, B, and C were the *Porphyromonadaceae* unclassified (12.27%–15.91%), *Prevotella* (0.84%–9.18%), *Ruminococcaceae* unclassified (4.15%–9.16%), *Lactobacillus* (4.71%–8.38%), *Phascolarctobacterium* (3.15%–4.68%), *Clostridiales* unclassified (2.65%–5.16%), *Oscillibacter* (0.75%–2.37%), *Bacteroidetes* unclassified (1.12%–2.29%), *Firmicutes* unclassified (2.15%–5.16%), and *Faecalibacterium* (0.00%–2.11%). In addition, relative abundance of OTUs grouped into genera positively correlated with variance for each trial comparison

Table 1. Pig weights, ADG, and α-diversity of colonic samples.

[1]Trial	[2]Weight at day 5 (kg)	[2]Weight at day 12 (kg)	[2]Weight at day 19 (kg)	[2]Weight at day 22 (kg)	[2]ADG (d5–d22) kg/day	[2]Inverse Simpson Diversity	[2]Chao Richness
A	2.48 (0.27)	4.58 (0.42)	6.97 (0.60)	7.75 (0.43)	0.31 (0.02)	14.29 (2.20)	1241.08 (171.68)
B	2.34 (0.52)	4.38 (0.87)	6.40 (1.36)	7.38 (1.63)	0.30 (0.07)	17.97 (12.00)	1794.48 (250.51)
C	2.45 (0.46)	4.43 (0.50)	6.57 (0.86)	7.25 (0.75)	0.28 (0.04)	20.20 (7.94)	1679.88 (395.45)

[1]Trial A conducted January–February, B April–May, and C October–November 2010.
[2]Values are means (SD). Means are not significantly different between trials (ANOVA, $P = .92$, $P = .92$, $P = .78$, $P = .84$, $P = .79$, $P = .70$, and $P = .10$, respectively). Mean weight significantly increased with time (ANOVA, D5–D12 $P = .002$, D12–19 $P < .001$, and D19–D22 $P < .001$).

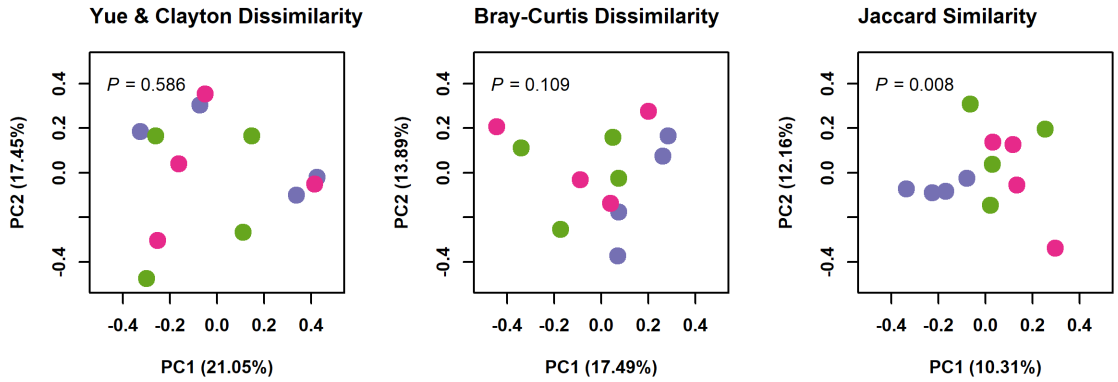

Figure 1. β-diversity of colonic samples from 22-day-old suckling pigs in three separate trials conducted at different times of year. Purple circles Trial A, pink Trial B, and green Trial C.

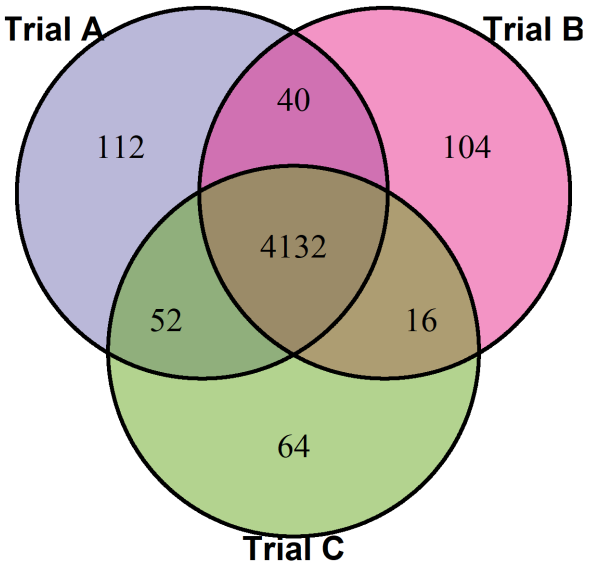

Figure 2. Venn diagram depicting unique and shared OTUs at the genus level in colonic samples from pigs in three separate trials, A, B, and C.

A–B, A–C, and B–C, $P < .001$ in each case. That is, the greater the relative abundance of named genera, the greater the variance lent to Bray–Curtis diversity measures (Figure S3, Supporting Information). Unclassified bacteria accounted for 2.57%–5.27% of the variation between trials. A total of 20 OTUs were identified by SIMPER as contributing variation to each trial comparison A–B, A–C, and B–C. However, their abundance across all three trials, A, B, and C was not significantly different following Kruskal–Wallis rank sum tests, $P > .05$ in each case. LEfSe did not identify differentially abundant OTUs occurring at ≥ 1% between trials with the exception of a greater abundance of one unclassified OTU at the genus level from trial A, $P = .01$.

Performance and diversity

Suckling pig weight at day 22 and Inverse Simpson Diversity correlated, where $r = 0.62$ (Pearson Correlation Coefficient), $R^2 = 0.38$ and $P = .032$ (linear modelling). Similarly, ADG and Inverse Simpson Diversity correlated, where $r = 0.59$, $R^2 = 0.35$, and $P = .042$ (Fig. 2). However, there was no correlation between weight at day 22 and Chao Richness where $r = 0.16$, $R^2 = 0.03$, and $P = .62$ or correlation between ADG and Chao Richness where $r = 0.10$, $R^2 = 0.01$, and $P = .75$ (Fig. 4).

Performance and abundance

Suckling pig weights at day 22 and ADG were correlated with abundance of phyla and OTUs at the genus level using Kendall rank correlations and assessed by subsequent linear modelling. The abundance of phyla did not correlate with weight or ADG where $P > .05$ in each case. However, the abundance of four OTUs at the genus level showed positive correlations with weight: *Terrisporobacter,* (Kendal Tau $\tau = 0.67$, $R^2 = 0.40$, and $P = .046$), *Ruminococcaceae* unclassified, ($\tau = 0.44$, $R^2 = 0.34$, and $P = .046$), *Intestinimonas,* ($\tau = 0.44$, $R^2 = 0.54$, and $P = .02$), and *Dorea*, ($\tau = 0.41$, $R^2 = 0.58$, and $P = .017$). A total of two OTUs at the genus level showed positive correlations with ADG: *Intestinimonas,* ($\tau = 0.27$, $R^2 = 0.53$, and $P = .024$) and *Dorea*, ($\tau = 0.36$, $R^2 = 0.51$, and $P = .024$; Fig. 5).

Discussion

The primary objective of this study was to compare variation in the microbiota of suckling pigs from three separate trials conducted at different times of year and to determine if common

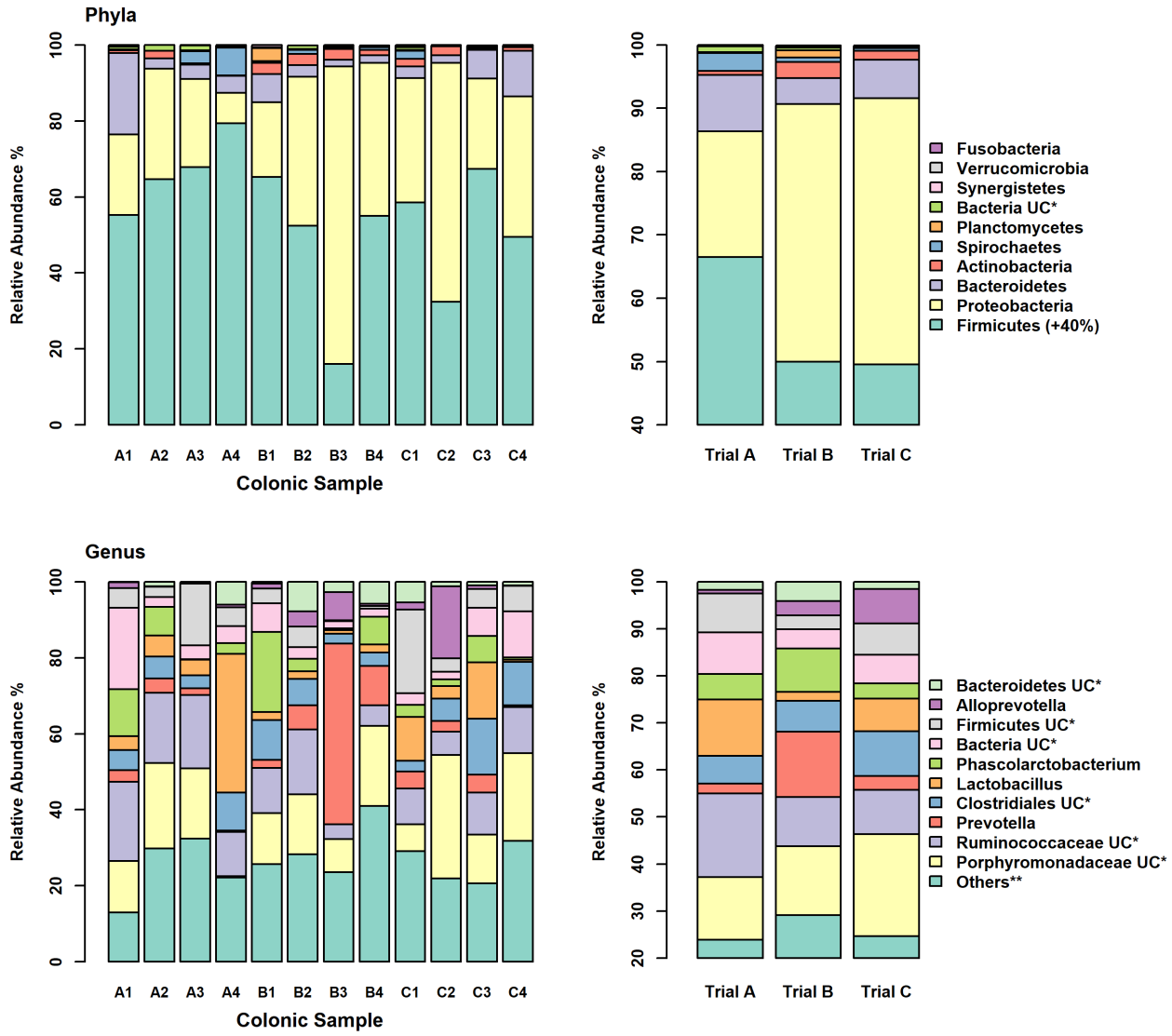

Figure 3. Relative abundance of bacterial taxa annotated to OTUs at the phyla and genus level as identified from colonic samples of 22-day-old suckling pigs in three separate trials conducted at different times of year. *UC = unclassified at the phylum or genus level. **Others = remaining 4490 OTUs comprising 4.31% of the total relative abundance.

colonic community indicators could be identified. Previous studies have analyzed pooled data from separate trials to establish community trends (Thompson *et al.* 2008; Kim *et al.* 2011; Pajarillo *et al.* 2015; Chen *et al.* 2017). This study has verified this approach with suckling pigs and demonstrated that whilst intertrial variation including significant differences in Jaccard Similarity exist, data may still be analyzed and compared to establish community relationships with attendant gains in statistical power. Whilst it is generally accepted that pig microbiota from different groups converge to a similar state over time (Kim *et al.* 2011; Bian *et al.* 2016) the variation needs to be more closely examined. In this study, each colonic sample was taken from suckling pigs in separate pens born from different sows. Cross-fostering for welfare issues may have introduced microbiota from other pens within the first 24 hours, but was limited to only one pen in trials A and B and two pens in Trial C. After this time, there was no physical contact between pigs in different pens. That is, neither sow nor piglet could influence one another across samples except for the direct effect of the nursing sow on the suckling piglet. This was probably the most influential factor for the development of neonatal bacteria during suckling (Bian *et al.* 2016). Other factors include the immediate early-life environment and the genetic background of the animals (Mulder *et al.* 2009; Merrifield *et al.* 2016), which were replicated, as far as possible, in these trials through use of the same facility, breed of sows, and batch of semen for artificial insemination. Long-term cohousing increases the similarity of pig faecal microbiota (Pajarillo *et al.* 2015), and there is a significant correlation between the intestinal microbiota of cohoused pigs particularly at 3–4 weeks postpartum (Thompson *et al.* 2008). These effects have been reduced in this study since samples relate to independently housed suckling pigs ($n = 12$ litters where "n" is the pen) rather than cohoused animals who might influence each other's microbiota as in other studies (Chen *et al.* 2017).

Results show that suckling pig weights at days 5–22 and ADG were normally distributed and not significantly different between trials indicating that animals could be compared. Furthermore, Inverse Simpson diversity and Chao Richness were normally distributed with no significant differences between trials (Table 1). In a meta-analysis of 91 pig colonic samples mean (SD), Inverse Simpson Diversity was reported to be 33.2 (22.6; Holman *et al.*

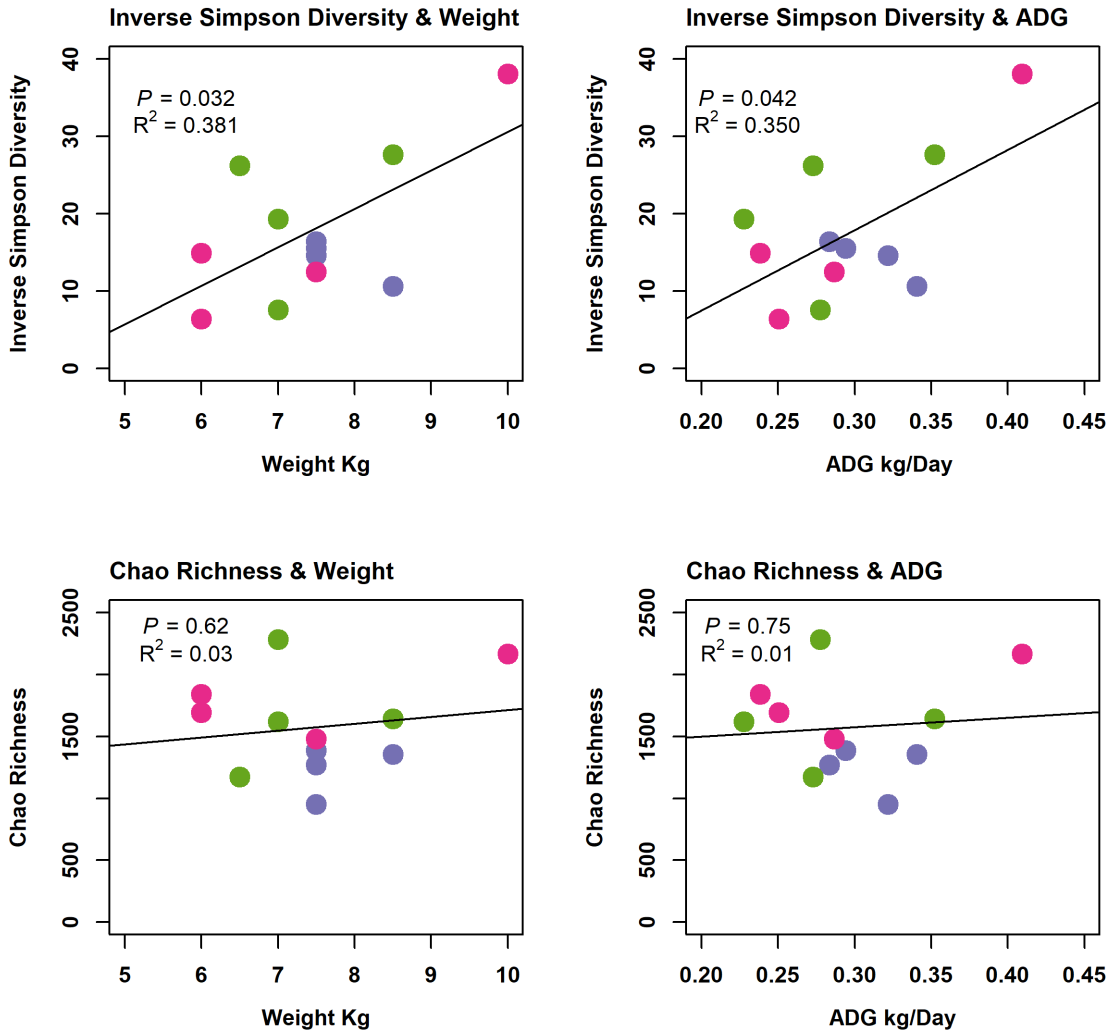

Figure 4. Correlations between performance and α-diversity of colonic samples from 22-day-old suckling pigs in three separate trials A, B, and C conducted at different times of year. Purple circles Trial A, pink Trial B, and green Trial C.

2017). However, these samples included older pigs, where the abundance and α-diversity of gut microbiota are known to significantly increase with age (Kim *et al.* 2011; Niu *et al.* 2015; Chen *et al.* 2017). Chao Richness results of other comparable studies with 21-day-old pigs are more variable, presumably due to differences in environmental conditions and/or breeds (Thompson *et al.* 2008; Bian *et al.* 2016). At suckling, Chao Richness was determined as 290.0 (Vo *et al.* 2017) and 1240.3 (Holman and Chénier 2014) for faecal samples and 1757 for colonic samples (Hoeflinger *et al.* 2015), the latter two in agreement with the present findings (Table 1). However, α-diversity metrics are highly dependent on the region sequenced, sequencing technology, depth, quality control postsequencing, and the reference database used. Thus, comparisons between studies may be confounded and difficult to compare.

β-diversity was modelled as Yue and Clayton Dissimilarity (θ_{YC}; Yue and Clayton 2005), Bray–Curtis Dissimilarity (Bray and Curtis 1957), and Jaccard Similarity (Jaccard 1901), with the model of best fit being θ_{YC} which explained 39% of the variance between trials in two dimensions. AMOVA of the θ_{YC} and Bray–Curtis metrics indicated no significant differences between trials, both metrics taking account of presence and abundance of OTUs. In contrast, there were significant differences between trials when using Jaccard Similarity as one of three metrics for analysis by AMOVA where $P = .008$ for overall comparisons. This metric compares samples based on the presence or absence of species and has revealed differences in colonic microbial community structure mainly between Trial A and Trials B and C. That is, Trial A was less similar to trials B and C, which had a greater similarity to each other (Fig. 1) in terms of species richness. This may be due to the greater abundance of *Proteobacteria* in colonic samples from trials B and C in contrast with Trial A as seen in Fig. 3. Nevertheless, community membership of the faecal microbiota, as measured by Jaccard Similarity and community structure as measured by θ_{YC}, significantly differ with pig age, underlying the importance of repeating studies with pigs of the same age if trials are to be compared (Slifierz *et al.* 2015). Likewise, interindividual Bray–Curtis distances between different pigs increased significantly during the suckling period and reduced postweaning, with no significant differences noted between two replicated trials (Chen *et al.* 2017).

In this study, a total of 4132 OTUs (91.4%) occurred in pigs in all trials A, B, and C, with *Firmicutes* and *Bacteroidetes* the dominant phyla, a result in keeping with previous studies of similarly aged suckling pigs for colonic (Jacobi *et al.* 2016; Zhang *et al.* 2016; Leblois *et al.* 2017) and faecal samples alike (Kim and Isaacson 2015; Chen *et al.* 2017; Vo *et al.* 2017). The fourth most abundant phylum were the *Proteobacteria* (1.64%), which are known to in-

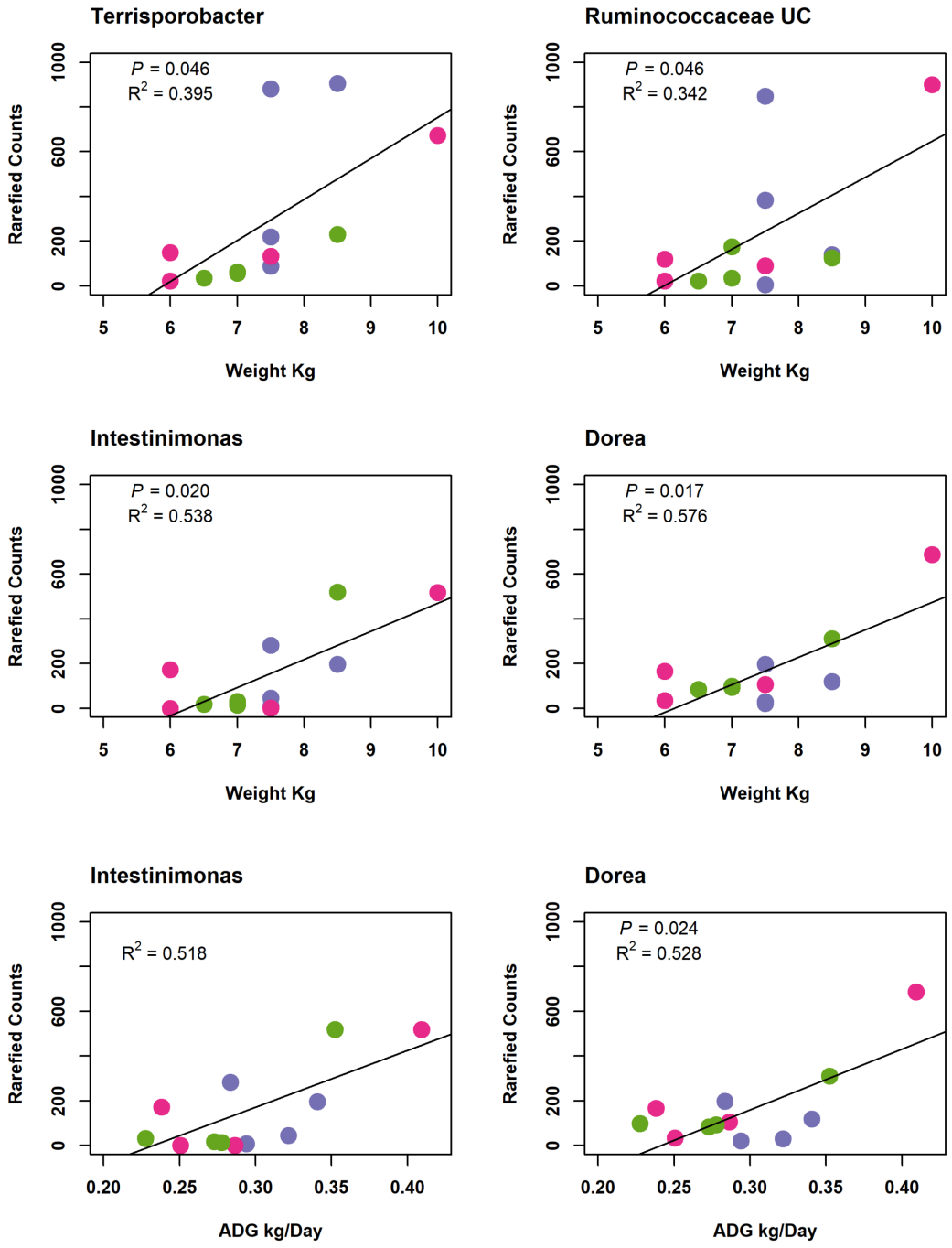

Figure 5. Correlations between performance and abundance of OTUs at the genus level of colonic samples from 22-day-old suckling pigs in three separate trials conducted at different times of year. Purple circles Trial A, pink Trial B, and green Trial C.

clude a wide variety of opportunistic, potentially pathogenic organisms such as *Campylobacter*, *Escherichia*, *Salmonella*, and *Helicobacter* (Madigan 2018). Although these OTUs occurred at very low relative abundances and may be commensal, their presence highlights the potential for the development of gut dysbiosis and the importance of a diverse microbiota at this stage of life.

Overall, there were no significant differences in the abundance of phyla between separate trials, further indicating the similarity of microbiota between trials. At the genus level, the most abundant taxa annotated to OTUs were the *Porphyromonadaceae* unclassified, *Ruminococcaceae* unclassified, *Prevotella*, *Clostridiales* unclassified, *Lactobacillus*, *Phascolarctobacterium*, and *Firmicutes* unclassified, which have been identified as predominant taxa in colonic (Hoeflinger *et al.* 2015; Zhang *et al.* 2016; Leblois *et al.* 2017) and faecal samples (Jacobi *et al.* 2016; Vo *et al.* 2017; Gaukroger *et al.* 2020) from preweaning pigs of a similar age to this study. Notably, these taxa were responsible for contributing the most variation between trials as analyzed by SIMPER (Figures S2 and S3, Supporting Information), but there were no significant differences in the abundance of the OTUs identified as contributing variation to each trial comparison. Neither did LEfSe identify differentially abundant OTUs occurring at ≥ 1% in each trial, except for a greater abundance of one unclassified OTU at the genus level in trial A, $P = .01$.

The secondary objective of this study was to determine if there were any associations between performance, microbial diversity, and abundance using pooled data from each trial. High diversity of GIT microbiota is considered beneficial for pig health (Gresse *et al*. 2017) and α-diversity expressed as Inverse Simpson Diversity correlated with weight at day 22 and ADG ($P = .032$ and $P = .042$, respectively). There were no correlations between performance and Chao Richness. This is probably explained by the difference in the two metrics used. Chao Richness estimates the total number of species, whereas Inverse Simpson Diversity estimates both richness and abundance of species (Morris *et al*. 2014). Thus, richness and abundance are possibly both factors relating to performance of suckling pigs of 22-days of age in this study. In contrast, α-diversity expressed as richness, evenness, and Shannon index were found not to be significantly different in colonic samples taken from low and high weight gain suckling piglets (Morissette *et al*. 2018). However, Shannon index was strongly correlated with back fat thickness and ADG in 15-week-old pigs (Lu *et al*. 2018), whereas abundance-based coverage and Chao richness estimators were significantly higher for weaned heavy pigs (~19 kg) compared with light pigs (~10 kg), but not Shannon and Simpson diversity indices (Han *et al*. 2017). These variations probably reflect the different ages of animals, environments, breeds, and evolution of the microbiota over time (Thompson *et al*. 2008; Kim *et al*. 2011; Bian *et al*. 2016).

This study identified four taxa from colonic samples, corresponding to OTUs at the genus level, which positively correlated with pig weight at day 22 and ADG. These were the *Ruminococcaceae* unclassified, *Terrisporobacter, Dorea*, and *Intestinimonas*, all of the order *Clostridiales* (Fig. 5). *Ruminococcaceae* are found in abundance in faecal samples from suckling pigs (Dou *et al*. 2017; Vo et al 2017) with their relative abundance positively linked to milk fat content of the nursing sow (Bian *et al*. 2016), better growth rates (Mach *et al*. 2015), and higher weight gain during the lactation period (Morissette *et al*. 2018). In adult pigs, OTUs annotated to *Ruminococcaceae* showed positive associations with fatness traits (He *et al*. 2016). *Ruminococcaceae* produce butyrate (Onrust *et al*. 2015), which is trophic to the colonic epithelium (Scheppach *et al*. 2001), their abundance in suckling pigs providing higher energy harvesting, prevention against pathogen infection and higher weight gain (Dou *et al*. 2017). *Terrisporobacter* is found in the ileum of adult pigs (Quan *et al*. 2018) and is a member of the family *Peptostreptococcaceae*, which are abundant in the GIT of suckling and weaning pigs (Li *et al*. 2017). These bacteria produce SCFAs from protein (Zhou *et al*. 2016) and their abundance in the GIT has been positively correlated with adult pig weight gain (Kim et al. 2016). *Dorea* belongs to the Lachnospiraceae family and ferments dietary carbohydrates to SCFAs (Vacca et al. 2020). *Intestinimonas* is a recently described bacterial genus with representative strains present in the GIT of humans and animals that produces SCFAs (Bui *et al*. 2016). Given that sows colostrum contains approximately 16% protein during the first 12 hours after parturition and milk 6%–7% 36 hours thereafter (Krogh *et al*. 2015), *Intestinimonas* and other protein fermenting, SCFA producers may have a nutritional role in the early, preweaning, suckling pig GIT that affects weight gain and development of the microbiota in later life and, in this respect, further research is required in the suckling pig. Notwithstanding the findings of this study, there are limitations. A larger sample size would have been preferable, but as discussed, this may be hard to achieve with large animals. Furthermore, only control samples and for one time-point have been analyzed. Future work may seek to address this by examining samples from test animals, e.g. those fed prebiotics and sampling postweaning to verify if common indicators can be identified across repeated trials in these conditions.

Conclusions

Reproducible small-scale suckling pig trials can be conducted in controlled environmental conditions, at different times of year without major differences in diversity, colonic microbiota composition, or OTU variation, except for a significant difference in Jaccard Similarity indicating species difference between trials. Regardless of intertrial variation, common colonic community indicators can be identified across repeated trials where pooling data supports the identification of performance related colonic microbiota. Correlations between α-diversity and performance show the abundance of common OTUs across trials are factors in the development of the suckling pig microbiota and weight gain. Correlation of the abundance of OTUs that relate to bacteria capable of protein digestion and SCFA production with performance, suggests a nutritional role for these community microbiota members in suckling pigs, which merits further investigation.

Authors' contributions

M.L.B. and K.H.M. conducted the animal research trials. A.L. performed the bioinformatics, analyzed the microbiota data, and wrote the manuscript. M.L.B., K.H.M., and I.F.C. designed the experiment and reviewed the data. All authors read and approved the final manuscript.

Supplementary data

Supplementary data is available at *FEMSEC* online.

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