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# Abstract

The postnatal environment, including factors such as weaning and acquisition of the

gut microbiota, has been causally linked to the development of later immunological diseases such as allergy and autoimmunity, and has also been associated with a predisposition to metabolic disorders. We show that the very early-life environment influences the development of both the gut microbiota and host metabolic phenotype in a porcine model of human infants. Farm piglets were nursed by their mothers for 1 day, before removal to highly controlled, individual isolators where they received formula milk until weaning at 21 days. The experiment was repeated, to create two batches, which differed only in minor environmental fluctuations during the first day. At one day after birth, metabolic profiling of serum by $^{1}$H NMR spectroscopy demonstrated significant, systemic, inter-batch variation which persisted until weaning. However, the urinary metabolic profiles demonstrated that significant inter-batch effects on 3-hydroxyisovalerate, trimethylamine-*N*-oxide and mannitol persisted beyond weaning to at least 35 days. Batch effects were linked to significant differences in the composition of colonic microbiota at 35 days, determined by 16S pyrosequencing. Different weaning diets modulated both the microbiota and metabolic phenotype independently of the persistent batch effects. We demonstrate that the environment during the first day of life influences development of the microbiota and metabolic phenotype and thus should be taken into account when interrogating experimental outcomes. In addition, we suggest that intervention at this

early time could provide ‘metabolic rescue’ for at-risk infants who have undergone aberrant patterns of initial intestinal colonisation.

## Introduction

There is a growing body of evidence which suggests that disease in adulthood can originate in early-life during ‘programming events’ (Huh et al 2012, Langley-Evans 2015, Vickers 2014). This term refers to critical points of developmental plasticity where environmental factors, whether stimulus or insult, can have long-term consequences on physiological development (Winick and Noble 1966). In adult humans, there is a clear association between the intestinal microbiota and metabolic disease (Bajaj et al 2012, Everard et al 2014, Koren et al 2011, Wen et al 2008). However, the links between the early-life environment and development of a healthy metabolic phenotype are not well understood. There is a great deal of evidence suggesting that microbial acquisition from the early-life environment determines development and function of the immune system (Cebra et al 1998, Lewis et al 2012, Mulder et al 2009). Given the recently identified importance of the interactions between the immune and metabolic systems, here we ask the question; do factors which occur during early-life affect metabolic phenotype in the host as well as the

composition of the intestinal microbiota? The long-term effects of differences in early metabolic development, influenced by early-life environment and host-microbial interactions in the gut, may be involved in the pathogenesis of many chronic conditions, including atherosclerosis, diabetes, inflammatory and cardiovascular disease and even obesity (Cox et al 2014, Prentice and Moore 2005, Wells 2012). Indeed evidence suggests that preterm and/or low birth weight and subsequent nutritional intervention predisposes individuals to greater risk of neurodevelopmental conditions and to metabolic syndrome and cardiovascular disease in later life (Barker 1999, Walhovd et al 2012, Wang et al 2012)

The pig is valuable model for human nutritional studies since it shares many common features of gastrointestinal physiology, microbiology, genetics and diet (Miller and Ullrey 1987, Pond and Houpt 1978). Pigs are particularly useful when assessing host microbe interactions since the gut microbiota is substantially more stable over time in both pigs and humans compared to rodent models. Moreover, inter-individual variability is more similar between humans and pigs than between humans and mice, thus in these respects the human population is better reflected in this outbred animal species (Thompson et al 2010). The porcine metabolic phenotype has recently been

58 characterised across a number of biological compartments (Merrifield et al 2011).
59 Coupled with numerous studies using metabolic profiling to identify the impact of
60 nutrition on health (Bertram et al 2006, He et al 2009, Larsen et al 2010), these
61 studies have validated the use of high-resolution spectroscopic techniques to assess
62 nutritional intervention in this tractable model species. Piglets are precocial; they can
63 be removed from the sow at birth and are therefore easily manipulated from a very
64 early age, unlike human and rodent equivalents.

65

66 Early-life and weaning are associated with major shifts in the microbial community of
67 the gut in both humans (Johnson and Versalovic 2012) and other mammals
68 (Konstantinov et al 2006). Here we examine the system-wide metabolic and
69 microbiological consequences of variation in the micro-environment during the first
70 day of life. We also ascertain the subsequent effect of nutritional intervention, in this
71 case weaning on to diets with different protein sources. We propose that this systems
72 approach to exploring early life environment could identify linked patterns of
73 microbial-mammalian dysbiosis and therefore, in the long-term, may provide a
74 platform to identify individual susceptibility to disease. In addition, a systems
75 medicine approach has the potential to assess methods of intervention during an
76 early window of opportunity in which to limit disease risk by preventing, or correcting,

early deviation from intestinal homeostasis.

# Materials and Methods

### *Animal Husbandry*

Animal work was performed according to local ethical guidelines under a UK Home Office License, approved by the local ethical review group. The six Large White hybrid, out-bred sows (which had previously had 1-2 litters) used in this experiment were chosen randomly from a commercial herd of approximately 250 animals which were housed all together in a single, open-ended barn. Normal practice on this farm is to artificially inseminate 20 appropriate sows on a weekly basis, and then to move them to one of 4 farrowing units (temperature controlled at 22$^{o}$C plus heat-mats for the piglets) just before the sows are due to give birth. The piglets are normally weaned during their 4$^{th}$ week of life and moved to alternative housing, while their mothers rejoin the herd and are artificially inseminated 4-5 days later. Consequently, farrowing units are run on a 4-week rotation and are steam-cleaned and left standing for 1 day before the next group of sows are brought in to give birth. During this time, the farrowing facility is sprinkled with a dry disinfectant (Naturclean®, SCA NuTec, Staffordshire, UK) to reduce damp and microbial load. Experimental piglets were taken from normal sows undergoing this process. There were no symptoms of disease, no antibiotic treatment was given and there were no differences in the diet fed to any of the farm sows during the pregnancy and lactation periods of the specific sows used for these experiments.

During the autumn, 3 sows (contributing to batch 1) were artificially inseminated using pooled semen from 3 boars. Eight weeks later, 3 additional sows (contributing to batch 2) were artificially inseminated using pooled semen from the same 3 boars. Shortly before sows contributing to batch 1 were due to farrow, they were removed to a farrowing unit and 2 piglets from each of the resulting litters (n=6) were removed to the University of Bristol's piglet isolator facility at one day old. This isolator provided a highly controlled, standardised environment: specific pathogen-free (SPF), HEPA filtered, positive pressure environment which is thoroughly cleaned and fumigated with formaldehyde gas (Alphagen Prills pellets, Antec AH International, Sudbury, UK) for 12 hours before each experimental run. The piglets were housed together within this isolator for one day while they learnt to drink bovine-based formula milk (Piggimilk®,Volac Ltd, Sleaford, Lincolnshire, UK). The nutritional composition of the formula is summarized in Table S1 and is similar to that given to human infants. In order to minimise the time taken for all of the piglets to learn to drink from bowls, piglets were housed in groups to learn by emulation. At two days old, the piglets were moved to individual units, within the same isolator, and fed hourly for 14 days. At 14 days of age, the piglets were litter- and gender-matched into two groups before weaning at 21 days, to separate the effects of weaning from those due to the stress of mixing. One group of piglets were weaned onto a diet in which the protein source was egg, whilst the other group received a soya-based diet. Both diets were nutritionally balanced and supplemented with appropriate levels of vitamins and minerals (Volac Ltd, Sleaford, Lincolnshire, UK.). The nutritional composition of each of these diets is detailed in Table S1. There were no traces of egg in the soya diet, nor soya in the egg diet; a biosecurity barrier was established between the two groups to avoid food crossover. Four weeks after batch 1 sows vacated the farrowing unit, batch 2 sows entered the same unit in preparation for farrowing. An

additional group of sows had occupied the unit between batch 1 and batch 2. Again, 2 piglets were removed from each of the 3 batch 2 sows at 1 day old and underwent exactly the same process as batch 1 piglets. A summary of the experimental design is depicted in Figure 1A and a more detailed schematic is available in Fig. S3.

This experimental design resulted in n=6, gender-matched, litter-matched piglets in the treatment groups (weaned onto either an egg-diet or soya diet) and n=6 gender-matched piglets in each replicate (batch 1 and batch 2). Replicates could not be litter-matched since they were born 8 weeks apart. Detailed weather conditions, including temperature, wind speed, rainfall, hours of sunshine and global radiation, throughout the whole experiment are shown in Fig. S4, with the start date of each replicate indicated.

All piglets were bled by venipuncture at one day (upon removal from the mother), 21 days (immediately before weaning) and 28 days old and samples were stored at -80$^{o}$C. In addition, fresh faecal samples were also obtained at 21 days, just prior to weaning, snap frozen in liquid nitrogen and stored at -80$^{o}$C. At 35 days, piglets were euthanized with an overdose of pentobarbitone (Euthatal®, Merial Animal Health, Essex, UK). At post-mortem, heart blood, colonic (descending colon, adjacent to the colo-rectal junction) contents and mucosal scrapings, mesenteric lymph node (MLN) draining the distal jejunum and liver (tip of the same lobe in each piglet) were recovered; tissues, colon content and mucosal scrapings were snap frozen at post-mortem and stored at -80$^{o}$C. Urine was removed directly from the bladder *via* a sterile syringe, snap frozen and stored at -40$^{o}$C pending NMR analysis.

***Sample Preparation for $^{1}$H NMR***

*Serum*

Blood serum samples were prepared by the addition of 350µl 0.9% saline solution

containing 10% $D_2O$ (to act as a spectrometer field frequency lock) to 200µl of serum. This mix was then vortexed, centrifuged at 12,000*g* for 20 minutes and transferred into 5mm outer diameter NMR tubes in preparation for analysis.

*Urine*

Urine samples were prepared by the addition of 220µl of a 1mM 3-trimethylsilyl-1-[2,2,3,3,-$^{-2}H_4$] propionate (TSP), 3mM sodium azide ($NaN_3$) and 80/20 (v/v) $H_2O/D_2O$ phosphate buffer solution (pH 7.4) to 440µl of urine. This mixture was vortexed, left to stand for 20 minutes and then centrifuged at 12,000*g* for 20 minutes. Six hundred µl of the supernatant was transferred to 5mm outer diameter NMR tubes (Beckonert et al 2007).

*Tissues*

For high-resolution magic angle spinning (HR-MAS) NMR spectroscopy, intact tissue samples from above (~15mg) were immersed in a 0.9% saline $D_2O$ solution within a zirconium oxide 4mm diameter rotor, and an insert was used to make a spherical sample volume up to 25µl.

## ***$^1$H NMR Spectroscopy***

*Serum*

Serum samples were collected longitudinally throughout the experiment as previously described. The $^1$H NMR metabolic profiles of these samples were acquired on an Avance 600 MHz NMR spectrometer (Bruker Biospin) and spectra were collected using a BACS autosampler with a Bruker 5mm TXI triple resonance probe at 300K. 256 scans (16 dummy scans) were collected into 64 k data points over a 20 ppm spectral width using a Carr-Purcell-Meiboom-Gill (CPMG) spin echo sequence,

incorporating an echo time of 200μs to highlight contributions from low-molecular weight moieties.

*Urine*

The metabolic profile of urine from each of the piglets (collected at 35 days) was obtained by NMR spectroscopy. All spectra were acquired on an Avance 600 MHz NMR spectrometer (Bruker Biospin) and spectra were collected using a BACS autosampler with a Bruker 5mm TXI triple resonance probe at 300K. 256 scans (16 dummy scans) were collected into 64 k data points over a 20 ppm spectral width using the first increment of a standard one dimensional experiment with water presaturation during the relaxation delay of 2 s and during the mixing time of 100 ms.

*Tissues*

Sections from the liver and the MLN were analysed by $^{1}$H HR-MAS-NMR as described previously (Merrifield et al 2011) using a 4mm g-HR MAS HPCD probe at 283K. A total of 256 scans (16 dummy scans) were collected into 64 k data points over a 20 ppm spectral width using a Carr-Purcell-Meiboom-Gill (CPMG) spin echo sequence, incorporating an echo time of 200μs to highlight contributions from low-molecular weight moieties.

***Quantification of the GI microbiota using 16S DNA pyrosequencing***

DNA was extracted from faecal and colonic content samples using the Qiagen EZ1 kit (Qiagen, Germany) after mechanical disruption as follows: for colon content, 100mg of material was placed in lysozyme buffer (50 mg/ml) with 0.3 g of glass beads, disrupted in a bead-beater at maximal speed for 1 min and then incubated at

37°C for 15 min; for colon tissue, 200mg were placed in Lysing Matrix Tubes D with lysozyme buffer and homogenized with FastPrep-24 (MP Biomedicals, Solon, OH 44139), 2x20 sec speed 6, with 5 min break. After centrifugation (5 min, 200g), supernatant was transferred into tubes with 0.3g of glass beads, disrupted in a bead-beater at 4$^{o}$C at maximal speed for 1 min and then incubated at 37$^{o}$C for 15 min.

The V1, V2 and V4 regions of the 16S genes contained in the DNA extracts were amplified, sequenced in multiplex using the GS FLX System (Roche, Switzerland), and analyzed as previously described (Claus et al 2011), except for the confidence threshold of the RDP Classifier which was set to 60%, as in previous published studies (Dominguez-Bello et al 2011, Koren et al 2011). All data analysis was conducted on the mean relative proportion of bacteria (%). Due to the high proportion (~35%) of unclassified bacteria of pig origin identified at the genus level, we have used the family level as a trade-off between the coverage of the microbial population and the depth of taxonomical analysis.

***Data Analysis***

An exponential window function with a line broadening of 0.3Hz was applied prior to Fourier transformation to all 1D NMR spectra. The resultant spectra were phased, baseline corrected and calibrated to TSP (δ 0.0, urine) or the α anomeric resonance of glucose (δ 5.23, blood serum and tissues) using an in-house routine (developed by Dr. J. M. Fonville), these were then manually checked using Topspin (2.0a, Bruker BioSpin 2006) as a visualiser. The spectra were subsequently imported into Matlab then down-sampled to 13k data points by linear interpolation (R2009b, The

MathsWorks inc.) and the regions containing the water resonance (δ 4.6 -5.2) and for urine, urea (δ 4.6-5.9), were removed. The spectra were aligned using an in-house algorithm (Veselkov et al 2009) to abrogate the effects of peak shifting due to variation in pH. The data were then normalized by the probabilistic quotient method, using the median spectra as the reference (Dieterle et al 2006) to minimise the effects of inter-sample variation due to phenomena such as dilution or sample volume. Orthogonal partial least squares discriminant analysis (O-PLS-DA) and Wilcoxon rank sum tests were performed in a Matlab environment using in-house algorithms (Cloarec et al 2005) and the Matlab statistical toolbox. To generate the serum trajectory, a series of pair-wise O-PLS-DA models was constructed for each time-point using mean-centered scaling and the cross validated scores (Tcv) extracted for each model. Results were then normalised by dividing each value by the total Tcv standard deviation for each model. The scores corresponding to the class definition in the pair-wise comparison at each time point were represented using their respective mean and standard deviations. For each O-PLS-DA model depicting urinary and microbial differences, it was determined whether the $Q^2Y$ value (predictive ability) of the model was significantly different to the $Q^2Y$ value calculated from up to 2,000 random permutations of Y using the cumulative probability value determined at the $95^{th}$ quartile (in-house algorithm written by Dr. O.Cloarec). Models were scaled to unit variance such that each variable had equal weight in the model, and were subject to a 7-fold cross-validation step. The spectral regions or bacteria highlighted by the discriminant analysis as having a correlation coefficient (*r*) of >+/- 0.6 were selected for display via heat maps to provide a global view of metabolic and bacterial variation in response to early-life environment and weaning diet. To assess the effects of diet independently of batch, the models investigating dietary differences include all animals from both batches (*n*=6 per group), and similarly, to assess the

effects of batch independently of diet, animals on each diet (*n*=6 per group) were included in the model irrespective of batch. For analysis of the tissue, betaine in the liver and MLN and taurine in the liver were found to be significantly different in the soya and egg-based diets; these metabolites were therefore integrated and analysed independently of the rest of the spectra. Integration of selected metabolite regions was performed in a Matlab environment using an in-house algorithm (Dr. R. Cavill). The microbial-metabolic interaction map was created using a hierarchical clustering and correlation script in Matlab using an in-house algorithm (Dr. J.T.M. Pearce) and fitted with a 50% false-discovery rate (FDR) to correct for multiple testing, the hierarchical clustering algorithm used the Pearson product-moment correlation coefficient. We acknowledge that this seems to be a high FDR but this is a deliberate compromise considering the small number of observations. Moreover, the false-discovery rate calculation assumes that variables are independent and without correlation. Biological process involves intrinsic correlation between metabolites and the nature of NMR spectroscopy brings another level of correlation between variables. All these reasons make the real FDR difficult to estimate and thus justify the acceptance of such a high FDR.

# Results

### *Differences in the first day of life caused a sustained shift in the metabolic phenotype of serum*

Serum profiles acquired for two experimental replicates (n=6) of piglets at 1 day

(immediately after removal from the mother) and 21 days old showed significant metabolic differences, despite the fact that from day 2 onwards all animals were raised in a standardised, controlled environment (Figure 1b). Orthogonal projection to latent structures discriminant analysis (O-PLS-DA) between the two experimental replicates showed that the spectral region of *N*-acetylglycoproteins, varied between the two batches at day 1 (Figure 1d) and that the serum *myo*-inositol concentration was also increased in batch 2 at this time point. At 21 days, the piglets in the two batches still exhibited significant differences in their serum metabolic profiles; one of the major discriminatory metabolites was galactose (Figure 1e) which was higher in batch 2. Additionally, serum formate levels were also increased in batch 2. However, after weaning (28 days), the metabolic profiles converged and at the end of the experiment (35 days) no significant metabolic differences between the two batches could be detected in serum. A summary of the O-PLS-DA loadings describing the batch-dependant variation of the metabolic serum profiles is provided in the supplementary information (Figure S1).

***Weaning onto different diets caused a transient divergence of metabolic phenotype in serum***

The effect of different weaning regimens (egg vs soya-based diet) was significant seven days after the introduction of the weaning diet (Figure 1c). This difference was

attributed to a transient increase in serum betaine in the egg-fed animals (Figure 1f). Importantly, by the end of the experiment (35 days) there were no significant differences in the serum metabolic profiles of these animals associated either with the early environmental variation (batch) or with weaning diet.

***Weaning diet and experimental batch confer significant, but differential, effects on the urinary metabolic phenotype at 35 days***

We observed clear and significant differences in urinary metabolite profiles between experimental replicates (Figures 2 c and d). This difference was apparent 34 days after environmental standardisation: animals in the second experimental batch excreted relatively more 3-hydroxyisovalerate, trimethylamine-*N*-oxide (TMAO) and mannitol (Figure 2f), suggesting a sustained alteration of the metabolic phenotype. Urine metabolites of animals weaned onto different diets were also significantly different (Figure 2a). O-PLS-DA analysis shows a difference at 35 days between the animals weaned onto an egg-based diet and those weaned onto a soya-based diet (Tcv, Figure 2b). These differences were generally distinct from differences relating to batch. Diet-induced differences included increased excretion of *p*-cresol glucuronide, phenylacetylglycine (PAG), 2-hydroxyisobutyrate, 3-

307 methylcrotonylglycine, betaine and unknown metabolites U3[1] and U4[2] in the egg-
308 based diet with a concomitant decrease in urinary allantoin, taurine, U1[3] and
309 cadaverine (Figure 2e).

310

311 ***Metabolic profiles in systemic tissues***

312 Pigs fed an egg-based diet had significantly ($p<0.001$) higher levels of betaine in the
313 MLN and liver than those fed a soya-based diet (Figure 2 g, h and i). In addition,
314 hepatic taurine levels were significantly ($p<0.001$) decreased in the egg-fed animals
315 compared to the soya-fed animals.

316

317 ***Significant differences are observable due to experimental batch in the faecal***
318 ***microbiota at 21 days.***

319 The fecal and mucosa-associated microbiota was determined by 16S
320 pyrosequencing. After quality filtering, a total of 233,342 sequencing reads were

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[1] U3 ($\delta^1$H 1.44; $\delta^{13}$C 16.5, (s))

[2] U4 ($\delta^1$H 1.88(s) & $\delta^1$H 2.04(s)) assigned to the same molecule by STOCSY. The $^{13}$C shifts could not be unambiguously assigned for these resonances.

[3] U1 ($\delta^1$H 7.09; $\delta^{13}$C 121 (d) & $\delta^1$H 7.27; $\delta^{13}$C 133.3 (d)) is likely to be a *para*-substituted phenolic compound but shows no clear similarity to any reported compounds.

obtained (254 bp average read length covering the V1-V2 and V4 regions of the 16S gene). Per sample, 1919 sequences (median value) were tentatively classified into 70 families. Analysis of faecal samples collected at 21d showed significant ($p<0.03$) microbial differences between the experimental batches (Figure S2). The primary difference in microbial composition was in levels of *Clostridia Incertae Sedis XN* which was significantly increased in batch 2 (*r* 0.75). At this stage in the experiment, all the animals were on a formula diet and no significant differences between the animals subsequently fed a soya-based or egg-based diet were observed at this time point (as shown by a negative $Q^2Y$). This is consistent with the batch differences pre-existing weaning.

***Weaning diet and first day environment significantly affect composition of the colonic microbiota at 35 days***

Bacteria belonging to the classes *Bacteroidia* and *Clostridia* were dominant in communities of all animals (Figure 3). Significant differences ($p<0.05$) between the two diets were clearly identifiable for each site; differences in bacterial abundance were observed for 3 bacterial families in the colonic content and for 5 families in the mucosa. Inter-batch differences were also observed; 9 bacterial families showed significant differences in the colonic content and 9 families had different abundances

340 in the mucosa (Figures 3 a and b). Supervised multivariate statistical comparison of
341 the recorded bacterial families was carried out using O-PLS-DA and those groups
342 found to be significantly different ($r$>+/-0.6) due to either weaning diet or batch are
343 summarised in figure 3c by a correlation coefficient heat map. Bacteria of the
344 families *Ruminococcaceae, Veillonellaceae, Erysipelotrichaceae* and *Marinilabiaceae*
345 were increased in both the content and mucosa of batch 1 compared to batch 2,
346 whereas *Prevotellaceae* was increased in the content and mucosa of batch 2. The
347 bacterial response to diet showed less concordance between colonic and mucosal
348 community changes than the batches did and included increased
349 *Porphyromonadaceae* in the colonic content of piglets fed an egg-based diet.
350 Additionally there were increased mucosal proportions of *Strepococcaceae* and
351 *Alcaligenaceae*.

352

353 ***Microbial-Metabolic Interactions affect a number of metabolic pathways***

354 We identified several statistically significant microbial-metabolic associations (Figure
355 4). An established gut microbial by-product of dissimilatory amino acid metabolism,
356 PAG, was found to be positively correlated with *Erysipelotrichaceae* isolated from the
357 colonic mucosa. Metabolites involved in methyl donation, such as betaine and
358 sarcosine, were positively correlated with mucosal and colonic *Prevotellaceae*, and

359 betaine was negatively correlated with colonic *Veillonellaceae* and *Lactobacillaceae*.
360 Importantly, 3-methylcrotonlyglycine, associated with biotin deficiency, was
361 negatively correlated with *Lachnospiraceae* in the colonic content whereas, 3-
362 Hydroxyisovalerate, also associated with biotin metabolism, was positively
363 associated with colonic mucosal *Rikenellaceae*.

364

## 365 Discussion

366 We have used a tightly controlled experimental system to explore the effect of the
367 environment during the first 24 hours of life and to interrogate the subsequent
368 weaning strategy on the microbiota and metabolism of newborn piglets. We show
369 that minor variations during the *first day* of life can have long-term effects on patterns
370 of intestinal colonisation and the urinary metabolic phenotype of piglets.
371 Furthermore, piglets subsequently weaned onto either egg or soya-based diets
372 displayed reliably altered microbiota and metabolic parameters despite the persistent
373 batch-effect observed between experimental replicates.

374 It is well documented that the microbiota is acquired from both the maternal vaginal
375 canal and the immediate neonatal external environment (Mulder et al 2011, Schmidt
376 et al 2011). Previous studies have used early-life interventions to demonstrate that

377 successive microbial colonisation can be affected during infancy (Krishnan and
378 Ramakrishna 1998) (Kirjavainen and Gibson 1999). The pattern of colonisation
379 appears to be a programming event, which influences both composition, and
380 function, of the adult microbiota, which subsequently impacts on metabolism
381 (Dominguez-Bello et al 2011). It is also known that variation in early-life events can
382 be linked to increased disease risk in later life (Aiken and Ozanne 2014, Dabelea and
383 Crume 2012). It is very rare, however, that a single bacterial species can be
384 identified as a specific marker or predictor for disease in the absence of a known
385 pathogen.

386

387 Significant metabolic effects between the two experimental batches were observed in
388 the serum metabolic profile at one day old, indicating that metabolic differences
389 between the experimental replicates were established during the first day of life.
390 Serum *N*-acetyl glycoprotein concentrations have been positively correlated with an
391 acute-phase reaction in humans (Bell et al 1987), so the observation that at one day
392 old, piglets in batch 2 had increased levels of *N*-acetyl glycoproteins despite having
393 different mothers could indicate that animals in this batch were undergoing greater
394 antigenic challenge than those in batch 1. No pathology was observed either on the
395 farm or in the isolator unit during either experimental run, so an acute phase

396 response may have been in response to minor environmental fluctuations. At 21
397 days after birth, batch differences were still evident in the serum metabolite profiles
398 and this was primarily due to galactose concentrations, which were virtually
399 undetectable in batch 1 but significantly elevated in batch 2. Since there was no
400 variation between batches in the time between feeding and serum sample collection,
401 this could indicate inter-batch variation in intestinal absorption or enzymatic
402 conversion of lactose to galactose (Holmes et al 1990). By the time the piglets were
403 35 days old, no difference in the metabolic profile of serum was observed due to
404 either experimental replicate or weaning diet, but strong batch and diet differences
405 were apparent in the microbiome and the urinary metabolic profile. Although the lack
406 of a significant difference in serum metabolic profiles between batches and diet
407 groups by the end of the experiment could indicate the lack of a sustained metabolic
408 effect of these parameters, blood serum is kept under tight homeostatic control. We
409 have shown previously that there is a comparative lack of NMR detectable
410 metabolites of microbial origin in porcine serum relative to urine (Merrifield et al 2011)
411 Additionally, we have observed differences in the urinary metabolic profiles in pigs
412 due to weaning diets up to 8 weeks after weaning, despite there being no differences
413 in the serum metabolic profile (Merrifield et al 2013). Thus the differences in microbial
414 composition observed at this time point are unlikely to be reflected in this serum, and

415 the observed differences early on in the experiment suggest marked systemic
416 differences between the batches.

417

418 It was not feasible for faecal material to be reliably collected from these piglets at a
419 similar time point on day one due to inherent differences in production of faecal
420 matter in the neonatal pig, therefore we can only speculate about differential
421 microbial composition at this time point, however, we suspect that the batch
422 differences observed throughout the experiment are likely to be due to differential
423 early microbial acquisition based on the differing microbial composition observed
424 between batches at later time points.

425

426 The significantly higher level of faecal *Clostridiae incertae sedis* XIV in batch 2 at 21
427 days supports the hypothesis that the two batches had differential microbial
428 composition, as these samples were collected before the animals were weaned onto
429 either an egg-based or soya-based diet. Additionally, both the batch and weaning
430 diet had a sustained effect on the urinary profile, the 16S DNA profile of both colonic
431 mucosal scrapings and on colonic content observed at 35 days. Gut microbiota can
432 be classified as either ‘autochthonous’ (residing in the gut) or ‘allocthonous’ (passing

433 through the gut). It is thought that autochthonous bacteria are more permanent
434 members of the microbiota, whereas allocthonous bacteria may be transient and
435 related to diet. The observation made in our experiment, that both the composition of
436 mucosal which may represent autochthonous bacteria, and the luminal content which
437 may represent allocthonous bacteria, were altered by both the batch and weaning
438 diet, suggests that both early life environment and weaning diet affect not just the
439 bacterial composition of the gut content, which might be expected, but also the
440 *resident* colonic bacterial population. These animals were kept under identical
441 environmental conditions following the first day of life and received the same batch of
442 feed between replicates, thus any changes between the batches are indicative of
443 differences in microenvironment during the first 24 hr of life, or in the maternal
444 microbiota passed on to the piglets. Given that the batch effect derived from two
445 groups, each containing three sows, it is unlikely that the individual vaginal
446 microbiota was a strong contributory factor to the batch differences. There was
447 minor seasonal variation in outside temperature during the study (supplementary
448 figure 2), but we cannot be certain that this is sufficient to affect the
449 maternal/neonatal metabolism of these animals.

450 Several of the metabolites associated with batch and weaning diet in this study
451 derive from either microbial metabolism or microbial-mammalian co-metabolism, and

several of these demonstrate a correlation with bacteria as measured by 16S DNA, suggesting that both the environment during the first day of life and the weaning diet can induce alteration of microbial composition. We have shown previously that weaning diet has a sustained effect on the urinary metabolic profile in the pig, despite subsequent dietary standardisation (Merrifield et al 2013) and here we demonstrate that the early-life environment creates a similar set-point in acquisition of microbiota and development of metabolic phenotype. Importantly the increase in *p*-cresol glucuronide and PAG excretion in the egg-fed animals implies a diet-induced alteration of gut microbial activity or ecology, since these metabolites are known end-products of proteolytic fermentation in the distal gut (Smith and Macfarlane 1996, Yokoyama et al 1982). Our previous work has demonstrated that piglets initially fed an egg-based diet excreted comparably more PAG and *p*-cresol glucuronide than animals initially fed a soya-based diet, even after four weeks of dietary standardisation by feeding a fish-based diet (Merrifield et al 2013), implying that these metabolites are a product of altered microbial metabolism rather than of differential dietary protein.

***Biotin metabolism correlates to differences in both first day environmental***

***exposure and diet***

Differential excretion patterns of the urinary organic acids 3-hydroxyisovalerate and 3-methylcrotonylglycine relating to diet and batch are of interest as these are putative markers of biotin deficiency. Biotin, also known as vitamin H or coenzyme R, is primarily involved in carbohydrate metabolism. Biotin is a cofactor for five mammalian carboxylases, and decreased activity of these leads to increased urinary excretion of 3-hydroxyisovalerate, 3-methylcrotonylglycine and other organic acids (Mock et al 2004). It is also a cofactor for fatty acid synthesis, gluconeogenesis (Sawamura et al 2007) and, since it is intimately involved with epigenetic regulation of epithelial proliferation (O'Keefe et al 2009), it has also been linked to the development of colorectal cancers. Biotin is a fermentation product of elements of the microbiome (Hill 1997) and can also be derived from dietary protein. Despite egg yolk containing high concentrations of biotin, feeding an egg-based diet is reported to reduce the bioavailability of biotin since egg white contains avidin, a protein with a strong affinity for biotin that inhibits uptake across the GI tract. However, the cooking process used to prepare the experimental diets destroys avidin activity and, in addition, these specifically formulated diets were supplemented with biotin (200mg, Volac Ltd).

In this experiment, increased urinary excretion of 3-methycrotonylglycine was

observed in the animals fed an egg-based diet, whereas increased urinary excretion of 3-hydroxyisovalerate was seen in batch 2, irrespective of diet. Together, these differences suggest that bioavailability of biotin is more likely to be due to differential microbial production and or microbial or mammalian metabolism than to dietary intake. Cross-correlation of bacteria and metabolic profiles in this study (Figure 4) also supports this idea. We demonstrate a positive correlation between 3-hydroxyisovalerate and mucosal *Rikenellaceae*, which belong in the order Bacteroidales. The Bacteroides are also included in this order and members of the Bacteroides genus are known to require biotin for growth (Macy and Probst 1979) so could theoretically reduce the bioavailability of biotin.

***Choline metabolism is affected by both diet and first day environmental exposure***

Several studies have suggested that gut bacteria affect the bioavailability of dietary choline to the host, and that microbially derived metabolites of choline have been implicated in important disease processes such as atherosclerosis (Koeth et al 2013, Spencer et al 2013). Betaine concentration in the serum was significantly increased in the egg-fed animals 7 days after weaning. Because eggs contain relatively more choline than soya beans (Zeisel et al 2003), and because plasma concentrations of

509 betaine have been found to correlate with oral doses of betaine (Atkinson et al 2009),
510 this is to be expected. In this study, although plasma betaine levels normalised 2
511 weeks after weaning, at the end of the experiment (35 days) hepatic mesenteric
512 lymph node and urinary betaine concentrations were significantly increased in the
513 animals weaned onto an egg-based diet. Whilst these observations are consistent
514 with increased bioavailability of betaine due to dietary intake, urinary betaine was
515 shown to be significantly associated with a number of colonic bacteria. Importantly,
516 urinary TMAO is a host metabolic product of trimethylamine, which is produced by
517 the microbiota from choline (al-Waiz et al 1992, Zeisel et al 1983, Zeisel et al 1989).
518 While urinary TMAO was found in higher concentrations in batch 2 animals, hepatic
519 levels of TMA and TMAO were not significantly different between batches, indicating
520 that this difference was not due to increased flavine monooxygenase (FMO) activity
521 in the liver. Increased levels of TMAO have been related to increased atherogenesis
522 (Koeth et al 2013). Animals in batch 2 had increased acute phase serum *N*-
523 acteylglycoproteins at one day old, so it would be of interest to investigate if
524 inflammation in early life predisposes to increased TMAO formation and hence
525 atherogenesis.

526 Choline metabolism in the gastrointestinal tract is intimately associated with gut
527 microbiota, and urinary metabolic end-products of choline degradation, including

528 betaine and sarcosine, were statistically strongly associated with bacteria in the
529 colonic mucosa and content. Urinary betaine and sarcosine were positively
530 correlated with Bacteroida *Prevotellaceae* in both the mucosa and colonic content.
531 The *Prevotellaceae* were also significantly increased in batch 2; these bacteria are
532 known to produce $H_2$ and have been found in high concentrations in the GI tracts of
533 obese humans (Zhang et al 2009).

534

535 ***Host-microbial metabolism is influenced by both diet and early life***
536 ***environmental exposure***

537 Overall, our results are consistent with the hypothesis that there are at least two
538 critical points during early life which affect establishment of gut microbial
539 communities: immediately following birth and at weaning. Our data show that the
540 batch effects initiated in the first day of life were still strongly represented in the
541 microbiota and in urinary metabolites of piglets at 35 days old, and that the effects of
542 different diets introduced at 21 days on the urinary metabolite profiles were also
543 sustained. These data also confirm that differences in metabolic phenotype attributed
544 to diet and intestinal microbiota are more readily detected in urine than in serum at
545 35 days. We have previously shown that weaning diet confers a sustained shift in
546 metabolic phenotype (Merrifield et al 2013). The results reported here suggest that

this may have resulted from an altered intestinal microbiota during the weaning period, as evidenced by the significant differences observed by 16S DNA sequencing. This is probably a result of the weaning diet and occurred independently of the batch effects.

In adults, the relative stability of the intestinal microbiota means that modulations such as those achieved by prebiotics, probiotics and some antibiotics, are dependent on continuous consumption since the microbiota tends to revert to its original profile on cessation of the intervention (Lozupone et al 2012, Moore and Moore 1995). Here we demonstrate that early-life events can cause persistent changes in the microbiome, of at least 5 weeks duration, with equally persistent effects on metabolic function. However, we have shown previously that weaning confers a sustained effect on the urinary metabolic phenotype of at least 8 weeks duration. We propose that variations in the early neonatal environment should be taken into account when designing experimental trials. Since batch, or differences in the microenvironment, has a significant effect on both the microbiome and metabolome, it is important to establish whether changes are truly a result of an intervention or a reflection of the early postnatal environment.

In conclusion, we suggest that the early post-natal period may represent a time of

physiological plasticity during which future therapeutic interventions could potentially minimise the effect of environmental perturbations, thus impacting positively on human and animal health. This may lower the risk of developing metabolic dysfunction in later life and is especially relevant to infants who have been exposed to known risk factors including premature birth, caesarean delivery, time in the neonatal ICU, and those born at term with low birth weights.

## Supplementary Information

Supplementary information is available at The ISME Journal's website.

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## Conflict of Interests

The authors declare no conflict of interests.

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# Figure Legends

***Figure 1: The temporal effects of early-life environment and weaning diet on the metabolic profile of porcine blood serum.***

(a) Represents the experimental setup, where Batch 1 and 2 correspond to the experimental replicate. Animals were fed formula at 1 day until 21 days, at which point they were weaned onto either an egg or soya-based diet (weaning is denoted by red arrow). At each time-point a pairwise comparison was made by O-PLS-DA (mean-centred scaling), between either the two experimental batches (b) (*n*=6 per group) or the two weaning diets (C) (*n*=6 per group) and the mean of the cross-validated scores (Tcv) from each model (+/- standard deviation) are plotted at 1, 21, 28 and 35 days. The spectral regions derived from 600MHz CPMG metabolic profiles of serum of selected discriminatory metabolites at each time-point have been plotted; (d) shows increased serum *N*-acetyl glycoproteins in batch 2 at 1 day; (e) shows increased galactose in batch 2 at 21 days and (F) depicts increased in betaine in the egg-fed animals at 28 days.

807 ***Figure 2: Weaning diet and early environment both affect the urinary metabolic***

808 ***phenotype of the young pig.***

809 Median spectra from each group fed either an egg-based (top, *n*=6) or soya-based

810 (bottom, *n*=6) diet (*A*) with numbers corresponding to the listed metabolites (E) and

811 (F). The groups underwent comparison by pairwise O-PLS-DA analysis conducted

812 with one predictive and one orthogonal component ($Q^2Y$: 0.81, $R^2Y$: 0.97, $R^2X$: 0.54)

813 and the cross-validated scores (Tcv) are shown (b), each point represents one

814 animal and the letters correspond to litter. Discriminating metabolites are shown in

815 (e). (c) represents median spectra from each experimental batch with numbers

816 corresponding to the listed metabolites in (e) and (f). The Tcv from an O-PLS-DA

817 model constructed with one predictive and one orthogonal component ($Q^2Y$: 0.72,

818 $R^2Y$: 0.92, $R^2X$: 0.50) are shown in (d). The discriminatory metabolites from this

819 model are highlighted in (f). Plots of integral regions calculated from 600MHz HR-

820 MAS-NMR spectra of tissues from each animal; (g) relative betaine concentration in

821 the mesenteric lymph node (MLN), where the points on the left represent the animals

822 on the soya diet and those on the right the egg diet. Animals in batch 1 are outlined

823 in black whereas those in batch 2 are outlined in red. Integral regions of hepatic

824 betaine (*H*) and hepatic taurine (*I*). ** Denotes $p<0.001$ (Wilcoxon rank sum).

825

826 ***Figure 3: Weaning diet and early environment affect the composition of***
827 ***microbiota in the colonic content and mucosa of the young pig.*** The mean
828 relative proportion (%) of bacterial families measured by 16S DNA sequencing
829 (V1+V2 and V4 regions) is summarised for each intervention in the colonic content
830 (a) and for the colonic mucosa (b). Each figure has a colour-coded representation of
831 the relative proportion of bacteria as summarised by the key and is separated into
832 animals on a soya-based diet (**S**, *n*=6), an egg-based diet (**E**, *n*=6), those in batch 1
833 (**B1**,*n*=6) and those in batch 2 (**B2**, *n*=6). The diets (soya vs egg) were statistically
834 compared using the Wilcoxon rank sum test for each bacterial family, as were the
835 batches (batch 1 vs batch 2). Statistical significance of $p<0.05$ is indicated by (*) and
836 $p<0.005$ by (**). The O-PLS-DA correlation coefficients for a series of four separate
837 O-PLS-DA models, constructed on the mean relative proportions of bacteria, denoted
838 at the top of the column (cmk. Full bacterial family names are: Actinobacteria
839 coriobacteriaceae; Bacteroidia bacteroidaceae; Bacteroidia porphyromonadaceae;
840 Bacteroidia prevotellaceae; Bacilli lactobacillaceae; Bacilli streptococcaceae;
841 Clostridia eubacteriaceae; Clostridia incerta sedis XN; Clostridia lachnospiraceae;
842 Clostridia ruminococcaceae; Clostridia veillonellaceae; Erysipelotrichi
843 erysipelotrichaceae; Erysipelotrichi campylobacteraceae; Epsilonproteobacteria
844 helicobactereaceae; Bacteroidia marinilabiaceae; Bacteroidia rickenellaceae;

845 Clostridia clostridiaceae; Clostridia incerta sedis XIII; Betaproteobacteria
846 alcaligenaceae; Deltaproteobacteria desulfovibrionaceae; Gammaproteobacteria
847 enterobacteriaceae

848

849 ***Figure 4: Microbial-metabolic interactions are visible across multiple metabolic***
850 ***pathways***

851 A hierarchical clustering and correlation map between bacterial families and selected
852 metabolic integrals. Positive correlations are shown in green and negative
853 correlations in red. Gut-microbial metabolites are highlighted in purple, metabolites
854 related to methyl donation in blue, metabolites related to biotin in grey and those
855 related to purine metabolism in red. The suffix '_C' denotes bacteria isolated from
856 the colonic content and the suffix '_M' denotes those isolated from the colonic