Gut transcriptome reveals differential gene expression and enriched pathways linked to immune activation in response to weaning in pigs.

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Abstract

Weaning represents one of the most critical periods in pig production associated with increase in disease risk, reduction in performance and economic loss. Physiological changes faced by piglets during the weaning period have been well characterised, however little is currently known about the underlying molecular pathways involved in these processes. As pig meat remains one of the most consumed sources of protein worldwide, understanding how these changes are mediated is critical to improve pig production and consequently sustainable food production globally. In this study, we evaluated the effect of weaning on transcriptomic changes in the colon of healthy piglets over time using an RNA-sequencing approach.

The findings revealed a complex and coordinated response to weaning with the majority of genes found to be rapidly differentially expressed within one day post weaning. Multiple genes and pathways affected by weaning in the colon were associated with immune regulation, cell signalling and bacterial defence. NOD-like receptors, Toll-like receptor and JAK-STAT signalling pathways were amongst the pathways significantly enriched. Immune activation was evidenced by the enrichment of pathways involved in interferon response, cytokines interactions, oxidoreductase activities and response to microbial invasion. Biosynthesis of amino acids, in particular arginine, was also amongst the most enriched KEGG pathways in weaned pigs, reinforcing the critical role of arginine in gut homeostasis under stress conditions.

Overall, transcriptomic and physiological results suggest that pigs going through the weaning transition undergo a transient period of inflammatory state with a temporary breakdown of barrier functions in the gut. These findings could provide valuable tools to monitor host response post weaning, and may be of particular relevance for the investigation and development of intervention strategies aimed to reduce antibiotic use and improve pig health and performance.

Keywords: Pig, Weaning, RNA-sequencing, Transcriptomic, Gut, Immune response.

Introduction

Livestock production is expected to produce more food than ever before. As the expanding world population is getting wealthier, the demand for safe and secure animal protein is increasing (Henchion et al., 2017). The challenge is to meet this

demand in ways that are environmentally, socially and economically sustainable. Together with poultry, pork is one of the fastest growing livestock sectors and also one of the most consumed meats world-wide (FAO, 2019). Pig production is also widely recognised as one of the most efficient in term of carbon footprint and climate change potential compared to other animal protein source (Macleod et al., 2013). Ensuring optimal production is critical to ensure animals can fulfil their genetic potential and contribute to sustainable food source.

As a result of abrupt dietary, social stresses, and environmental changes, weaning is recognised as the most critical period in modern pig production associated with increase in disease risks, reduction in performance and welfare leading to significant economic loss (Gresse et al., 2017; Nowland et al., 2019). At weaning, the pig gastrointestinal tract (GIT) undergoes rapid changes in size, protein turnover rates, microbiome composition, and detrimental alterations in digestive and barrier functions (Pluske et al., 2018). Although the physiological changes faced by piglets over weaning have been well characterised, little is known about the underlying genes and pathways involved in these processes. Understanding how these changes are regulated or mediated is critical to improve pig production and consequently sustainable food production globally.

Furthermore, due to similarities in anatomy and physiology, the pig is widely recognised and utilised as a translational animal model to study human gastrointestinal diseases and to understand biological pathways related to mucosal function, development and nutritional regulation (Roura et al., 2016; Sciascia et al., 2016; Zhang et al., 2013). Previous studies have highlighted the importance of

improving the knowledge on molecular mechanism responsible for phenotypic differences especially at an early ages with the dual purpose of improving production and providing adequate models for human studies (Ayuso et al., 2015).

Recent advances in sequencing technologies now provides novel opportunities to comprehensively explore the complex gut ecosystem of humans and animals. RNA-sequencing (RNA-Seq) is a powerful high-throughput approach to profile gene expression that, in contrast with microarray-based technologies, allows for the characterisation and quantification of both known and unknown transcripts (Mach et al., 2014). Fundamental understanding of the host response to stress and its environment is paramount for the optimisation and development of application to improve pig health, productivity and welfare. To date, RNA-Seq has been used to study production traits of livestock animals but transcriptomic study in pigs using this technology is relatively scarce and have mainly focused on disease response or regulation mechanism of fat deposition and muscle development to evaluate growth and meat quality between pig genotypes (Piórkowska et al., 2018; Xu et al., 2019).

Much attention and focus has been given to the gut microbiome and its taxonomic and metabolic changes through pig development and weaning (Bian et al., 2016; Frese et al., 2015; Guevarra et al., 2018a) but we are still lacking understanding about the host gene expression change in response to weaning. The current study aims to investigate the transcriptomic changes in the pig gut through weaning over time.

Material and methods

Animals and experimental design

All animals were treated in accordance with the University of Nottingham ethical guidelines and codes of practice applying to care and management of animals. Twenty-four litters (Landrace x Large White) over three batches were used in the study and housed at the School of Biosciences, Sutton Bonington Campus, University of Nottingham, UK. For prevention of iron deficiency and coccidiosis, all piglets received a 1 ml IM injection of Gleptosil (Alstoe Ltd, York, UK) 24 h after birth, and 0.7 ml of Baycox (Bayer, Newbury, UK) orally 3 d after birth. At 21 days of age, 6 weight-matched piglets per litter were randomly allocated by random selection of coded balls to treatment (baseline average weight at day 19: 6.77 ± 0.189 kg). Piglets allocated to the weaned treatment were separated from their dam, moved and mixed with non-littermates in pens of 4 individuals and received ad lib commercial diet (wheat, whey powder and soya based) containing: 21.25% protein, 7.5% fat, 2.00% fibre, 5% ash, 1.70% lysine, 13.80% moisture). Weaned piglets did not receive creep feed supplementation before weaning. Piglets allocated to the unweaned treatment as control remained with their dam and littermate up to 35 days of age with access to creep feed from day 25d of age (same commercial diet as above). No antibiotic or anthelmintic treatment were used during the trial. At day 1, 4 and 14 post-weaning: one weaned and one unweaned piglet from each litter were weighed euthanised by intraperitoneal injection of Dolethal (1 ml.kg-1 body weight; 20% w/v Pentobarbitone Sodium, Vétoquinol, Buckingham, UK). At slaughter, body lesion was scored for each pig on a 3 point scale with 1 for no lesion, 2 for moderate scratches on back, flank, head, ear and tail, and 3 for intense, deep or bleeding scratches on back, flank, head, ear and tail.

Intestinal measurements

All sample processing and analysis was blinded using randomly generated numerical codes. Tissue samples from the 0.5 section of the colon were immediately collected post-slaughter, rinsed in sterile buffered saline solution and preserved in RNA later (Ambion, CA USA) at 4^{o}C for 24-48h to allow tissue penetration then stored at -80^{o}C. Tissue samples of the 0.5 small intestine (as proportions) along from the gastric pylorus to the ileocecal valve were fixed in Bouin's solution, embedded in paraffin and cut in 5 µm transverse sections. Histological section were stained with H&E for histometric measurement of villus length and crypt depth, with Periodic Acid Schiff stain for goblet cell counts (Matsuo et al., 1997) and with Toluidine Blue for quantification of mast cells (Moeser et al., 2007).

Secretory IgA (sIgA) was measured in ileal flushes using the methods previously described by Lessard et al. (2009). At slaughter, a 20 cm segment of ileum taken upstream from the cecum was flushed using 5 ml of sterile PBS, and centrifuged for 10 min at 500 g. The supernatant was collected and stored at -80^{o}C. Secretory IgA was measured in duplicate using a sandwich Porcine IgA ELISA Quantitation Kit (Bethyl Laboratories, TX, USA) according to the manufacturer protocol.

Data and Bioinformatics analysis

Statistical analysis was performed in IBM SPSS v24 to determine the effect of weaning treatment at different time point treatment on pig weight, intestinal and blood measurement using linear mixed model analysis.

Gene expression cluster analysis was performed and revealed that sex was the only factor that showed a grouping effect. Therefore sex was included in the model as a confounder for the gene expression analysis.

Sequence alignment and read quantification was performed using the pseudo-alignment-based tool Kallisto v0.43. (Bray et al., 2016). Differential expression was determined using the Wald test in Sleuth v0.28.1 (Pimentel et al., 2017) with sex as a confounder in the model. Transcripts with a false detection rate corrected p-value < 0.05 and a log2 fold change (log2FC) greater than 1 and less than minus 1 were considered to be differentially expressed, unless otherwise stated. False detection rate correction was performed using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Results

Phenotypic data

All piglets were found in good health during the trial and displayed no clinical signs of disease or scour. As expected, weaning caused a number physiological changes which were found to be time-dependant. Significant differences were observed in pig weight, blood or plasma measurements, body lesion scores and intestinal measurement (Table 1). These preliminary results also indicate the significant effect of time on almost all variables measured between day 1 and day 14 post weaning. This highlights that the pigs are undergoing rapid period of development at this age and the importance of designing studies using age-matched controls when evaluating the effect of weaning in pigs as opposed to using pre-weaning values as controls.

Plasma analysis revealed that cortisol levels remained stable over time in the unweaned group but increased almost 4-fold at day 1 and 2-fold at day 4 post weaning

175 in the weaned pigs indicating an activation of the HPA axis under weaning stress
176 (Martínez-Miró et al., 2016). Interestingly, while most parameters measured in this
177 study showed a rapid spike followed by a progressive return to the unweaned level by
178 day 14, cortisol level still remained significantly higher at 14 days post weaning
179 although to a lesser magnitude.
180
181 Haematology and biochemistry profiles are used as indicators of health status to
182 evaluate the metabolic, nutritional and energy state of the pig. In blood, circulating
183 granulocyte levels, haemoglobin and haematocrit were significantly increased in
184 weaned pigs, while platelet counts decreased. In plasma, cholesterol, triglycerides
185 and globulin levels decreased at day 4 and day 14 in the weaned group compared to
186 unweaned pigs.
187
188 Measurement of intestinal architecture were also affected by weaning as previously
189 reported, with significant reduction in jejunal villus height and villus/crypt ratio.
190 However, villus width remains unaffected by weaning and crypt depth was increased.
191 Goblet cell numbers in crypt and in villus remained unaffected in this study, however
192 there was a significant decrease in mucosal mast cells at day 4 with an overall
193 statistical trend for weaned pigs to show reduced mast cell count compared to
194 unweaned controls.
195
196 Ileal sIgA was also greatly reduced at all time points post weaning, suggesting a
197 decrease in immune protection from maternal milk. IgA plays an important role in the
198 protection of mucosal surfaces against pathogens and is the principal immunoglobulin
199 secreted in sow's milk (40% of the total whey protein) (Klobasa et al., 1987). sIgA is

200 considered the first line of defence and one of the most important factor for piglet
201 growth and survival (Salmon et al., 2009). At weaning, removal of the piglets from the
202 sow causes dramatic drop in gut IgA levels as observed in the current study and by
203 others (Lessard et al., 2009), increasing the immune vulnerability of piglets post
204 weaning.
205
206 *Transcriptomic analysis*
207 RNAseq (HiSeq) was used to identify deferentially expressed transcripts (DET)
208 between weaned and unweaned pigs in colonic tissue. An average of 52.3 million
209 trimmed paired reads were obtained for each sample. Reads mapped as pairs (83.0-
210 85.3%) to the porcine genome ref sequence (sus scrofa 10.2). The Wald test was used
211 on TPM to establish the total number of DET between weaned and unweaned pigs for
212 each time point and identified a total of 239 transcripts at q value ≤ 0.1 and FC ≥ 2.
213 The volcano plots visually represent significant DET for each time point and show an
214 even distribution between up- and down-regulated genes (Figure 1).
215
216 The majority of transcripts 171 (71.6%) were found to be differentially expressed after
217 1 day post weaning. After 4 days and 14 days post weaning only 67 (28.0%) and 1
218 (0.4%) genes were differentially expressed between weaned and unweaned pigs,
219 respectively (Figure 2). Among DET with the cut off values of Qval ≤ 0.1 & FC ≥ 2, two
220 transcripts were identified in common between day 1 and day 4 post weaning:
221 ENSSSCT00000014128 and ENSSSCT00000017308 which are of unknown function
222 (Figure 3). They were no DET common at all three time point or shared between day
223 4 and 14 or day 1 and 14 post weaning. Transcription according to time point and

224 weaning treatment are shown in PCA plots and revealed distinct clusters between
225 weaned and unweaned pigs across time points (Figure 4).
226
227 The full list of differentially expressed transcripts at Qval ≤ 0.1 & FC ≥ 2 is provided in
228 Supplementary Table S1. DETs with FC>2 across all time points were subject to Gene
229 Ontology (GO) and KEGG pathway analysis using NIPA
230 https://github.com/richarddemes/NIPA to identify significantly enriched pathways
231 between weaned and unweaned pigs at Qval ≤ 0.05 and minimum number of gene in
232 term of two (Supplementary Table S2). Top 10 significantly enriched GO terms and
233 KEGG pathways are shown in Figure 5.
234
235 **Discussion**
236 In this study, the typical characteristics previously reported in other studies were
237 observed validating our weaning model. Piglet weights were negatively affected by
238 weaning at day 1 and day 4 but recovered at day 14. Post-weaning growth check is a
239 commonly reported problem in pigs with detrimental impact on life-time growth
240 performances representing a large economic loss to the industry (Collins et al., 2017)
241
242 Evidence of stress was also observed in this study with elevated plasma cortisol and
243 increased lesion scores in the weaned pigs. Following post-weaning mixing, intense
244 aggressive patterns especially directed to facial, belly and anogenital regions has been
245 reported in previous studies and were shown to be linked with the establishment of
246 social hierarchy between unacquainted pigs (Meese and Ewbank, 1973; Turner et al.,
247 2009). In agreement with previous studies, the majority of lesions found in this study
248 were found on the head, ears and shoulders (data not shown), suggesting aggressive

249 frontal confrontation, however no direct behavioural observation were performed so
250 other behaviour which could have caused lesions could not be excluded. Interestingly,
251 lesion scores in the unweaned piglets were found at 14 d, when pigs were 35 day of
252 age and had reached over 12 kg, suggesting that despite creep feed available, they
253 may have been some possible agonistic behaviour between littermates in competition
254 for food source as the milk yield from the sow starts to decrease at this stage of
255 lactation (Hansen et al., 2012). Weaning age is a controversial topic of discussion
256 regarding performance and welfare, with typical weaning age varying between
257 production systems and countries. Most studies agree that weaning at an early age
258 (<21 days old) exacerbate the effect of weaning stress (Smith et al., 2010; Xun et al.,
259 2018) but our results suggest that later weaning at 35d could also have negative
260 impact on the piglets welfare.
261
262 Measurements of intestinal structures such as villus and crypt have been commonly
263 used to evaluate gut health as a measure of absorptive capacities for nutrients
264 (Bontempo et al., 2006; Le Bon et al., 2010). As reported by others, the current study
265 shows that villus morphometry is impaired by weaning. Although feed intake was not
266 measured in this trial, low feed intake post-weaning is considered one of the main
267 aetiological factors for morphological changes in gut physiology due to the importance
268 of enteral stimulation for mucosal homeostasis (Spreeuwenberg et al., 2001). Villus
269 atrophy during the first few days post-weaning has been commonly reported and is
270 often associated with loss in barrier function, decreased enzyme activity and
271 decreased performance such as seen in this study (Lallès, 2008). Interestingly, the
272 effect observed on the crypt depth may indicate an increased level of cellular activity
273 and turnover in the intestinal mucosa in response to weaning.

This study has revealed a large number of genes up and down regulated as consequences of weaning in the pig colon highlighting the profound physiological impact of weaning on healthy pigs. As the genome of the domestic pig is relatively recent in comparison with other model species (Groenen, 2016), many genes and transcripts still have limited annotation and identified function to this date. Differential gene expression in the gut of weaned pigs has previously been investigated using microarray-based techniques (Inoue et al., 2015; Wang et al., 2008), however the number of genes and pathways identified were limited to known transcripts, while RNA seq platform offers a more comprehensive view of the transcriptome including genes of unknown functions. Freeman et al. (2012) documented the first transcriptomic atlas of the domestic pig describing tissue-specific gene expression and emphasised the importance to generate and increase our knowledge of transcriptomic expression to document and understand the function of many genes with limited functional annotation. The transcriptomic profile of pig along the small intestine has been previously evaluated in the duodenum, ileum, jejunum and ileal Peyer's Patches of healthy growing pigs (Mach et al., 2014), but relatively little is known about the colon in pigs. The current study identified many genes associated with weaning with and without known function, contributing to advancing out fundamental understanding of the pig genome.

The large majority of differentially expressed transcripts were found to peak on day 1 post weaning and progressively returned to unweaned level by day 14, emphasising the abrupt response of pigs to weaning (Lallès et al., 2007). There was little overlap in transcripts between time points suggesting a coordinated regulation of the host in

299 response to weaning. These findings could inform selection of appropriate time points
300 for further trials in weaned pigs.
301
302 Due to the large number of DET identified in this study, the main focus of this
303 discussion is on pathways significantly affected by weaning rather that individual
304 genes. Metabolic, immune and barrier function activities have previously been
305 implicated with weaning response in both the small and large intestine but the
306 underlying mechanism for these changes have not been previously identified. In the
307 current study, we found several pathways related to microbial response and immune
308 functions activated post-weaning; specifically, NOD-like receptors, Toll-like receptor
309 and JAK-STAT signalling pathways are shown to be significantly activated. These
310 signalling pathways indicate a cellular response to Microbial Associated Molecular
311 Patterns (MAMPs) that drives the activation innate immune response. As such, we
312 also observe positive regulation of Interferon alpha production and Interferon gamma
313 mediated pathways.
314
315 In agreement with these results, Wang et al. (2008) also reported that weaning at 21
316 days of age showed an increase in expression of genes associated with oxidative
317 stress and immune activation but decreased expression of genes related to nutrient
318 utilisation and cell proliferation in the jejunum of pigs using microarray analysis. A
319 previous study also reported that pigs weaned at a later age (28 days) showed
320 increase in pro-inflammatory cytokines expression in the small intestine and colon
321 during the first two days post weaning before returning to pre-weaned level after 8
322 days post weaning (Pié et al., 2004). The interferon response following weaning stress
323 in pigs has previously been characterised and showed that weaning causes the

release of IFN-α and the transient shut-off of the corresponding gene transcriptions in PBMC (Razzuoli et al., 2011). Bailey (2009) also demonstrated that weaned pigs develop an early and transient immune response to novel dietary antigens at weaning before establishing tolerance (Everaert et al., 2017).

In addition, Moeser et al. (2017) demonstrated that colonic and jejunal transepithelial resistance was increased resulting in impaired permeability of the gut barrier as a result of weaning, which may facilitate infiltration of luminal component such as bacteria or bacterial products. Here we see at least two GO biological terms that would agree with this hypothesis: “cellular responses to LPS” and “response to bacterium” where genes such as NOS2, CD274, IRF3, CXCL11 and ACOD1, are upregulated.

The immune regulation observed through our transcriptomic results also reflects the significant increase in granulocyte levels found in blood, suggesting both a local and systemic activation of immunity in response to weaning. The combined effect of immune and stress activation can lead to an energy cost whereby growing animals divert their energy resource towards these responses instead of growth which can explain reduced performance observed in weaned pigs. In growing pigs, transcriptomic multi-tissue analysis revealed that activated immune response, protein metabolism, defence against pathogens and oxidative stress were the main biological pathways associated with feed efficiencies (Gondret et al., 2017). The authors suggested that dietary intervention with anti-inflammatory or antioxidant properties could be evaluated to improve efficiencies in growing pigs. As feed accounts for more than 60% of the cost of food production, improving feed efficiency is a major target to improve profitability of the pig industry (Jing et al., 2015).

In recent years, significant progress has been made in characterising the complex network of communication and signals between the nervous, immune and endocrine systems of the gut. The role of the gut-brain axis in neurological disorders is well recognised but its mechanism poorly understood. Activation of the gut brain axis under stressful stimuli has been shown to stimulates inflammatory pathways, alteration of gut barrier function and has been associated with changes in intestinal microbiota in humans and animal models (Martin et al., 2018). The multifactorial challenges faced by pigs at weaning have provoked changes in behavioural response and elevated corticoids levels as shown in previous studies (Campbell et al., 2013; Flynn and Wu, 1997; Li et al., 2016). These could be in part responsible for the immune activation observed at the transcriptomic level in the colon but would require further investigation to determine if these changes can be attributed to brain.

The colon also harbours the richest and most diverse microbial population of the gut. In recent years, a large number of studies have documented the development of the gut microbiota of the pig over time (Frese et al., 2015; Guevarra et al., 2018b; Han et al., 2018; Ke et al., 2019; Mach et al., 2015; Wang et al., 2019). As a transition from a milk-based to a plant-based diet, weaning is typically associated with a decrease in gut microbial diversity and major shifts in bacterial taxa composition (Guevarra et al., 2019). In line with other inflammatory gut disorders, such as IBD or Crohn's disease, the causal relation effect between inflammation and microbiota is unclear. Recently, several studies have provided evidence to suggest that inflammation in gut tissue is conducive to the proliferation of gut bacteria that contribute to pathogenesis or disease development (Zeng et al., 2017). In the current study, we observe activation of immune

374 signalling pathways and pro-inflammatory cytokines in the early days post weaning,
375 and we also observe regulation of pathways involved in oxido-reducatase activity.
376 Amongst the activated pathways related to anti-microbial defence, genes involved in
377 oxidative burst such as NOS2 (Nitric oxide synthesase 2) and NOX1 (NAPDH oxidase
378 1) are upregulated which are involved in the production of nitic oxide and superoxide
379 radicals. These nitrogen rich compounds are rapidly converted into nitrate (NO3-) in
380 the lumen providing favourable condition for the growth and proliferation of gut bacteria
381 that carry nitrate reductase genes such as *Enterobacteriaceae* including *E. coli* and
382 *Salmonella* (Winter et al., 2013). In addition, inflammatory conditions provide
383 increased level of luminal oxygen due to elevated blood flow and haemoglobin, this
384 favours aerobic respiration of *Enterobacteriaceae* while inhibiting the growth of
385 obligate anaerobes such as *Bacterioides* and *Clostridia* (Zeng et al., 2017).
386 *Enterobacteriaceae* have a detrimental effect on pig health and growth and is one of
387 the leading cause of diarrhoea in pig production (Rhouma et al., 2017). *E. coli* is highly
388 prevalent post-weaning and can lead to mortality and zoonosis (Luppi, 2017). As a
389 result, antibiotic usage is commonly used to treat and prevent *E.coli* infection and have
390 led to increasing reports in colistin-resistant *E. coli* found in pigs (Rhouma *et al.*, 2017).
391 Tackling post weaning inflammatory response could represent a step towards
392 reduction in antimicrobial use in pig production.
393
394 At weaning, pigs are abruptly transitioned from sow's milk to a complex plant-based
395 diet with distinct nutritional profile and as such transcriptomic modulation of pathways
396 involved in nutritional metabolism would be expected. Whilst the majority of nutrient
397 digestion and absorption takes place in the small intestine, the main role of the colon
398 in digestive functions is to reabsorb water and electrolytes. However, in the current

399 study, biosynthesis of amino acids, in particular arginine biosynthesis was the second
400 most significant KEGG pathway enriched in weaned pigs via the up regulation of
401 argininosuccinate synthase 1 gene (ASS1). Arginine is an essential amino acid that
402 play key roles in nutrition and metabolism. Young mammals, including piglets, have a
403 particularly high requirement of arginine for growth and metabolic function. As
404 enterocytes actively transport and metabolise arginine, the gut is an important organ
405 for maintaining body arginine homoeostasis (Wu et al., 2018). L-Arginine is also
406 the biological precursor of nitric oxide (NO), and alteration of arginine uptake and
407 metabolism has been found to be associated with inflammatory bowel diseases, such
408 as ulcerative colitis (Coburn et al., 2016; Luiking et al., 2012; Stuehr, 2004). In post-
409 weaning pigs, arginine metabolism in the gut is critical to maintain normal intestinal
410 physiology and for efficient utilisation of dietary protein (Wu et al., 2004). In addition,
411 glucocorticoids plays an important role in regulating the enhanced arginine metabolism
412 in the enterocytes of post-weaning pigs (Flynn and Wu, 1997). Arginine availability in
413 the digestive tract plays a key role in maintaining intestinal immune homeostasis under
414 conditions of inflammation and infection (Das et al., 2010; Fritz, 2013; Singh et al.,
415 2019). A number of studies have reported that arginine administration significantly
416 attenuate intestinal inflammation under physiological and pathological conditions
417 including intestinal dysfunction in weaned piglets, and enhancement of growth
418 performance and survival (Che et al., 2019; Wu et al., 2010; Zheng et al., 2018). Other
419 models have reported that arginine supplementation also down-regulated JAK-STAT
420 signalling pathway and attenuated the inflammatory response, which exerted
421 protective effects on the intestine of chickens challenged with *C. perfringens* ((Zhang
422 et al., 2019). Arginine has been shown to exert anti-inflammatory and antioxidant
423 effects in IPEC-J2 cells challenged with LPS (Qiu *et al.*, 2019). Reports of reduced

arginine availability in conditions of acute and chronic stress, often associated with increase in NOS2 activity are in agreement with the results of the current study (Luiking et al., 2012). The specific mechanisms of regulation and interaction between cortisol, NOS, immune response, and arginine metabolism observed in this study remain unknown, but could provide further evidence to suggest that arginine requirement should be carefully evaluated when designing diet to support pigs during the weaning transition.

Finally, although the current study has identified a large number of transcripts and pathways regulated at the mRNA level, it is likely that post-transcriptional and post translational regulatory mechanisms also regulate the host response to weaning and should be investigated in future studies as comprehensive and complementary approach to transcriptomic methods.

Conclusion

Weaning is a multifactorial event that results in complex interactions between gut, brain and metabolism. Understanding these responses and the molecular mechanisms that underpins these changes is critical to improve sustainable pig production. This study has identified multiple genes and pathways differentially regulated by weaning. These results revealed that pigs going through the weaning transition undergo a transient period of inflammatory state with temporary breakdown of barrier functions in the gut. The condition of the inflamed gut have been previously shown to provide favourable growth advantage for the expansion of *Enterobacteriaceae*, a leading cause of enteric disease in pigs. Under the experimental and controlled conditions of this trial, differential gene expression

449 returned to unweaned control levels by day 14 post weaning. However, the translation
450 of the study results to commercial production setting remains to be explored.
451
452 Indicators of weaning stress and response have previously been used including
453 histology, systemic markers of immunity and characterisation of the microbiota
454 composition. Here, we have identified a number of target gene and pathways that
455 could also be used as biomarker of intestinal inflammation to complement these
456 measures. Together, these could provide valuable tools to monitor host response post-
457 weaning, especially in context of intervention strategies aimed to reduce antibiotic use
458 and improve pig health and performance. Finally, as weaning in pigs have been used
459 as a model for stress-related bowel dysfunction in humans, it would be of interest to
460 investigate if the similar transcriptomic changes are involved in these disorders.
461
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467
468 **Declaration of interest**
469 The authors declare no conflict of interest.
470
471 **References**
472 Ayuso, M., Fernández, A., Núñez, Y., Benitez, R., Isabel, B., Barragán, C.,
473 Fernández, A.I., Rey, A.I., Medrano, J.F., Cánovas, Á., González-Bulnes, A.,

López-Bote, C., Ovilo, C., 2015. Comparative analysis of muscle transcriptome between pig genotypes identifies genes and regulatory mechanisms associated to growth, Fatness and metabolism. PLoS One. https://doi.org/10.1371/journal.pone.0145162

Bailey, M., 2009. The mucosal immune system: Recent developments and future directions in the pig. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2008.07.003

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E.G., Mackie, R., Liu, J., Mu, C., Huang, R., Smidt, H., Zhu, W., 2016. Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. Environ. Microbiol. https://doi.org/10.1111/1462-2920.13272

Bontempo, V., Di Giancamillo, A., Savoini, G., Dell'Orto, V., Domeneghini, C., 2006. Live yeast dietary supplementation acts upon intestinal morpho-functional aspects and growth in weanling piglets. Anim. Feed Sci. Technol. https://doi.org/10.1016/j.anifeedsci.2005.12.015

Bray, N.L., Pimentel, H., Melsted, P., Pachter, L., 2016. Near-optimal probabilistic RNA-seq quantification. Nat. Biotechnol. https://doi.org/10.1038/nbt.3519

Campbell, J.M., Crenshaw, J.D., Polo, J., 2013. The biological stress of early weaned piglets. J. Anim. Sci. Biotechnol. https://doi.org/10.1186/2049-1891-4-19

Che, D., Adams, S., Zhao, B., Qin, G., Jiang, H., 2019. Effects of Dietary L-arginine

499 Supplementation from Conception to Post- Weaning in Piglets. Curr. Protein
500 Pept. Sci. 20, 736–749. https://doi.org/10.2174/1389203720666190125104959
501 Coburn, L.A., Horst, S.N., Allaman, M.M., Brown, C.T., Williams, C.S., Hodges, M.E.,
502 Druce, J.P., Beaulieu, D.B., Schwartz, D.A., Wilson, K.T., 2016. L-Arginine
503 Availability and Metabolism Is Altered in Ulcerative Colitis. Inflamm. Bowel Dis.
504 22, 1847–1858. https://doi.org/10.1097/MIB.0000000000000790
505 Collins, C.L., Pluske, J.R., Morrison, R.S., McDonald, T.N., Smits, R.J., Henman,
506 D.J., Stensland, I., Dunshea, F.R., 2017. Post-weaning and whole-of-life
507 performance of pigs is determined by live weight at weaning and the complexity
508 of the diet fed after weaning. Anim. Nutr. (Zhongguo xu mu shou yi xue hui) 3,
509 372–379. https://doi.org/10.1016/j.aninu.2017.01.001
510 Das, P., Lahiri, Amit, Lahiri, Ayan, Chakravortty, D., 2010. Modulation of the arginase
511 pathway in the context of microbial pathogenesis: A metabolic enzyme
512 moonlighting as an immune modulator. PLoS Pathog.
513 https://doi.org/10.1371/journal.ppat.1000899
514 Everaert, N., Van Cruchten, S., Weström, B., Bailey, M., Van Ginneken, C.,
515 Thymann, T., Pieper, R., 2017. A review on early gut maturation and
516 colonization in pigs, including biological and dietary factors affecting gut
517 homeostasis. Anim. Feed Sci. Technol.
518 https://doi.org/10.1016/j.anifeedsci.2017.06.011
519 FAO, 2019. Meat market review. Food Agric. Organ. United Nations.
520 Flynn, N.E., Wu, G., 1997. Glucocorticoids Play an Important Role in Mediating the
521 Enhanced Metabolism of Arginine and Glutamine in Enterocytes of Postweaning
522 Pigs. J. Nutr. https://doi.org/10.1093/jn/127.5.732
523 Freeman, T.C., Ivens, A., Baillie, J.K., Beraldi, D., Barnett, M.W., Dorward, D.,

524 Downing, A., Fairbairn, L., Kapetanovic, R., Raza, S., Tomoiu, A., Alberio, R.,
525 Wu, C., Su, A.I., Summers, K.M., Tuggle, C.K., Archibald, A.L., Hume, D.A.,
526 2012. A gene expression atlas of the domestic pig. BMC Biol.
527 https://doi.org/10.1186/1741-7007-10-90
528 Frese, S.A., Parker, K., Calvert, C.C., Mills, D.A., 2015. Diet shapes the gut
529 microbiome of pigs during nursing and weaning. Microbiome.
530 https://doi.org/10.1186/s40168-015-0091-8
531 Fritz, J.H., 2013. Arginine cools the inflamed gut. Infect. Immun.
532 https://doi.org/10.1128/IAI.00789-13
533 Gondret, F., Vincent, A., Houée-Bigot, M., Siegel, A., Lagarrigue, S., Causeur, D.,
534 Gilbert, H., Louveau, I., 2017. A transcriptome multi-tissue analysis identifies
535 biological pathways and genes associated with variations in feed efficiency of
536 growing pigs. BMC Genomics. https://doi.org/10.1186/s12864-017-3639-0
537 Gresse, R., Chaucheyras-Durand, F., Fleury, M.A., Van de Wiele, T., Forano, E.,
538 Blanquet-Diot, S., 2017. Gut Microbiota Dysbiosis in Postweaning Piglets:
539 Understanding the Keys to Health. Trends Microbiol.
540 https://doi.org/10.1016/j.tim.2017.05.004
541 Groenen, M.A.M., 2016. A decade of pig genome sequencing: A window on pig
542 domestication and evolution. Genet. Sel. Evol. https://doi.org/10.1186/s12711-
543 016-0204-2
544 Guevarra, R.B., Hong, S.H., Cho, J.H., Kim, B.R., Shin, J., Lee, J.H., Kang, B.N.,
545 Kim, Y.H., Wattanaphansak, S., Isaacson, R.E., Song, M., Kim, H.B., 2018a.
546 The dynamics of the piglet gut microbiome during the weaning transition in
547 association with health and nutrition. J. Anim. Sci. Biotechnol. 9, 1–9.
548 https://doi.org/10.1186/s40104-018-0269-6

549 Guevarra, R.B., Hong, S.H., Cho, J.H., Kim, B.R., Shin, J., Lee, J.H., Kang, B.N.,
550 Kim, Y.H., Wattanaphansak, S., Isaacson, R.E., Song, M., Kim, H.B., 2018b.
551 The dynamics of the piglet gut microbiome during the weaning transition in
552 association with health and nutrition. J. Anim. Sci. Biotechnol.
553 https://doi.org/10.1186/s40104-018-0269-6
554 Guevarra, R.B., Lee, J.H., Lee, S.H., Seok, M.J., Kim, D.W., Kang, B.N., Johnson,
555 T.J., Isaacson, R.E., Kim, H.B., 2019. Piglet gut microbial shifts early in life:
556 Causes and effects. J. Anim. Sci. Biotechnol. https://doi.org/10.1186/s40104-
557 018-0308-3
558 Han, G.G., Lee, J.Y., Jin, G.D., Park, J., Choi, Y.H., Kang, S.K., Chae, B.J., Kim,
559 E.B., Choi, Y.J., 2018. Tracing of the fecal microbiota of commercial pigs at five
560 growth stages from birth to shipment. Sci. Rep. 8.
561 https://doi.org/10.1038/s41598-018-24508-7
562 Hansen, A. V., Strathe, A.B., Kebreab, E., France, J., Theil, P.K., 2012. Predicting
563 milk yield and composition in lactating sows: A Bayesian approach. J. Anim. Sci.
564 https://doi.org/10.2527/jas.2011-4788
565 Henchion, M., Hayes, M., Mullen, A., Fenelon, M., Tiwari, B., 2017. Future Protein
566 Supply and Demand: Strategies and Factors Influencing a Sustainable
567 Equilibrium. Foods. https://doi.org/10.3390/foods6070053
568 Inoue, R., Tsukahara, T., Nakatani, M., Okutani, M., Nishibayashi, R., Ogawa, S.,
569 Harayama, T., Nagino, T., Hatanaka, H., Fukuta, K., Romero-Pérez, G.A.,
570 Ushida, K., Kelly, D., 2015. Weaning markedly affects transcriptome profiles and
571 Peyer's patch development in Piglet ileum. Front. Immunol.
572 https://doi.org/10.3389/fimmu.2015.00630
573 Jing, L., Hou, Y., Wu, H., Miao, Y., Li, X., Cao, J., Michael Brameld, J., Parr, T.,

574 Zhao, S., 2015. Transcriptome analysis of mRNA and miRNA in skeletal muscle
575 indicates an important network for differential Residual Feed Intake in pigs. Sci.
576 Rep. https://doi.org/10.1038/srep11953
577 Ke, S., Fang, S., He, M., Huang, X., Yang, H., Yang, B., Chen, C., Huang, L., 2019.
578 Age-based dynamic changes of phylogenetic composition and interaction
579 networks of health pig gut microbiome feeding in a uniformed condition. BMC
580 Vet. Res. 15, 1–13. https://doi.org/10.1186/s12917-019-1918-5
581 Klobasa, F., Werhahn, E., Butler, J.E., 1987. Composition of sow milk during
582 lactation. J. Anim. Sci. https://doi.org/10.2527/jas1987.6451458x
583 Lallès, J.-P., 2008. Nutrition and gut health of the young pig around weaning : what
584 news ? Arch. Zootech. 11, 5–15.
585 Lallès, J.P., Bosi, P., Smidt, H., Stokes, C.R., 2007. Nutritional management of gut
586 health in pigs around weaning. Proc. Nutr. Soc.
587 https://doi.org/10.1017/S0029665107005484
588 Le Bon, M., Davies, H.E., Glynn, C., Thompson, C., Madden, M., Wiseman, J.,
589 Dodd, C.E.R., Hurdidge, L., Payne, G., Le Treut, Y., Craigon, J., Tötemeyer, S.,
590 Mellits, K.H., 2010. Influence of probiotics on gut health in the weaned pig.
591 Livest. Sci. https://doi.org/10.1016/j.livsci.2010.06.058
592 Lessard, M., Dupuis, M., Gagnon, N., Nadeau, É., Matte, J.J., Goulet, J., Fairbrother,
593 J.M., 2009. Administration of Pediococcus acidilactici or Saccharomyces
594 cerevisiae boulardii modulates development of porcine mucosal immunity and
595 reduces intestinal bacterial translocation after Escherichia coli challenge. J.
596 Anim. Sci. https://doi.org/10.2527/jas.2008-0919
597 Li, L.A., Yang, J.J., Li, Y., Lv, L., Xie, J.J., Du, G.M., Jin, T.M., Qin, S.Y., Jiao, X.L.,
598 2016. Effect of weaning age on cortisol release in piglets. Genet. Mol. Res.

https://doi.org/10.4238/gmr.15027693

Luiking, Y.C., Ten Have, G.A.M., Wolfe, R.R., Deutz, N.E.P., 2012. Arginine de novo and nitric oxide production in disease states. Am. J. Physiol. - Endocrinol. Metab. https://doi.org/10.1152/ajpendo.00284.2012

Luppi, A., 2017. Swine enteric colibacillosis: Diagnosis, therapy and antimicrobial resistance. Porc. Heal. Manag. https://doi.org/10.1186/s40813-017-0063-4

Mach, N., Berri, M., Esquerré, D., Chevaleyre, C., Lemonnier, G., Billon, Y., Lepage, P., Oswald, I.P., Doré, J., Rogel-Gaillard, C., Estellé, J., 2014. Extensive expression differences along porcine small intestine evidenced by transcriptome sequencing. PLoS One. https://doi.org/10.1371/journal.pone.0088515

Mach, N., Berri, M., Estellé, J., Levenez, F., Lemonnier, G., Denis, C., Leplat, J.J., Chevaleyre, C., Billon, Y., Doré, J., Rogel-Gaillard, C., Lepage, P., 2015. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ. Microbiol. Rep. https://doi.org/10.1111/1758-2229.12285

Macleod, M., Gerber, P., Mottet, A., Tempio, G., Falcucci, A., Opio, C., Vellinga, T., Henderson, B., Steinfeld, H., 2013. Greenhouse gas emissions from pig and chicken supply chains - A global life cycle assessment, Food and Agriculture Organization of the United Nations (FAO).

Martin, C.R., Osadchiy, V., Kalani, A., Mayer, E.A., 2018. The Brain-Gut-Microbiome Axis. CMGH. https://doi.org/10.1016/j.jcmgh.2018.04.003

Martínez-Miró, S., Tecles, F., Ramón, M., Escribano, D., Hernández, F., Madrid, J., Orengo, J., Martínez-Subiela, S., Manteca, X., Cerón, J.J., 2016. Causes, consequences and biomarkers of stress in swine: An update. BMC Vet. Res. https://doi.org/10.1186/s12917-016-0791-8

Matsuo, K., Ota, H., Akamatsu, T., Sugiyama, A., Katsuyama, T., 1997.

624 Histochemistry of the surface mucous gel layer of the human colon. Gut.
625 https://doi.org/10.1136/gut.40.6.782
626 Meese, G.B., Ewbank, R., 1973. The establishment and nature of the dominance
627 hierarchy in the domesticated pig. Anim. Behav. https://doi.org/10.1016/S0003-
628 3472(73)80074-0
629 Moeser, A.J., Pohl, C.S., Rajput, M., 2017. Weaning stress and gastrointestinal
630 barrier development: Implications for lifelong gut health in pigs. Anim. Nutr.
631 https://doi.org/10.1016/j.aninu.2017.06.003
632 Moeser, A.J., Ryan, K.A., Nighot, P.K., Blikslager, A.T., 2007. Gastrointestinal
633 dysfunction induced by early weaning is attenuated by delayed weaning and
634 mast cell blockade in pigs. Am. J. Physiol. - Gastrointest. Liver Physiol.
635 https://doi.org/10.1152/ajpgi.00304.2006
636 Nowland, T.L., Plush, K.J., Barton, M., Kirkwood, R.N., 2019. Development and
637 Function of the Intestinal Microbiome and Potential Implications for Pig
638 Production. Anim. an open access J. from MDPI 9.
639 https://doi.org/10.3390/ani9030076
640 Pié, S., Lallès, J.P., Blazy, F., Laffitte, J., Sève, B., Oswald, I.P., 2004. Weaning Is
641 Associated with an Upregulation of Expression of Inflammatory Cytokines in the
642 Intestine of Piglets. J. Nutr. https://doi.org/10.1093/jn/134.3.641
643 Pimentel, H., Bray, N.L., Puente, S., Melsted, P., Pachter, L., 2017. Differential
644 analysis of RNA-seq incorporating quantification uncertainty. Nat. Methods.
645 https://doi.org/10.1038/nmeth.4324
646 Piórkowska, K., Żukowski, K., Ropka-Molik, K., Tyra, M., Gurgul, A., 2018. A
647 comprehensive transcriptome analysis of skeletal muscles in two Polish pig
648 breeds differing in fat and meat quality traits. Genet. Mol. Biol.

https://doi.org/10.1590/1678-4685-gmb-2016-0101

Pluske, J.R., Turpin, D.L., Kim, J.C., 2018. Gastrointestinal tract (gut) health in the young pig. Anim. Nutr. https://doi.org/10.1016/j.aninu.2017.12.004

Razzuoli, E., Villa, R., Sossi, E., Amadori, M., 2011. Characterization of the interferon-α response of pigs to the weaning stress. J. Interf. Cytokine Res. https://doi.org/10.1089/jir.2010.0041

Rhouma, M., Fairbrother, J.M., Beaudry, F., Letellier, A., 2017. Post weaning diarrhea in pigs: Risk factors and non-colistin-based control strategies. Acta Vet. Scand. https://doi.org/10.1186/s13028-017-0299-7

Roura, E., Koopmans, S.J., Lallès, J.P., Le Huerou-Luron, I., De Jager, N., Schuurman, T., Val-Laillet, D., 2016. Critical review evaluating the pig as a model for human nutritional physiology. Nutr. Res. Rev. https://doi.org/10.1017/S0954422416000020

Salmon, H., Berri, M., Gerdts, V., Meurens, F., 2009. Humoral and cellular factors of maternal immunity in swine. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2008.07.007

Sciascia, Q., Daş, G., Metges, C.C., 2016. Review: The pig as a model for humans: Effects of nutritional factors on intestinal function and health. J. Anim. Sci. https://doi.org/10.2527/jas2015-9788

Singh, K., Gobert, A.P., Coburn, L.A., Barry, D.P., Allaman, M., Asim, M., Luis, P.B., Schneider, C., Milne, G.L., Boone, H.H., Shilts, M.H., Washington, M.K., Das, S.R., Piazuelo, M.B., Wilson, K.T., 2019. Dietary arginine regulates severity of experimental colitis and affects the colonic microbiome. Front. Cell. Infect. Microbiol. https://doi.org/10.3389/fcimb.2019.00066

Smith, F., Clark, J.E., Overman, B.L., Tozel, C.C., Huang, J.H., Rivier, J.E.F.,

674 Blisklager, A.T., Moeser, A.J., 2010. Early weaning stress impairs development
675 of mucosal barrier function in the porcine intestine. Am. J. Physiol. -
676 Gastrointest. Liver Physiol. https://doi.org/10.1152/ajpgi.00081.2009
677 Spreeuwenberg, M.A.M., Verdonk, J.M.A.J., Gaskins, H.R., Verstegen, M.W.A.,
678 2001. Small Intestine Epithelial Barrier Function Is Compromised in Pigs with
679 Low Feed Intake at Weaning. J. Nutr. https://doi.org/10.1093/jn/131.5.1520
680 Stuehr, D.J., 2004. Enzymes of the L-arginine to nitric oxide pathway. J. Nutr. 134,
681 2748S-2751S; discussion 2765S-2767S.
682 https://doi.org/10.1093/jn/134.10.2748S
683 Turner, S.P., Roehe, R., D'Eath, R.B., Ison, S.H., Farish, M., Jack, M.C., Lundeheim,
684 N., Rydhmer, L., Lawrence, A.B., 2009. Genetic validation of postmixing skin
685 injuries in pigs as an indicator of aggressiveness and the relationship with
686 injuries under more stable social conditions. J. Anim. Sci.
687 https://doi.org/10.2527/jas.2008-1558
688 Wang, J., Chen, L., Li, P., Li, X., Zhou, H., Wang, F., Li, D., Yin, Y., Wu, G., 2008.
689 Gene Expression Is Altered in Piglet Small Intestine by Weaning and Dietary
690 Glutamine Supplementation. J. Nutr. https://doi.org/10.1093/jn/138.6.1025
691 Wang, W., Hu, H., Zijlstra, R.T., Zheng, J., Gänzle, M.G., 2019. Metagenomic
692 reconstructions of gut microbial metabolism in weanling pigs. Microbiome 7.
693 https://doi.org/10.1186/s40168-019-0662-1
694 Winter, S.E., Winter, M.G., Xavier, M.N., Thiennimitr, P., Poon, V., Keestra, A.M.,
695 Laughlin, R.C., Gomez, G., Wu, J., Lawhon, S.D., Popova, I.E., Parikh, S.J.,
696 Adams, L.G., Tsolis, R.M., Stewart, V.J., Bäumler, A.J., 2013. Host-derived
697 nitrate boosts growth of E. coli in the inflamed gut. Science (80-.).
698 https://doi.org/10.1126/science.1232467

699 Wu, G., Bazer, F.W., Johnson, G.A., Hou, Y., 2018. Board-invited review: Arginine
700 nutrition and metabolism in growing, gestating, and lactating swine. J. Anim. Sci.
701 https://doi.org/10.1093/jas/sky377
702 Wu, G., Knabe, D.A., Kim, S.W., 2004. Arginine Nutrition in Neonatal Pigs. J. Nutr.
703 https://doi.org/10.1093/jn/134.10.2783s
704 Wu, X., Ruan, Z., Gao, Y., Yin, Y., Zhou, X., Wang, L., Geng, M., Hou, Y., Wu, G.,
705 2010. Dietary supplementation with L-arginine or N-carbamylglutamate
706 enhances intestinal growth and heat shock protein-70 expression in weanling
707 pigs fed a corn-and soybean meal-based diet. Amino Acids.
708 https://doi.org/10.1007/s00726-010-0538-y
709 Xu, X., Mishra, B., Qin, N., Sun, X., Zhang, S., Yang, J., Xu, R., 2019. Differential
710 Transcriptome Analysis of Early Postnatal Developing Longissimus Dorsi
711 Muscle from Two Pig Breeds Characterized in Divergent Myofiber Traits and
712 Fatness. Anim. Biotechnol. https://doi.org/10.1080/10495398.2018.1437045
713 Xun, W., Shi, L., Zhou, H., Hou, G., Cao, T., 2018. Effect of weaning age on
714 intestinal mucosal morphology, permeability, gene expression of tight junction
715 proteins, cytokines and secretory IgA in Wuzhishan mini piglets. Ital. J. Anim.
716 Sci. https://doi.org/10.1080/1828051X.2018.1426397
717 Zeng, M.Y., Inohara, N., Nuñez, G., 2017. Mechanisms of inflammation-driven
718 bacterial dysbiosis in the gut. Mucosal Immunol.
719 https://doi.org/10.1038/mi.2016.75
720 Zhang, B., Gan, L., Shahid, M.S., Lv, Z., Fan, H., Liu, D., Guo, Y., 2019. In vivo and
721 in vitro protective effect of arginine against intestinal inflammatory response
722 induced by Clostridium perfringens in broiler chickens. J. Anim. Sci. Biotechnol.
723 https://doi.org/10.1186/s40104-019-0371-4

724 Zhang, Q., Widmer, G., Tzipori, S., 2013. A pig model of the human gastrointestinal
725 tract. Gut Microbes. https://doi.org/10.4161/gmic.23867
726 Zheng, P., Song, Y., Tian, Y., Zhang, H., Yu, B., He, J., Mao, X., Yu, J., Luo, Y., Luo,
727 J., Huang, Z., Tian, G., Chen, H., Chen, D., 2018. Dietary Arginine
728 Supplementation Affects Intestinal Function by Enhancing Antioxidant Capacity
729 of a Nitric Oxide-Independent Pathway in Low-Birth-Weight Piglets. J. Nutr.
730 https://doi.org/10.1093/jn/nxy198

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735 **Table 1:** *Phenotypic measurements between weaned and unweaned pigs at 1, 4 and 14 days post weaning. Data shown are*

736 *means and the pooled SEM for weaned and unweaned pigs for each time points.*

Time point (days post weaning)	1		4		14			p values		
Treatment	Unwean	wean	unwean	wean	unwean	Wean	SEM	time	weaning	time*weaning
Pig performance										
Pig weights (kg)	7.16	6.60	8.31	7.20	12.68	12.41	0.37	<0.001	0.002	0.238
Body lesion scores	1.3	2.4	1.7	2.0	1.6	1.4	0.2	0.007	<0.001	<0.001
Blood and plasma measurement										
RBC (10^6/µl)	5.83	5.92	6.04	6.15	6.78	6.67	0.12	<0.001	0.726	0.432
HCT (%)	35.56	35.78	34.32	37.16	35.14	39.89	1.06	0.035	<0.001	0.019
HGB (g/dl)	11.43	11.55	11.19	12.00	11.16	12.61	0.37	0.309	<0.001	0.045
PLT (10^3/µl)	552.6	551.9	579.4	491.0	595.5	436.0	28.8	0.356	<0.001	0.009
WBC (10^3/µl)	9.41	9.40	10.08	12.33	13.40	13.30	0.84	<0.001	0.202	0.152
Granulocytes (10^3/µl)	2.650	3.854	3.504	4.958	4.096	4.933	0.38	0.003	<0.001	0.722
Total plasma protein (g/l)	45.700	48.688	46.937	45.420	45.663	43.875	0.74	0.005	0.860	0.002
Plasma globulin (g/l)	16.254	17.418	16.528	15.757	14.693	13.262	0.47	<0.001	0.368	0.018
Plasma cholesterol (mmol/l)	3.925	3.687	3.910	2.441	3.681	2.432	0.13	<0.001	<0.001	<0.001
Plasma triglyceride (mmol/l)	0.848	0.872	0.971	0.470	0.939	0.511	0.06	0.001	<0.001	<0.001
Plasma cortisol (ng/ml)	15.886	57.744	16.125	30.884	10.999	17.767	4.18	<0.001	<0.001	<0.001
Intestinal measurement										
Villus length (µm)	0.372	0.293	0.373	0.341	0.403	0.418	0.018	<0.001	0.023	0.026
Crypt depth (µm)	0.151	0.145	0.155	0.171	0.175	0.192	0.005	<0.001	0.018	0.013
Villus width (µm)	0.100	0.098	0.115	0.114	0.128	0.135	0.004	<0.001	0.572	0.217
V/C ratio	2.508	2.106	2.500	2.054	2.368	2.276	0.137	0.944	0.005	0.355
Mast cells (/mm^2 submucosa)	100.0	109.8	96.4	89.6	182.3	161.2	13.6	<0.001	0.582	0.517
Mast cells (/mm^2 mucosa)	138.2	167.3	188.3	106.2	222.3	204.7	20.0	<0.001	0.089	0.005

Goblet cells in villus (/100µm^2)	1931.1	2087.6	763.8	993.0	863.5	908.5	152.8	<0.001	0.260	0.831
Goblet cells in crypt (/100µm^2)	4204.1	3959.8	1699.4	1684.0	1815.0	1770.1	601.4	<0.001	0.643	0.899
Goblet cells total (/100µm^2)	6136.7	6047.4	2463.2	2677.0	2678.5	2678.5	451.4	<0.001	0.898	0.925
Ileal flush sIgA (µg/ml)	596.3	22.3	169.6	10.8	72.5	12.7	88.9	0.007	<0.001	0.011

RBC: red blood cells, HCT: haematocrit, HGB: Haemoglobin, PLT: platelet, WBC: white blood cells, V/C: Villus/Crypt, sIgA: Secretory Immunoglobulin A

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738 **Figure captions**

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740 **Figure 1**: Volcano plots of differentially expressed transcripts between weaned and
741 unweaned pigs at 1, 4 and 14 days post weaning. Red points indicates genes
742 significant at Qval ≤ 0.1.

743

744 **Figure 2:** Number of up and downregulated transcripts between weaned and
745 unweaned pigs at 1, 4 and 14 days post weaning (Qval ≤ 0.1 & FC ≥ 2).

746

747 **Figure 3:** Venn diagram of the number of differentially expressed transcripts between
748 weaned and unweaned pigs for each time point (Qval ≤ 0.1 & FC ≥ 2). Venn diagram
749 was plotted using Venny, an interactive tool for comparing list.

750

751 **Figure 4:** Principal Component Analysis (PCA) of gene expression data from pigs
752 across time point and weaning status.

753

754 **Figure 5:** Gene ontology (GO) and KEGG pathway enrichment analysis showing top
755 10 enriched terms between weaned and unweaned pigs (all time points). Size of
756 circles represents number of gene in each term. A: GO Biological Process, B: GO
757 Molecular Function, C: GO Cellular Compartment, D: KEGG.

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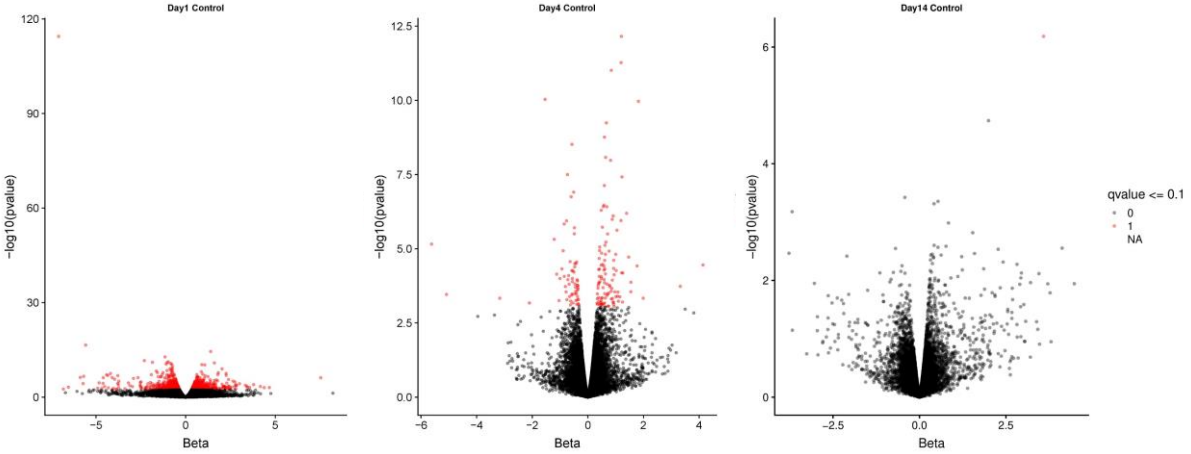
761 **Figure 1**



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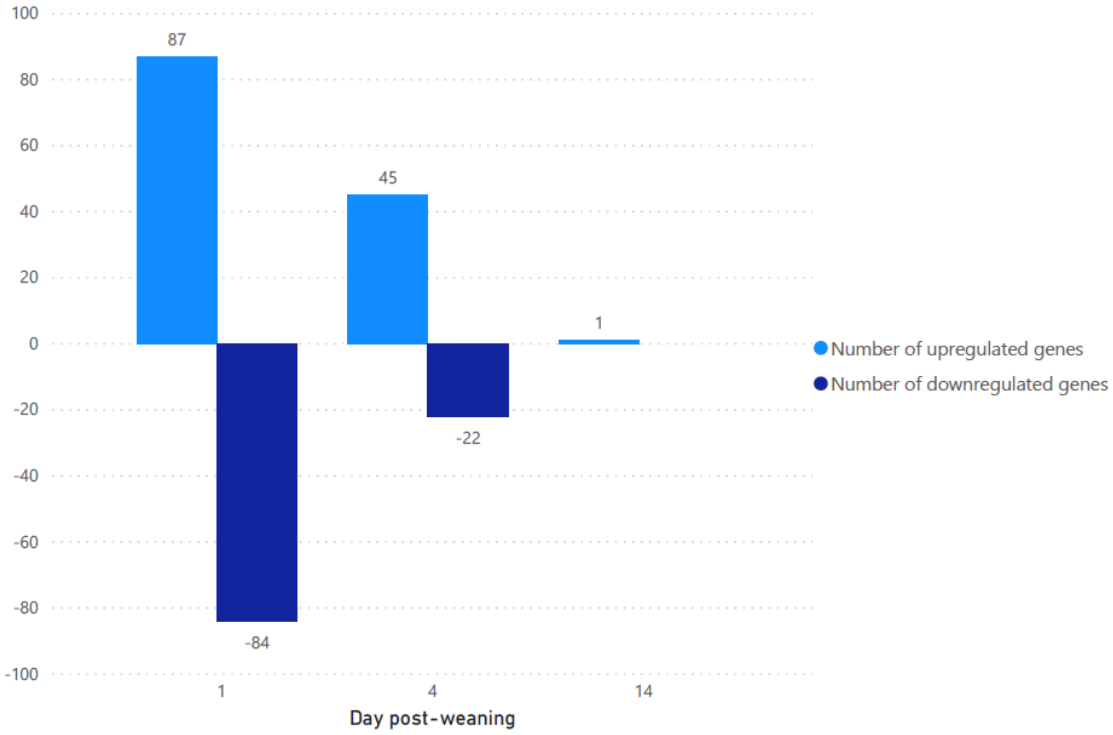
764 **Figure 2**



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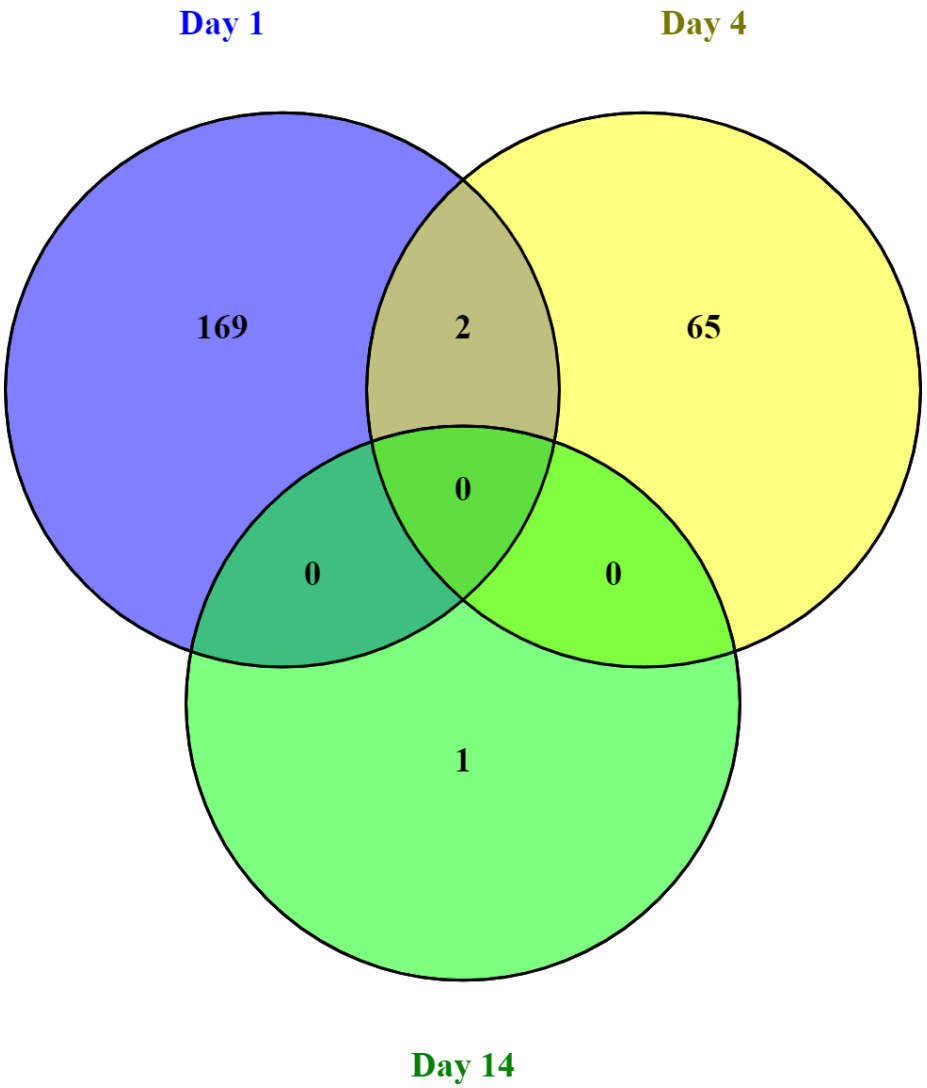
767 **Figure 3**



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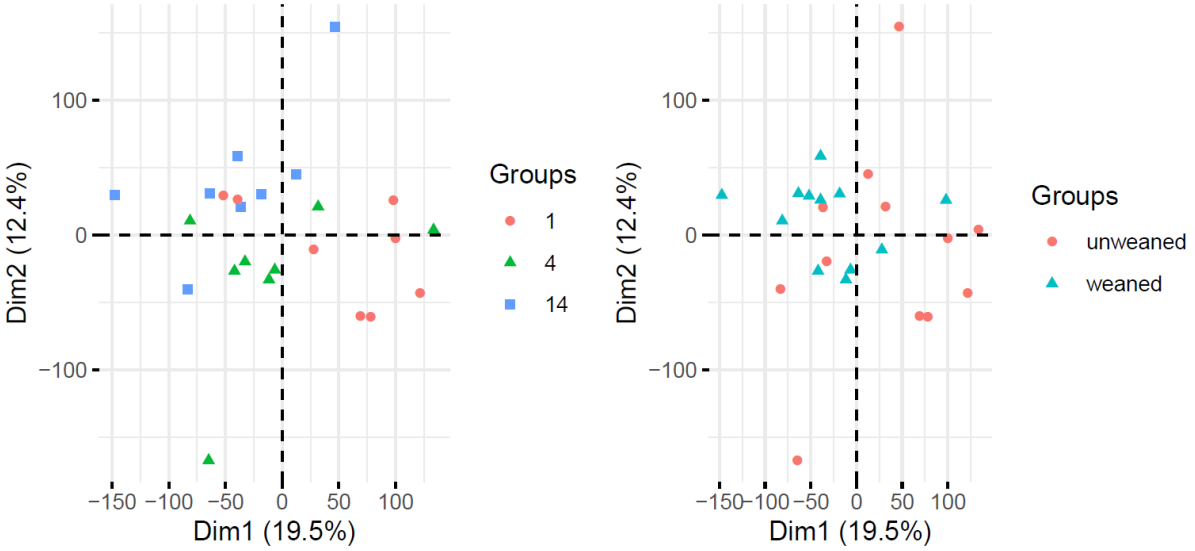
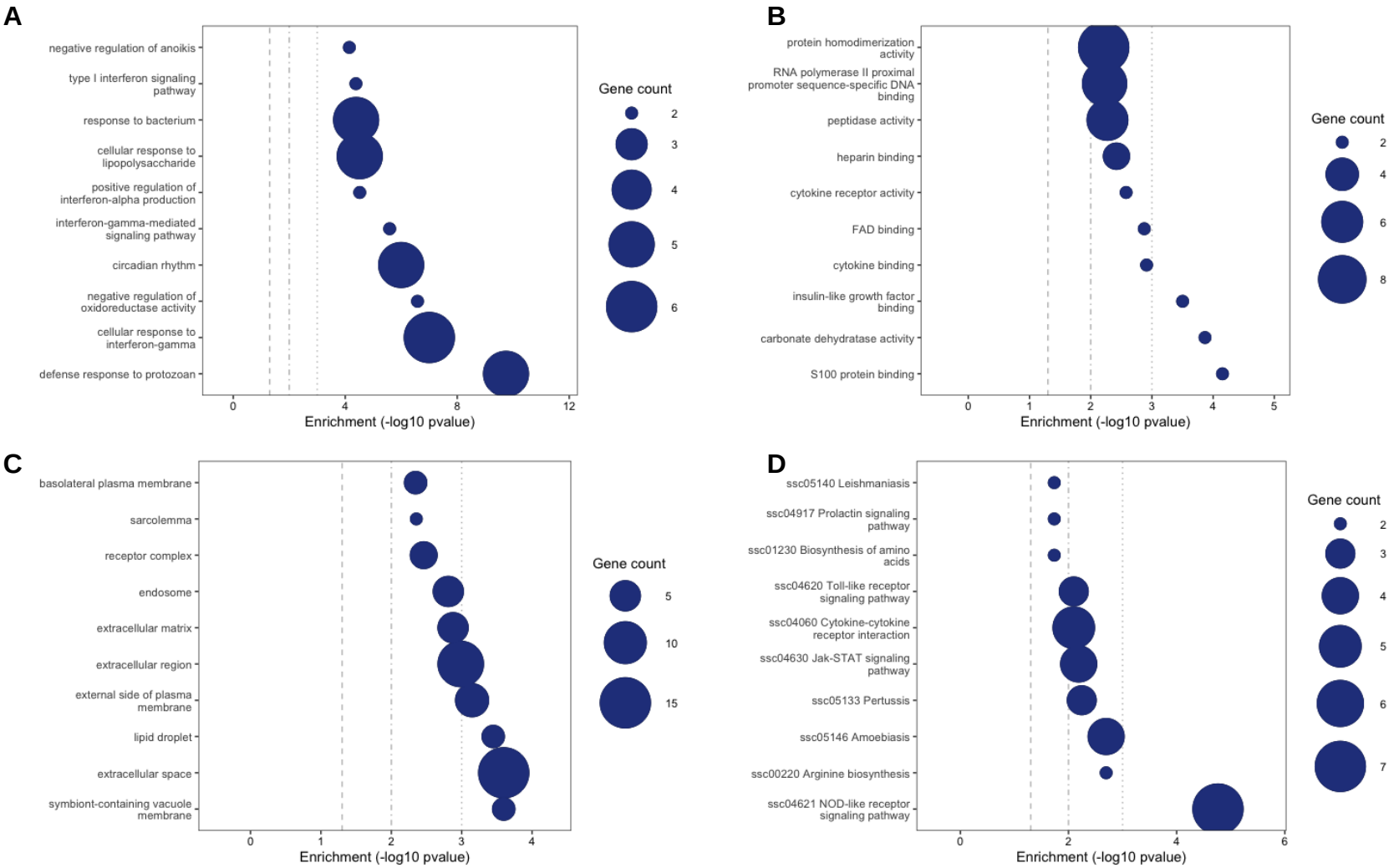
770 **Figure 4**



771
772

773

Figure 5


A
negative regulation of anoikis
type I interferon signaling pathway
response to bacterium
cellular response to lipopolysaccharide
positive regulation of interferon-alpha production
interferon-gamma-mediated signaling pathway
circadian rhythm
negative regulation of oxidoreductase activity
cellular response to interferon-gamma
defense response to protozoan
0
4
8
12
Enrichment (-log10 pvalue)
Gene count
2
3
4
5
6
B
protein homodimerization activity
RNA polymerase II proximal promoter sequence-specific DNA binding
peptidase activity
heparin binding
cytokine receptor activity
FAD binding
cytokine binding
insulin-like growth factor binding
carbonate dehydratase activity
S100 protein binding
0
1
2
3
4
5
Enrichment (-log10 pvalue)
Gene count
2
4
6
8
C
basolateral plasma membrane
sarcolemma
receptor complex
endosome
extracellular matrix
extracellular region
external side of plasma membrane
lipid droplet
extracellular space
symbiont-containing vacuole membrane
0
1
2
3
4
Enrichment (-log10 pvalue)
Gene count
5
10
15
D
ssc05140 Leishmaniasis
ssc04917 Prolactin signaling pathway
ssc01230 Biosynthesis of amino acids
ssc04620 Toll-like receptor signaling pathway
ssc04060 Cytokine-cytokine receptor interaction
ssc04630 Jak-STAT signaling pathway
ssc05133 Pertussis
ssc05146 Amoebiasis
ssc00220 Arginine biosynthesis
ssc04621 NOD-like receptor signaling pathway
0
2
4
6
Enrichment (-log10 pvalue)
Gene count
2
3
4
5
6
7