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Targeted metabolomic profiles of piglet plasma reveal physiological changes over the suckling period

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21 Highlights

- A targeted metabolomic profile was obtained on plasma of piglets over the suckling phase.
- Twenty-three metabolites differed significantly between the analysed time points.
- Plasma threonine, serine and tyrosine levels were lower at 21 days of age.
- Plasma creatinine and acylcarnitine levels indicated an increased muscle deposition at 21 days
 of age.
- Metabolomics in suckling piglets can disclose the biological mechanisms of this fast-growing
 phase.

30 Abstract

31 The suckling phase is a critical period for the piglets due to their incomplete immune system 32 development and their rapid growth rates. In this study, we analysed the metabolomic profiles of 33 piglets over this period. Eighteen piglets (nine males and nine females) from three different litters 34 were included in the study. Body weight was recorded at birth (T0), 12 (T1) and 21 (T2) days after 35 birth. Plasma samples were collected at two critical time points of the suckling phase (T1 and T2) 36 and about 180 metabolites of five different biochemical classes (glycerophospholipids, amino acids, 37 biogenic amines, hexoses and acylcarnitines) were analyzed using a target metabolomics approach 38 based on Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Metabolites whose 39 levels could discriminate the plasma profiles at T1 and T2 were identified using the sparse version of Multilevel Partial Least Squares Discriminant Analysis (sMLPLS-DA), coupled with a stability test 40 41 based on a Leave One Out (LOO) procedure. The level of twenty-three metabolites differed 42 significantly (P < 0.1; both for stability and the effect size) between the two time points. Higher levels 43 of six acylcarnitine (C14:1, C14:1-OH, C16-OH, C4, C5 and C5-OH), serine, threonine and tyrosine, 44 and one phosphatidylcholine (PC ae C42:3) were observed at T1, whereas one biogenic amine 45 (creatinine), eight phosphatidylcholines including PC aa C30:2, PC ae C30:0, PC ae C32:1, PC ae C38:4, PC ae C40:4, PC ae C42:4, PC ae C42:5 and PC ae C44:6, and four sphingomyelins, including 46 47 SM (OH) C22:1, SM C16:0, SM C16:1 and SM C18:0, were more abundant at T2. The Metabolite 48 Set Enrichment Analysis and the Pathway Analysis modules suggested a perturbation of the "glycine 49 and serine metabolism" and the "sphingolipid metabolism". Differences of these metabolites between 50 these two time points might be related to the rapid growth and immunological maturation phases of 51 the piglets in this period. Our results provided new information that could describe the biological 52 changes of the piglets over the suckling period. The identified metabolites may be useful markers of 53 the developmental processes occurring in the piglets over this critical pre-weaned phase.

54 Keywords: growing phase, MLPLS-DA, metabolite, pig, plasma.

55

56 1. Introduction

57 Metabolomics is an omics discipline that describes the metabolome which is defined as the 58 catalog of the metabolites (i.e. small chemical compounds) present in a biological system, usually 59 obtained by analysing different biofluids and tissue types (Psychogios et al., 2011; Hollywood et al., 60 2006). Blood is a tissue that serves as a liquid highway for the molecules that are secreted or discarded 61 by different tissues and organs in the body, and blood serum and plasma are commonly evaluated to 62 test the physiological state and response to different conditions (Psychogios et al., 2011). Metabolomics applied to livestock species is emerging as a powerful tool for the investigation of the 63 64 biological mechanisms and physiological changes underlying adaptations and responses of the 65 animals to different environmental and stressing conditions, treatments and developmental stages, 66 including the interaction between these factors and different genetic backgrounds (Fontanesi, 2016; 67 Goldansaz et al., 2017). In pigs, metabolomic analyses have been applied in finishing animals to study the impact of diets (e.g. Ingerslev et al., 2015; Metzler-Zebeli et al., 2015a, 2105b; Soumeh et al., 68 69 2016, Christensen et al., 2012), sexual dimorphisms (Bovo et al. 2015) and differences between 70 breeds (Bovo et al. 2016) for the detection of novel biomarkers. Moreover, metabolomic studies were 71 carried out in weaning and growing pigs to characterize the effects of different factors such as 72 environment (Solberg et al., 2010, 2016; Dou et al., 2017), feeding (Luise et al., 2019a; Getty et al., 73 2015), malnutrition (Jiang et al., 2016), pathogen challenges (e.g. Sugiharto et al., 2014; Gong et al., 74 2017) and genetics (Luise et al., 2019b; Poulsen et al., 2018).

The suckling period of the piglets is a crucial part of the pig life cycle that is characterized by rapid morphological and physiological modifications, which include the development of the gastrointestinal tract (GIT), significant muscle protein deposition, growth of bones and parenchymatous organs, and the beginning of fat deposition, among several other important changes, that will subsequently affect the performances of the weaned animals (e.g. Mahan and Shields, 1998; 80 Widdowson, 1971; Zhang et al., 1997). Large profit losses in the swine industry can be attributed to 81 morbidity and mortality of piglets occurring during this period. Moreover, suckling piglets, for their 82 similarity with human infants and their easy availability, have been also proposed as premier models 83 for the study of pediatrics' metabolism, nutrition and toxicology (Odle et al., 2014). Owing to its 84 relevance, the suckling phase has been extensively investigated in piglets, particularly to define basic 85 nutritional needs (e.g. Gu and Li, 2003; Rezaei et al., 2013), the structural and functional 86 modifications which occur in the GIT, including the development of the Gut-Associated Lymphoid 87 Tissue (GALT) (e.g. Bailey et al., 2005; Lallès et al. 2007; Stokes 2017), the establishment of a 88 microbial community (Chen et al., 2017; Motta et al., 2019) and the cross-talk between host and the 89 microbiota that drives the settlement of the gut ecosystem that can affect the physiology of the piglets 90 (Bailey et al., 2005; Mach et al., 2015; Motta et al., 2019; Schokker et al., 2014; Stokes, 2017). 91 Despite these works have obtained a good characterization of this first part of the piglet life span, a 92 detailed description of the physiological and biochemical changes over the piglet suckling period has 93 not been completely achieved yet. The application of metabolomic approaches to investigate piglet's 94 maturation in this phase under normal healthy condition remains scarce and limited to the description 95 of plasma or serum amino acid profiles (Flynn et al., 2000; Yin et al., 2011).

96 In this study, we used a targeted metabolomic approach based on mass spectrometric detection 97 of about 180 metabolites to disclose the metabolic determinants that could describe the physiological 98 developments of suckling pigs in two well-defined maturation phases (1-2 and 2-4 weeks of age; 99 Stokes et al., 2004). The results provided a first overview of the plasma metabolome changes that 100 occur over this rapid growth phase in healthy piglets.

101

102 **2. Materials and methods**

103 The procedures complied with the Italian regulations pertaining to experimental animals and 104 were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of 105 Bologna and the Italian Ministry of Health with the approval number 551/2018-PR, 16th July 2018. 106

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107 **2.1. Animals and sampling**

108 The experiment was conducted in a commercial piggery in the North of Italy. A total of 18 109 healthy crossbred [(Italian Landrace x Italian Large White) x Italian Duroc] piglets were selected 110 from 3 different litters (3 different sows). Each litter counted six piglets balanced per sex (three males 111 and three females). Piglets were followed from farrowing (initial body weight 1.50 ± 0.2 kg) until 112 weaning, occurring on day 21. Over this period, all piglets were housed with their respective sow in 113 farrowing cages located in the same room with automated control of temperature (24 °C) and 114 ventilation. Farrowing cages were equipped with a nest and red-light lamp to guarantee an adequate 115 thermal comfort for the new-borns. During the trial, piglets had free access to water through nipple 116 and suckled the milk from their respective mother. No creep feed was administrated. During the 117 lactation period, sows had free access to feed and water. Sows were fed with the same lactation diet 118 which was formulated to meet or exceed the National Research Council (NRC, 2012) nutrient 119 requirements for lactating sows. Ingredients and calculated composition of the sows' diet are reported in Table S1. 120

121 Piglets were then individually weighed at day 12 (T1) and at day 21 (T2) after birth. Average 122 body weights were 4.16 ± 0.76 kg and 6.26 ± 1.2 kg, respectively. All animals remained healthy over 123 the trial period.

For each piglet, blood was collected in 9 mL K3 EDTA-containing vacutainers (Vacutest Kima Srl, Arzergrande PD, Italy) by venipuncture of *vena cava* at the two time points (T1 and T2). In order to reduce circadian rhythm variability, blood was collected in the morning between 7.00 am and 8.00 am. Blood was centrifuged at 4 °C at $3000 \times g$ for 10 min to obtain plasma following the procedure already described (Bovo et al., 2016). Plasma was snap-frozen in liquid nitrogen and stored
at -80 °C until further analysis.

- 130
- 131 2.2. Analysis of plasma metabolites

Targeted metabolic profiling of plasma samples was performed using the AbsoluteIDQ p180 Kit, (Biocrates Life Science AG, Innsbruck, Austria). The assay allows the simultaneous quantification of 186 metabolites belonging to five biological classes, including amino acids (n. = 21), biogenic amines (n. 19), hexose (n. 1), carnitines (n. 40; divided in acylcarnitines, hydroxylacylcarnitines and dicarboxylacylcarnitines), sphingomyelins (n. 15), phosphatidylcholines (n. 76) and lysophosphatidylcholines (n. 14). Table S2 lists all analysed metabolites with full biochemical names and abbreviations.

Preparation of the samples required $10 \ \mu L$ plasma, processed according to the manufacturer's instructions (Biocrates Life Science AG). All samples were measured in a single assay together with a mixed porcine plasma sample, obtained from 10 pigs and were analysed in triplicate as quality control (QC).

143 The analytical platform included a Series 200 high-performance liquid chromatography system (Perkin Elmer, Waltham, Massachusetts, USA) coupled with an API 4000 QTrap mass 144 145 spectrometer (AB-Sciex, Foster City, California, USA). Metabolites belonging to the amino acid and 146 biogenic amine classes were analyzed by liquid chromatography-tandem mass spectrometry (LC-147 MS/MS) and quantified by isotopic dilution on a seven-point calibration curve. Metabolites belonging 148 to the acylcarnitine, phospho- and sphingolipid and hexose classes were analyzed by flow injection 149 analysis (FIA) - MS/MS and quantified by their relative intensity over the chosen isotopically-150 labelled internal standards. The quantification of a subset of compounds determined by FIA-MS/MS 151 was considered to be as semi-quantitative according to the kit manufacturer supplier (Biocrates Life 152 Science AG). The complete analytical process was performed using the MetIQ software packages,

which is an integral part of the AbsoluteIDQ Kit (Biocrates Life Science AG). Results were expressed
in micromolar unit (µM).

155

156 **2.3. Statistical analyses**

Quality assessment of the metabolomic datasets obtained at the two time points was carried 157 158 out in two steps, according to the procedures described in Bovo et al (2015, 2016): (i) only metabolites 159 with an intra-plate coefficient of variation (CV%) ≤ 20 (and CV% \neq NA, that means not obtained for 160 missing values) over QC samples and with less than 5% of missing values (NAs) were retained and (ii) samples were considered as outliers and removed if the metabolite concentrations measured for 161 162 that sample lied 1.5 times the interquartile range below or above the corresponding median for 30% of the data columns, i.e. metabolites. Full intra-plate CV% and excluded metabolites are reported in 163 164 Table S2.

165 Multilevel Partial Least Squares Discriminant Analysis (MLPLS-DA, Westerhuis et al., 2010) was used to investigate differences between T1 and T2 plasma metabolomic profiles. This statistical 166 167 approach considers the paired structure of the dataset taking advantage of PLS-DA which is a 168 multivariate tecnique that can model the dependences among metabolites and that is more suitable when dealing with small sample size, many irrelevant predictors and high co-linearity among them 169 170 (Lê Cao et al., 2011; Antonelli et al., 2019). This allows to address the biological complexity of the 171 analysed system with a proper and correct methodology. In order to detect a limited number of metabolites related to the main response variable (time points), we applied the sparse version of this 172 technique (sPLS-DA; Chung and Keles, 2010). As described by Westerhuis et al. (2010), the 173 174 multilevel approach has the advantage to separate the total variation into between-subject variation 175 and within-subject variation, the last describing the net differences in each of the measured variables 176 for each subject (paired observations). Observations on day 12 (T1) and day 21 (T2) were used to

177 compute and structure: (i) the within subject variation as $\begin{bmatrix} -D \\ +D \end{bmatrix}$, with D = [T2 - T1], and (ii) the 178 between subject variations as $\begin{bmatrix} M \\ M \end{bmatrix}$, with $M = \frac{1}{2}[T1 + T2]$. The two matrices were then inspected via 179 Principal Component Analysis (PCA) in order to identify sub-structures of data. Principal Component 180 Analysis was performed without data scaling to identify larger factors influencing the data. Further 181 to the identification of data sub-structures, linear regression models were fitted on raw data in order 182 to completely remove the factors causing sub-structures. Linear mixed models included sex, litter and 183 the piglet weight as covariates. The applied linear mixed model was:

184
$$y_{ij} = \beta_0 + \beta_T T_{ij} + \beta_S S_{ij} + \beta_L L_{ij} + \beta_W W_{ij} + \beta_{0i} + \varepsilon_{ij} \quad (I)$$

where, for the *i*th piglet on the *j*th time occasion, *y* was the concentration of a metabolite; β_0 185 186 was a fixed intercept term; T was a dummy variable distinguishing T1 and T2 data points; S was a 187 dummy variable representing the sex of the animal (female or castrated male); L was a dummy variable representing the litter (three levels); W was a continuous variable representing the animal 188 189 weight; β_T , β_S , β_L , β_w were the related corresponding regression coefficient; β_{0i} was a random intercept term which was specific for the ith piglet, and ε_{ii} was a random error term (Pinheiro and Bates, 2009). 190 191 Confounding effects were removed by computing the residuals of model (I) which represented 192 the part of the metabolite concentration that cannot be explained by the confounding factors included

in the model:

$$e_{ij} = y_{ij} - (b_0 + b_T T_{ij} + b_S S_{ij} + b_L L_{ij} + b_W W_{ij} + b_{0i}) \quad (II)$$

195 where b_0 , b_T , b_S , b_L , b_W and b_{0i} were the maximum likelihood estimates of β_0 , β_t , β_s , β_L , β_W and 196 b_{0i}.

197 Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was applied on the between 198 subject variation obtained from residuals of data (see equation II), by using as response variable a 199 dummy variable distinguishing the two time points (T1 and T2). The sPLS-DA sparseness coefficient eta (ranging from 0.1 to 0.9) and the number of hidden components K (ranging from 1 to 3) were automatically selected by using an internal 10-fold cross-validation procedure.

Metabolites contributing with non-null coefficients to discriminant dimensions (i.e. $\beta \neq 0$) were selected and validated by applying a permutation test coupled with a Leave One Out (LOO) procedure as detailed in Bovo et al., 2015, 2016. The purpose of this approach was to evaluate both the stability (P_{st}) and the effect size (P_{si}) of selected metabolites by testing the null hypothesis that metabolite concentration is unrelated to piglet maturation. Metabolites having P < 0.1 for both stability and effect size, simultaneously, were considered stable and significant (Bovo et al., 2016).

The relative time points-specific difference in the metabolite quantification (Δ %) was calculated as the difference between the mean metabolite concentration at T1 and the mean metabolite concentration at T2, divided by the mean metabolite concentration at T1 and expressed as percentage.

All analyses have been performed in R v. 3.3.1 (R Development Core Team, 2018) using the "lme4" packages for the computation of metabolite residuals, the "spls" packages (function *cv.splsda* and *splsda*) for the sPLS-DA analysis and the function "prcomp" for PCA analysis.

214

215 **2.4. Metabolomic enrichment and pathway analyses**

Metabolites identified using the statistical pipeline described above were mapped onto the 216 217 Human Metabolome Database 4.0 (HMDB; Wishart et al., 2018). Metabolite enrichment analysis, 218 performed via over-representation analysis (ORA), was used to interpret the metabolic pathways 219 involving the selected metabolites. Analyses were carried out with the Metabolite Set Enrichment 220 Analysis (MSEA) and with the Pathway Analysis modules of MetaboAnalyst 4.0 221 (http://www.metaboanalyst.ca/MetaboAnalyst/; Chong et al., 2018). Metabolite Set Enrichment 222 Analysis module identifies biologically meaningful patterns that are significantly enriched in 223 metabolomic data using human and mammalian libraries. This module was applied setting the pathway-associated metabolite sets, by interrogating a total of 99 metabolite sets, and using only 224

225 those containing at least two compounds. The Pathway Analysis module exploits the Kyoto 226 Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/; 80 metabolite sets 227 interrogated) and The Small Molecule Pathway Database (SMPDB; http://smpdb.ca/; 99 metabolite 228 sets interrogated) metabolic pathways with pathway enrichment and pathway topology methods. 229 Pathway topology analysis estimates the node relative importance (or role) in the metabolite network. 230 Here, it was applied with the human pathway libraries of KEGG and SMPDB using the relative 231 betweenness centrality as measure of metabolite importance. Over-representation analyses were 232 based on the hypergeometric test. The False Discovery Rate (FDR) procedure was adopted to counteract the problem of multiple testing. Only metabolite sets presenting a FDR adjusted P < 0.05, 233 234 and characterized by at least two different input metabolites, were considered significantly over-235 represented.

236

3. Results

238 **3.1. Metabolomic data**

239 Quality assessment defined the final dataset that included metabolomic data from all 18 piglets 240 (no outliers were detected) and for a total of 167 out of 186 analysed (89.8%) metabolites (Table S2). 241 Mean and standard deviation (SD) of raw data for the retained 167 metabolites [36 acylcarnitines, 19 242 amino acids, 10 biogenic amines, 87 phosphatidylcholines (among them 14 lysophosphatidylcholines), 14 (hydroxyl) sphingomyelins and 1 monosaccharide (or exose)] at the 243 244 two investigated time points (T1 = day 12; and T2 = day 21) are reported in Table S3.

245

246 **3.2. Effects of litter and sex on metabolomic profiles**

Multilevel Partial Least Squares Discriminant Analysis is a statistical approach that allows analysing paired data in a multivariate way, by dividing the total variation in the data into between subject variation and within subject variation. To identify potential confounding effects acting on the 250 metabolomic profiles, we studied the between subject variation via PCA before fitting the sPLS-DA 251 on the within variation matrix. Then, after the identification of the confounding effects, we included them in a linear mixed model to correct the original data. We identified the influence of litter and sex 252 253 on the plasma metabolomic profiles. Indeed, the combination of principal components (PC) two and PC3 highlighted the effect of litter (Figure S1), while PC4 and PC6 clustered the samples by sex 254 255 (Figure S2). Values of the explained total variance for litter and sex were 21.78% and 14.81%, 256 respectively. After removing these effects, the PCA on the new between subject variation did not 257 evidence any residual effect.

258

259 **3.3.** Metabolomic profile differences between the two developmental neonatal phases

Differences in the plasma metabolic profiles between T1 and T2 were investigated by 260 261 applying sPLS-DA on the within variance (after the removal of the confounding effects). This 262 approach was coupled with a statistical procedure aimed at evaluating the stability and significance of the metabolite selected as differentially abundant between the two time points. Since multilevel 263 264 sPLS-DA focuses on the within subject variation (variation similar among the subjects), the first 265 component mainly describes the difference between the analysed classes due to a main acting factor 266 (i.e. time points), whereas the other components describe the within subject variation that is different 267 between the subjects by reflecting these differences at the level of the main acting factor (Westerhuis 268 et al., 2010). Figure 1 demonstrates that the metabolomic profiles clearly cluster into two groups 269 which represent T1 and T2. Therefore, the selected metabolites can discriminate the piglet profiles at 270 the two different ages. Table S4 reports the stability, the effect size obtained with the multilevel sPLS-271 DA analysis and the relative time points-specific difference in the metabolite quantification (Δ %) for 272 all quantified metabolites. According to the stability test, 23 metabolites (13.8%) had a $P_{st} < 0.1$ 273 (threshold of significance based on the validation procedure). All the metabolic classes, except 274 hexoses, were included in this stability test results list. Considering the effect size (significance test), 275 34 metabolites (~ 20.4%) had a $P_{si} < 0.1$ (Table S4). A total of 32 out of the 34 identified metabolites 276 resulted significantly selected ($P_{si} < 0.05$), with two of them, PC as C40:1 and PC as C42:4, selected 277 with $P_{si} < 0.01$.

A total of 23 metabolites resulted both stable and significant ($P_{st} < 0.10$ and $P_{si} < 0.1$; Table 1). At 278 279 T1, piglets had higher levels of: (i) six acylcarnitines including C14:1 (Δ % = 68.45%), C14:1-OH 280 $(\Delta\% = 41.18\%)$, C16-OH ($\Delta\% = 14.31\%$), C4 ($\Delta\% = 76.99\%$), C5 ($\Delta\% = 130.01\%$) and C5-OH (C3-281 DC-M) ($\Delta\% = 39.88\%$), (ii) three amino acids: serine ($\Delta\% = 59.86\%$), threonine ($\Delta\% = 60.75\%$) and 282 tyrosine ($\Delta\% = 28.07\%$), and (iii) one phosphatidylcholines: PC as C42:3 ($\Delta\% = 42.25\%$) compared to piglets at T2. At T2, piglets had higher levels of: (i) one biogenic amine: creatinine (Δ % = -283 284 20.20%), (ii) eight phosphatidylcholines including PC as C30:2 (Δ % = -27.73%), PC as C30:0 (Δ % = -48.24%), PC ae C32:1 (Δ % =-37.82%), PC ae C38:4 (Δ % = -29.89%), PC ae C40:4 (Δ % = -285 29.22%), PC ae C42:4 ($\Delta\%$ = -31.01%), PC ae C42:5 ($\Delta\%$ = -19.64%) and PC ae C44:6 ($\Delta\%$ = -286 287 36.88), and (iii) four sphingomyelins including SM (OH) C22:1 ($\Delta\%$ = -31.06%), SM C16:0 ($\Delta\%$ = -23.89%), SM C16:1 (Δ % = -25.75%) and SM C18:0 (Δ % = -23.85%) compared to piglets at T1. 288

289

290 **3.4. Metabolomic pathways**

All the 23 metabolites selected by the sMLPLS-DA were mapped on the HMDB (Table 1). Then, to obtain an overview of the metabolic pathways that are altered during the suckling period, Metabolite Set Enrichment Analysis and Pathway Enrichment analyses were run through the MetaboAnalyst tool. The metabolite set enrichment analysis, performed according to MSEA module, suggested the involvement of the "Glycine and Serine Metabolism" and confirmed the "Sphingolipid Metabolism" (Table S5). However, none of these pathways was significantly over-represented.

Figure 2 shows the metabolome landscape perturbations that occur over this period as defined by mapping the metabolites on the human KEGG and SMPDB libraries. Table S6 reports detailed information of the metabolite enrichment analyses obtained with the two modules. Over the KEGG 300 database, a total of three pathways ("Aminoacyl-tRNA biosynthesis"," Sphingolipid metabolism" 301 and "Glycine, serine and threonine metabolism") were over-represented (P < 0.01). In the SMPDB 302 library-based Pathway Enrichment analysis, only one pathway ("Sphingolipid metabolism") was 303 marginally over-represented (P < 0.2). None of these pathways was significantly enriched when the 304 FDR-based adjustment for multiple comparisons was applied.

305

306 **4. Discussion**

307 The growth rate in piglets during the neonatal period is greater than at any other stages of 308 postnatal life. For this reason, the suckling phase represents a critical period for the piglets that express 309 their highest growth and developmental potentials, which affect the subsequent performances of the 310 animals and resilience against sub-optimal post-weaning conditions (Pluske, 2016). Most of the 311 previous studies that described blood metabolite levels in suckling piglets included different 312 treatments to the animals and evaluated a limited number of small chemical compounds, mainly 313 amino acids (e.g. Flynn et al., 2000; Yin et al., 2011). It is clear that a more detailed description of 314 the metabolomic profiles of the piglets in this phase could provide new opportunities to understand 315 the physiological mechanisms underlying particularly the high muscle mass deposition rate, bone 316 formation and GIT development, that are part of the critical biological growth waves of the suckling 317 period (Pluske, 2016; Rezaei et al., 2013; Stokes et al., 2004). This study investigated plasma 318 metabolomic profiles of suckling piglets providing information for 167 metabolites covering five 319 main metabolite classes. The obtained results aimed at understanding the physiological dynamics of 320 the maturation of suckling piglets in two well-defined immunological maturation stages, before and 321 after the first two weeks of age (Stokes et al., 2004). These stages were captured in the study by two 322 time points (at day 12 and day 21) that were used for a first comparative analysis of their metabolomic 323 profiles. sML-PLSDA, a multivariate approach incorporating the dependences among metabolites, 324 was used to investigate differences between T1 and T2 plasma metabolomic profiles. This approach

was applied to better address the biological complexity of the investigated question by considering multiple response variables modelled jointly, overcoming the limits of the univariate techniques which cannot consider dependencies among metabolites that are clearly present in the investigated system. Multivariate analysis has also other advantages especially in the setting of a large number of variables (the analysed metabolites) in a small sample size that in univariate analysis would suffer a limited statistical power.

331 During the first two weeks of life, the intestine of the piglets starts to be colonized by lymphoid 332 cells. At this stage, Peyer's patches do not show a mature structure yet, whereas from day 14 to day 28, intestine is characterized by the initial colonization of CD4⁺ T and few B cells and Peyer's patches 333 334 develop an adult structure (Stokes et al., 2004). Furthermore, during the suckling period a continuous 335 variation of the intestinal microbiota community is observed due to its un-stable colonization, 336 potentially impacting the host metabolism (Chen et al., 2017). According to these considerations, the 337 study clearly showed that the metabolomic profiles of the piglets at the two time points were different, 338 mimicking, to some extent, the changes that occurred in these animals over the nine days that 339 separated the two sampling points where animals grew on average more than two kg (about 50% of 340 the average weight at T1).

341 The sMLPLS-DA allowed to disclose a total of 23 discriminant metabolites that can describe 342 the physiological changes over this period. Day 12 showed higher concentrations of three amino acids 343 (threonine, tyrosine and serine) and six acylcarnitines (C4, C5, C5-OH, C14:1, C14:1-OH and C16-344 OH). The decreased level of threonine during the suckling period is in compliance with the results reported by Flynn et al. (2000). Threonine is one of the key amino acid needed to support optimum 345 346 growth and immune function of the piglets. It plays a critical role in the regulation of the intestinal 347 mucosal integrity, as it is required for the production of mucins (Van Klinken et al., 1995), and is one 348 of the major amino acids in plasma immunoglobulins (Smith and Greene, 1977). Since both mucin 349 and immunoglobulin productions increase over the suckling period (Brown et al., 2006; Martin et al.,

350 2005), the lower threonine level at T2 could reflect, at least in part, the increased use of this amino 351 acid for the anabolism of these proteins (Bertolo et al., 1998; Stoll et al., 1998). The same decreasing direction of the other two amino acids (i.e. tyrosine and serine) was not in agreement with the 352 353 observations of Flynn et al. (2000), but it matched what reported by Yin et al. (2011) who however analysed amino acid levels in serum over the suckling period. These differences among studies might 354 355 be due to the composition of the milk that is taken by the piglets, which can vary depending on the 356 breed and parity of the sow, on her feeding and on environmental conditions (Picone et al., 2018; 357 Leonard et al., 2010). The contemporary change of serine and threonine could depend by the increased need of proteins that can receive post-translationally functional adaptation because both amino acids 358 359 are important for N-glycosylation in the endoplasmic reticulum and O-glycosylation of the Golgi 360 apparatus (Blom et al., 2004).

361 Serine plays a central role in cell proliferation, as it is the predominant source of one-carbon 362 groups for the *de novo* synthesis of purine nucleotides and deoxythymidine monophosphate (de Koning et al., 2003). Plasma serine could derive from different sources: (i) feed intake, (ii) 363 364 degradation of protein and phospholipid, (iii) biosynthesis from the glycolytic intermediate 3-365 phosphoglycerate and (iv) conversion of glycine operated either by the glycine cleavage system (GCS) or by the serine hydroxymethyltransferase enzymes (EC 2.1.2.1; KEGG orthology K00600). 366 367 The recent observation of the efficacy of serine supplementation in weaning pigs, to improve villus 368 health (Zhou et al., 2018), would suggest that this non-essential amino acid can be nevertheless 369 limiting growth also before weaning.

Plasma tyrosine might derive from both phenylalanine catabolism and diet intake. This amino acid is a precursor of catecholamines (e.g. epinephrine, norepinephrine and dopamine) and it is well known to promote lipid metabolism (Korner et al., 2019). The lower level of serine and tyrosine in the T2 plasma samples might highlight an increased requirement of these amino acids in the latter weeks of the suckling period (Cynober, 2002). The amino acid composition of a milk-based diet or 375 the available amount of milk per piglets could not be enough to cover the nutritional requirement of 376 21 days-old piglets. It is well known that the imbalance of amino acid intake could limit growth and development of the piglets at this stage (Le Floc'h et al., 2018; Rezaei et al., 2013). The common 377 378 practice of offering pre-starter feed during the suckling period is applied with the aim to integrate a 379 milk-based diet and to improve piglet preparation to weaning (Pluske et al., 1997). Therefore, the 380 plasma amino acid differences between the two time points could help to better define the amino acid 381 pre-starter integration. Additional studies are needed to better evaluate the application of this 382 information.

383 Pathway analysis identified the "Glycine, serine and threonine metabolism" as a perturbed 384 biological mechanism in the suckling period. Glycine is considered a non-essential amino acid in pigs 385 for its endogenous synthesis in the body. It represents a major constituent of extracellular structural 386 proteins (mainly collagen and elastin). At T1, the "Glycine, serine and threonine metabolism" 387 pathway is characterized by a higher plasma level of serine and threonine and by a numerically lower 388 level of glycine, compared to the T2. Since both serine and threonine are implicated in the formation 389 of glycine, via serine hydroxymethyltransferase (SHMT) and the threonine dehydrogenase pathway, 390 respectively, our results might suggest that the formation of glycine via serine and threonine could be 391 perturbed or that plasma glycine could be converted to serine or used for protein formation in younger 392 pigs (Lamers et al., 2007; le Floc'h, 1994). Additional investigations of the "Glycine, serine and 393 threonine metabolism" pathway in young piglets are needed to define the glycine and serine required 394 levels to fully cover the physiological functions of the involved amino acids (Wang et al., 2013).

395 Six acylcarnitine (C4, C5, C5-OH, C14:1, C14:1-OH and C16-OH) had higher levels at day 396 12 than at day 21. Short-chain acylcarnitine, including C4 and C5 acylcarnitines, derives from 397 glucose, amino acids (including isoleucine, leucine, valine, tyrosine, and phenylalanine) and fatty 398 acid degradation, while medium- and long-chain acylcarnitines are derived from the oxidative 399 catabolism of fatty acid. Acylcarnitines are involved in fatty acids (C2-C26) transportation and can 400 be utilized for energy production in mitochondria or for the synthesis of endogenous molecules 401 (Rinaldo et al., 2008). Therefore, medium and long acylcarnitines are considered markers for 402 mitochondrial fatty acid oxidation. Their higher plasma concentrations at T1 may reflect an 403 incomplete long-chain fatty acid catabolism and β-oxidation processes and a reduced bioavailability 404 of acyl-CoA in the mitochondria at this stage (Adams et al., 2009; Meyburg et al., 2001). On the other 405 hand, these results indicate that piglets have a more efficient fatty acid catabolism than younger 406 piglets immediately before weaning. Taking into account that fatty acids are mainly utilized for 407 skeletal muscle deposition, which increases according to piglets age, the higher acylcarnitine level 408 observed in T1 can reflect the different muscle deposition rates at the two investigated time points.

At day 21 the metabolic profile was discriminated by a higher concentration of nine phosphatidylcholines and four sphingomyelins, indicating a clear influence of the age on lipid metabolism. Pathway Enrichment analysis with the KEGG and SMPD libraries indicated that a lipid related pathway, the sphingolipid metabolism, can capture the modified metabolism over the analysed suckling period.

414 Phosphatidylcholines, mainly derived from choline via the cytidine diphosphate (CDP)-415 choline pathway, represent the major phospholipid component of mammalian cell membranes 416 (Nohturfft and Zhang, 2009). They are involved in proliferative growth and programmed cell death 417 processes. Their biosynthesis is required for the physiological secretion of very-low-density 418 lipoprotein (VLDL) by the hepatocytes (Li and Vance, 2008). Phosphatidylcholine dysregulations 419 might be associated with disturbed inflammation homeostasis (Vorkas et al., 2015). As 420 sphingomyelins can be synthesized from phosphatidylcholines via sphingomyelin synthases 421 (Gibellini and Smith, 2010), it is not surprising that compounds of this class have a higher 422 concentration at the same time point in which several phosphatidylcholines had a higher level. 423 Sphingomyelins are considered rate-limiting precursors for other sphingolipids classes, such as 424 ceramides, that are directly involved in a variety of cell-signalling events, regulation of cells 425 endocytosis and functionality of ion channel and G-protein coupled receptor (Slotte, 2013; Milhas et al., 2010). Furthermore, aged and adult CD4⁺ T cells produce more sphingomyelins than young CD4⁺ 426 427 T cells (Molano et al., 2012). Piglets start to display CD4⁺ T cells in the lamina propria between the 428 second and fourth weeks after birth (Stokes et al., 2004). Based on these considerations, even if our 429 study did not follow the maturation of intestinal CD4⁺ T, the obtained results on the level of several sphingomyelins might indirectly provide information on the piglet immunological maturation process 430 431 (referred to CD4⁺ T cells) over the suckling period. Inspecting in details the results obtained for the 432 sphingomyelin class, it seems that an elongation step (converting SM C16:0 into SM C18:0) and only a desaturation/saturation step (separating SM C16:0 from SM C16:1) might be stimulated at T2. It 433 434 will be interesting to evaluate if these biochemical processes could also reflect subsequent different composition of lipids and lipoproteins whose blood transportation depends on sphingomyelins 435 436 (Nilsson and Duan, 2006).

In addition to the described phosphatidylcholines and sphingomyelins, T2 had a higher level of plasma creatinine. Creatinine is a chemical by-product of creatine that is used to supply energy mainly to muscles. Under physiological conditions, creatine and creatine phosphate spontaneously and irreversibly break down to creatinine, which is subsequently excreted in the urine (Van Niekerk et al., 1963). A high level of creatinine at T2 is in line with the other metabolite profiles, including the mentioned acylcarnitine levels, indicating an increased muscle deposition rate at T2.

443

444 **4.** Conclusions

In this work, the metabolomic profiles of suckling piglets were investigated to obtain a first overview of metabolic modifications that occur in plasma over this period. The overall picture clearly indicated that the used targeted metabolomic approach can describe the physiological maturation of piglets over the analysed time points, highlighting a modification in the plasma level of threonine, tyrosine and serine, several acylcarnitines and sphingomyelins. The obtained results could also

450	improve the usefulness of the suckling pig as an animal model for the study of pediatric metabolism.
451	Further investigations are needed to clarify the connection of the observed metabolomic profiles with
452	the host microbiota and the immune system development that, in turn, could be relevant to establish
453	relationships with the post-weaning survival rates of the animals.
454	
455	Conflict of interest
456	The authors declare that there is no conflict of interests regarding the publication of this
457	article.
458	
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462	
463	Reference
464	Adams, S.H., Hoppel, C.L., Lok, K.H., Zhao, L., Wong, S.W., Minkler, P.E., Hwang, D.H., Newman,
465	J.W., Garvey, W.T., 2009. Plasma acylcarnitine profiles suggest incomplete long-Chain fatty
466	acid b-Oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic african-
467	american women. J. Nutr. 139, 1073–1081.
468	Antonelli, J., Claggett, B.L., Henglin, M., Kim, A., Ovsak, G., Kim, N., Deng, K., Rao, K., Tyagi,
469	O., Watrous, J.D., Lagerborg, K.A., Hushcha, P.V., Demler, O.V., Mora, S., Niiranen, T.J.,
470	Pereira, A.C., Jain, M., Cheng, S. (2019) Statistical Workflow for Feature Selection in Human
471	Metabolomics Data. Metabolites. Jul 12;9(7). pii: E143.
472	Bailey, M., Haverson, K., Inman, C., Harris, C., Jones, P., Corfield, G., Miller, B., Stokes, C., 2005.
473	The development of the mucosal immune system pre- and post-weaning: balancing regulatory
171	
4/4	and effector function. Proc. Nutr. Soc. 64, 451–457.

- Bertolo, R.F., Chen, C.Z., Law, G., Pencharz, P.B., Ball, R.O., 1998. Threonine requirement of
 neonatal piglets receiving total parenteral nutrition is considerably lower than that of piglets
 receiving an identical diet intragastrically. J. Nutr. 128, 1752–1759.
- Blom, N., Sicheritz-Pontén, T., Gupta, R., Gammeltoft, S., Brunak, S., 2004. Prediction of posttranslational glycosylation and phosphorylation of proteins from the amino acid sequence.
 Proteomics 4, 1633-1649.
- Bovo, S., Mazzoni, G., Calò, D.G., Galimberti, G., Fanelli, F., Mezzullo, M., Schiavo, G., Scotti, E.,
 Manisi, A., Samorè, A.B., Bertolini, F., Trevisi, P., Bosi, P., Dall'Olio, S., Pagotto, U.,
 Fontanesi, L., 2015. Deconstructing the pig sex metabolome: Targeted metabolomics in heavy
 pigs revealed sexual dimorphisms in plasma biomarkers and metabolic pathways. J. Anim.
 Sci. 93, 5681–5693.
- Bovo, S., Mazzoni, G., Galimberti, G., Calò, D.G., Fanelli, F., Mezzullo, M., Schiavo, G., Manisi,
 A., Trevisi, P., Bosi, P., Dall'Olio, S., Pagotto, U., Fontanesi, L., 2016. Metabolomics
 evidences plasma and serum biomarkers differentiating two heavy pig breeds. Animal 10,
 1741–1748.
- Brown, D.C., Maxwell, C. V, Erf, G.F., Davis, M.E., Singh, S., Johnson, Z.B., 2006. The influence
 of different management systems and age on intestinal morphology, immune cell numbers and
 mucin production from goblet cells in post-weaning pigs. Vet. Immunol. Immunopathol. 111,
 187–198.
- Chen, L., Xu, Y., Chen, X., Fang, C., Zhao, L., Chen, F., 2017. The maturing development of gut
 microbiota in commercial piglets during the weaning transition. Front. Microbiol. 8, 1688.
- 496 Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D.S., Xia, J., 2018.
 497 MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic
 498 Acids Res. 46, W486–W494.

- Christensen, K.L., Hedemann, M.S., Jørgensen, H., Stagsted, J., Knudsen, K.E., 2012. Liquid
 chromatography-mass spectrometry based metabolomics study of cloned versus normal pigs
 fed either restricted or ad libitum high-energy diets. J. Proteome Res. 11, 3573–3580.
- 502 Chung, D., Keles S., 2010. Sparse partial least squares classification for high dimensional data. Stat.
 503 Appl. Genet. Mol. Biol. 9, 17.
- 504 Cynober, L.A., 2002. Plasma amino acid levels with a note on membrane transport: characteristics,
 505 regulation, and metabolic significance. Nutrition 18, 761–766.
- de Koning, T.J., Snell, K., Duran, M., Berger, R., Poll-The, B.-T., Surtees, R., 2003. L-serine in
 disease and development. Biochem. J. 371, 653–661.
- 508 Dou, S., Villa-Vialaneix, N., Liaubet, L., Billon, Y., Giorgi, M., Gourdine, J., Riquet, J., Renaudeau,
- 509 D., 2017. NMR-Based metabolomic profiling method to develop plasma biomarkers for 510 sensitivity to chronic heat stress in growing pigs. PLoS ONE 12, e0188469.
- 511 Flynn, N.E., Knabe, D.A., Mallick, B.K., Wu, G., 2000. Postnatal changes of plasma amino acids in
 512 suckling pigs. J. Anim. Sci. 78, 2369–2375.
- Fontanesi, L., 2016. Metabolomics and livestock genomics: Insights into a phenotyping frontier and
 its applications in animal breeding. Anim. Front. 6, 73–79.
- Getty, C.M., Almeida, F.N., Baratta, A.A., Dilger, R.N., 2015. Plasma metabolomics indicates
 metabolic perturbations in low birth weight piglets supplemented with arginine. J. Anim. Sci.
 93, 5754–5763.
- 518 Gibellini, F. Smith, T.K., 2010. The Kennedy pathway-de novo synthesis of 519 phosphatidylethanolamine and phosphatidylcholine. IUBMB Life 62, 414–428.
- Goldansaz, S.A., Guo, A.C., Sajed, T., Steele, M.A., Plastow, G.S., Wishart, D.S., 2017. Livestock
 metabolomics and the livestock metabolome: A systematic review. PLoS ONE 12, e0177675.

- Gong, W., Jia, J., Zhang, B., Mi, S., Zhang, L., Xie, X., Guo, H., Shi, J., Tu, C., 2017. Serum
 Metabolomic profiling of piglets infected with virulent classical swine fever virus. Front.
 Microbiol. 8, 731.
- Gu, X., Li, D., 2003. Fat nutrition and metabolism in piglets: a review. Anim. Feed Sci. Technol. 109,
 151–170.
- Hollywood, K., Brison, D.R., Goodacre, R., 2006. Metabolomics: current technologies and future
 trends. Proteomics. 6, 4716–4723.
- Ingerslev, A.K., Karaman, I., Bağcıoğlu, M., Kohler, A., Theil, P.K., Bach Knudsen, K.E.,
 Hedemann, M.S., 2015. Whole grain consumption increases gastrointestinal content of
 sulfate-conjugated oxylipins in pigs A multicompartmental metabolomics study. J.
 Proteome Res. 14, 3095–3110.
- Korner, J., Cline, G.W., Slifstein, M., Barba, P., Rayat, G.R., Febres, G., Leibel, R.L., Maffei, A.,
 Harris, P.E., 2019. A role for foregut tyrosine metabolism in glucose tolerance. Mol. Metabol.
 23, 37-57.
- Jiang, P., Stanstrup, J., Thymann, T., Sangild, P.T., Dragsted, L.O., 2016. Progressive changes in the
 plasma metabolome during malnutrition in juvenile pigs. J. Proteome Res. 15, 447–456.
- Lallès, J.P., Bosi, P., Smidt, H., Stokes, C.R., 2007. Nutritional management of gut health in pigs
 around weaning. Proc. Nutr. Soc. 66, 260–268.
- Lamers, Y., Williamson, J., Gilbert, L.R., Stacpoole, P.W., Gregory, J.F., 2007. Glycine turnover and
 decarboxylation rate quantified in healthy men and women using primed, constant infusions
- 542 of [1,2-(13)C2]glycine and [(2)H3]leucine. J. Nutr. 137, 2647–2652.
- Le Floc'h, N., Sève, B., Henry, Y., 1994. The addition of glutamic acid or protein to a threoninedeficient diet differentially affects growth performance and threonine dehydrogenase activity
 in fattening pigs. J. Nutr. 124:1987–1995.

- Lê Cao, K., Boitard, S. & Besse, P. 2011. Sparse PLS discriminant analysis: biologically relevant
 feature selection and graphical displays for multiclass problems. BMC Bioinformatics 12,
 253, doi:10.1186/1471-2105-12-253
- Le Floc'h, N., Wessels, A., Corrent, E., Wu, G., Bosi, P., 2018. The relevance of functional amino
 acids to support the health of growing pigs. Anim. Feed Sci. Tech. 245, 104–116.
- Leonard, S.G., Sweeney, T., Bahar, B., Lynch, B.P., O'Doherty, J.V., 2010. Effect of maternal fish
 oil and seaweed extract supplementation on colostrum and milk composition, humoral
 immune response, and performance of suckled piglets. J. Anim. Sci. 88, 2988–2997.

Li, Z., Vance, D.E., 2008. Phosphatidylcholine and choline homeostasis. J. Lipid Res. 49, 1187–1194.

- Luise, D., Bertocchi, M., Motta, V., Salvarani, C., Bosi, P., Luppi, A., Fanelli, F., Mazzoni M.,
 Archetti, I., Maiorano G., Nielsen, B.K.K., Trevisi, P. 2019a. Bacillus sp. probiotic
 supplementation diminish the Escherichia coli F4ac infection in susceptible weaned pigs by
 influencing the intestinal immune response, intestinal microbiota and blood metabolomics. J.
 Anim. Sci. Biotechnol. 10(1), 74.
- Luise, D., Motta, V., Bertocchi, M., Salvarani, C., Clavenzani, P., Fanelli, F., Pagotto, U., Bosi, P.,
 Trevisi, P. 2019b. Effect of Mucine 4 and Fucosyltransferase 1 genetic variants on gut
 homoeostasis of growing healthy pigs. J. Anim. Physiol Anim. Nutr. 103(3), 801-812.
- 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. 7, 554–569.
- Mahan, D.C., Shields, R.G., 1998. Essential and nonessential amino acid composition of pigs from
 birth to 145 kilograms of body weight, and comparison to other studies. J. Anim. Sci. 76, 513–
 568 521.
- Martin, M., Tesouro, M.A., González-Ramón, N., Piñeiro, A., Lampreave, F., 2005. Major plasma
 proteins in pig serum during postnatal development. Reprod. Fertil. Dev. 17, 439–445.

571	Metzler-Zebeli, B.U., Ertl, R., Klein, D., Zebeli, Q., 2015a. Explorative study of metabolic
572	adaptations to various dietary calcium intakes and cereal sources on serum metabolome and
573	hepatic gene expression in juvenile pigs. Metabolomics 11, 545–558.

- Metzler-Zebeli, B.U., Eberspacher, E., Grüll, D., Kowalczyk, L., Molnar, T., Zebeli, Q., 2015b.
 Enzymatically modified starch ameliorates postprandial serum triglycerides and lipid
 metabolome in growing pigs. PLoS ONE 10, e0130553.
- 577 Meyburg, J., Schulze, A., Kohlmueller, D., Linderkamp, O., Mayatepek, E., 2001. Postnatal changes
 578 in neonatal acylcarnitine profile. Pediatric Res. 49, 125–129.
- 579 Milhas, D., Clarke, C.J., Hannun, Y.A., 2010. Sphingomyelin metabolism at the plasma membrane:
 580 Implications for bioactive sphingolipids. FEBS Lett. 584, 1887–1894.
- 581 Molano, A., Huang, Z., Marko, M.G., Azzi, A., Wu, D., Wang, E., Kelly, S.L., Merrill, A.H., Bunnell,
- 582 S.C., Meydani, S.N., 2012. Age-dependent changes in the sphingolipid composition of mouse
- 583 cd4+ t cell membranes and immune synapses implicate glucosylceramides in age-related t cell
 584 dysfunction. PLoS ONE 7, e47650.
- Motta, V., Luise, D., Bosi, P., Trevisi, P., 2019. Faecal microbiota shift during weaning transition in
 piglets and evaluation of AO blood types as shaping factor for the bacterial community profile.
 PLoS ONE 14, e0217001
- 588 National Research Council (NRC), 2012. Nutrient requirements of swine: Eleventh Revised Edition.
 589 The National Academies Press. Washington DC, USA.
- Nilsson, A., Duan, R.D., 2006. Absorption and lipoprotein transport of sphingomyelin. J. Lipid Res.
 47, 154–171.
- Nohturfft, A., Zhang, S.C., 2009. Coordination of lipid metabolism in membrane biogenesis. Annu.
 Rev. Cell Dev. Biol. 25, 539-566.

- Odle, J., Lin, X., Jacobi, S.K., Kim, S.W., Stahl, C.H., 2014. The suckling piglet as an agrimedical
 model for the study of pediatric nutrition and metabolism. Annu. Rev. Anim. Biosci. 2, 419–
 444.
- 597 Picone, G., Zappaterra, M., Luise, D., Trimigno, A., Capozzi, F., Motta, V., Davoli, R., Nanni Costa,
 598 L., Bosi, P., Trevisi, P., 2018. Metabolomics characterization of colostrum in three sow breeds

and its influences on piglets' survival and litter growth rates. J. Anim. Sci. Biotechnol. 9, 23.

- 600 Pinheiro, J., Bates, D., 2009. Mixed-effects models in S and S-PLUS. Springer-Verlag, New York.
- Pluske, J.R., 2016. Invited review: Aspects of gastrointestinal tract growth and maturation in the preand postweaning period of pigs. J. Anim. Sci. 94, 399–411.
- Pluske, J.R., Hampson, D.J., Williams, I.H., 1997. Factors influencing the structure and function of
 the small intestine in the weaned pig: a review. Livest. Sci. 51, 215–236.
- Poulsen, A.R., Luise, D., Curtasu, M.V., Sugiharto, S., Canibe, N., Trevisi, P., Lauridsen, C., 2018.
 Effects of alpha-(1,2)-fucosyltransferase genotype variants on plasma metabolome, immune
 responses and gastrointestinal bacterial enumeration of pigs pre- and post- weaning. PLoS
 ONE 13, e0202970.
- 609 Psychogios, N., Hau, D.D., Peng, J., Guo, A.C., Mandal, R., Bouatra, S., Sinelnikov, I.,
 610 Krishnamurthy, R., Eisner, R., Gautam, B., Young, N., Xia, J., Knox, C., Dong, E., Huang,
- 611 P., Hollander, Z., Pedersen, T.L., Smith, S.R., Bamforth, F., Greiner, R., McManus, B.,
- Newman, J.W., Goodfriend, T., Wishart, D.S., 2011. The human serum metabolome. PLoS
 One 6, e16957.
- R Core Team. R: A language and environment for statistical computing. 2018, R Foundation for
 Statistical Computing, Vienna, Austria. URL <u>https://www.r-project.org/</u>
- Rezaei, R., Wang, W., Wu, Z., Dai, Z., Wang, J., Wu, G., 2013. Biochemical and physiological bases
 for utilization of dietary amino acids by young pigs. J. Anim. Sci. Biotech. 4, 7.

- Rinaldo, P., Cowan, T.M., Matern, D., 2008. Acylcarnitine profile analysis. Genet. Med. 10, 151–
 156.
- 620 Schokker, D., Zhang, J., Zhang, L.L., Vastenhouw, S.A., Heilig, H.G.H.J., Smidt, H., Rebel, J.M.J.,
- 621 Smits, M.A., 2014. Early-life environmental variation affects intestinal microbiota and 622 immune development in new-born piglets. PLoS ONE 9, e100040.
- 623 Slotte, J.P., 2013. Biological functions of sphingomyelins. Prog. Lipid Res. 52, 424-437.
- Smith, E.L., Greene, R.D., 1977. Further studies on the amino acid composition of immune protein.
 J. Biol. Chem. 171, 355–362.
- Solberg, R., Enot, D., Deigner, H.P., Koal, T., Scholl-Bürgi, S., Saugstad, O.D., Keller, M., 2010.
 Metabolomic analyses of plasma reveals new insights into asphyxia and resuscitation in pigs.
 PLoS ONE 5, e9606.
- Solberg, R., Kuligowski, J., Pankratov, L., Escobar, J., Quintás, G., Lliso, I., Sánchez-Illana, Á.,
 Saugstad, O.D., Vento, M., 2016. Changes of the plasma metabolome of newly born piglets
 subjected to postnatal hypoxia and resuscitation with air. Pediatr. Res. 80, 284–292.
- 632 Soumeh, E.A., Hedemann, M.S., Poulsen, H.D., Corrent, E., van Milgen, J., Nørgaard, J.V., 2016.
- Nontargeted LC–MS metabolomics approach for metabolic profiling of plasma and urine
 from pigs fed branched chain amino acids for maximum growth performance. J. Proteome
 Res. 15, 4195–4207.
- 636 Stokes, C.R., 2017. The development and role of microbial-host interactions in gut mucosal immune
 637 development. J. Anim. Sci. Biotech. 8, 12.
- Stokes, C.R., Bailey, M., Haverson, K., Harris, C., Jones, P., Inman, C., Piéc S., Oswaldc, I.P.,
 Williamsd, B.A., Akkermansd, A.D.L., Sowae, E., Rothköttere H.J., Miller, B.G., 2004.
 Postnatal development of intestinal immune system in piglets: implications for the process of
 weaning. Anim. Res. 53, 325–334.

- Stoll, B., Henry, J., Reeds, P.J., Yu, H., Jahoor, F., Burrin, D.G., 1998. Catabolism dominates the
 first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. J.
 Nutr. 128, 606–614.
- Sugiharto, S., Hedemann, M.S., Lauridsen, C., 2014. Plasma metabolomic profiles and immune
 responses of piglets after weaning and challenge with E. coli. J. Anim. Sci. Biotech. 5, 17.
- Van Klinken, B.J., Dekker, J., Buller, H.A., Einerhand, A.W., 1995. Mucin gene structure and
 expression: protection vs. adhesion. Am. J. Physiol. 269, 613–627.
- Van Niekerk, B.D., Reid, J.T., Bensadoun, A., Paladines, O.L., 1963. Urinary creatine as an index of
 body composition. J. Nutr. 79, 463–473.
- Vorkas, P.A., Isaac, G., Holmgren, A., Want, E.J., Shockcor, J.P., Holmes, E., Henein, M.Y., 2015.
 Perturbations in fatty acid metabolism and apoptosis are manifested in calcific coronary artery
 disease: An exploratory lipidomic study. Int. J. Cardiol. 197, 192–199.
- Wang, W., Wu, Z., Dai, Z., 2013. Glycine metabolism in animals and humans: implications for
 nutrition and health. Amino Acids 45, 463–477.
- Westerhuis, J.A., van Velzen, E.J.J., Hoefsloot, H.C.J., Smilde, A.K., 2010. Multivariate paired data
 analysis: Multilevel PLSDA versus OPLSDA. Metabolomics 6, 119–128.
- Widdowson, E.M., 1971. Intra-uterine growth retardation in the pig. I. Organ size and cellular
 development at birth and after growth to maturity. Neonatology 19, 329–340.
- 660 Wishart, D.S., Feunang, Y.D., Marcu, A., Guo, A.C., Liang, K., Rosa, V., Sajed, T., Johnson, D., Li,
- 661 C., Karu, N., Sayeeda, Z., Lo, E., Assempour, N., Berjanskii, M., Singhal, S., Arndt, D., Liang,
- 662 Y., Badran, H., Grant, J., Serra-Cayuela, A., Liu, Y., Mandal, R., Neveu, V., Pon, A., Knox,
- 663 C., Wilson, M., Manach, C., Scalbert, A., 2018. HMDB 4.0: the human metabolome database
- 664 for 2018. Nucleic Acids Res. 46, 608–617.
- Yin, F., Yin, Y., Li, T., Ren, W., Blachier, F., Huang, R., 2011. Developmental changes of serum
 amino acids in suckling piglets. J. Food Agric. Environ. 9, 322–327.

- 667 Zhang, H., Malo, C., Buddington, R.K., 1997. Suckling induces rapid intestinal growth and changes
 668 in brush border digestive functions of newborn pigs. J. Nutr. 127, 418–426.
- 669 Zhou, X., Zhang, Y., Wu, X., Wan, D., Yin, Y., 2018. Effects of dietary serine supplementation on
- 670 intestinal integrity, inflammation and oxidative status in early-weaned piglets. Cell. Physiol.
- 671 Biochem. 48, 993–1002.

Figure 1. Multilevel sPLS-DA (sMLPLS-DA) plot of the within subject variation of the piglet
metabolomic profiles at the two analysed time points (T1 and T2). Each point represents a piglet
profile at T1 (day 12) and at T2 (day 21). The same piglets are analyzed at T1 and T2 (paired structure
of the dataset).



680 Figure 2. Metabolic pathway analysis of the 23 metabolites selected by the sMLPLS-DA. Analyses run over the human KEGG and human SMPDB databases. Each point represents a metabolic 681 682 pathway. Size and color mirror the pathway impact and significance, respectively. The impact measures the importance (or role) of the metabolites in the metabolite network (pathway) estimated 683 684 from the pathway topology analysis. Pathways included in the plot are: 1) Aminoacyl-tRNA 685 biosynthesis (KEGG); 2) Sphingolipid metabolism (KEGG); 3) Glycine, serine and threonine metabolism (KEGG) and 4) Sphingolipid metabolism (SMPD). More details are reported in Table 686 687 S6.



688

690 Table 1. Metabolites selected by the multilevel sPLS-DA (sMLPLS-DA) pipeline that distinguished the piglet metabolomic profiles at the two

691 investigated time points (T1 = day 12; T2 = day 21).

		T1				T2					Stabili	ty ⁷	I	Effect Si			
Metabolite (short name) ¹	HMDB ²	Mean _R ³	$\mathrm{SD}_{\mathrm{R}}^4$	Mean _r ⁵	SD_r^6	Mean _R ³	$\mathrm{SD}_{\mathrm{R}}^{4}$	Mean _r ⁵	SD_r^6	t	N(t)	P_{st}	t	N(t)	P_{si}	Dir ⁹	$\Delta\%^{10}$
Tetradecenoylcarnit ine (C14:1)	HMDB0002014	0.08	0.01	0.09	0.01	0.05	0.01	0.06	0.01	18	49	0.098	0.21	6	0.012	T1	68.45
Hydroxytetradeceno ylcarnitine (C14:1-OH)	HMDB0013330	0.02	0	0.02	0	0.01	0	0.01	0	18	45	0.09	0.18	14	0.028	T1	41.18
Hydroxyhexadecan oylcarnitine (C16-OH)	HMDB0013336	0.02	0	0.01	0	0.02	0	0.01	0	18	48	0.096	0.21	30	0.060	T1	14.31
Butyrylcarnitine (C4)	HMDB0002013	0.25	0.04	0.25	0.03	0.14	0.02	0.16	0.03	18	43	0.086	- 0.22	10	0.020	T1	76.99
Valerylcarnitine (C5)	HMDB0013128	0.14	0.03	0.16	0.02	0.06	0.01	0.09	0.01	18	45	0.09	0.27	8	0.016	T1	130.01
Hydroxyvalerylcarn itine [C5-OH (C3-DC- M)]	HMDB0013130	0.26	0.04	0.27	0.04	0.19	0.02	0.2	0.01	18	47	0.094	0.23	8	0.016	T1	39.88
Serine	HMDB0000187	309.24	76.2	260.01	67.75	193.44	44.35	131.02	41.5	18	49	0.098	- 0.22	19	0.038	T1	59.86
Threonine	HMDB0000167	216.88	39.48	145.08	34.07	134.92	46.45	43.74	34.03	18	49	0.098	-0.3	16	0.032	T1	60.75
Tyrosine	HMDB0000158	154.59	17.85	107.16	18.35	120.71	32.34	59.05	26.26	18	49	0.098	- 0.22	18	0.036	T1	28.07
Creatinine	HMDB0000562	62.61	7.49	51.82	6.18	78.45	11.85	64.02	9.88	18	38	0.076	0.2	16	0.032	T2	-20.2
Phosphatidylcholine diacyl C30:2 (PC aa C30:2)	HMDB0007999	0.77	0.14	0.89	0.13	1.06	0.23	1.24	0.22	18	32	0.064	0.17	6	0.012	T2	-27.73
Phosphatidylcholine acyl-akyl C30:0 (PC ae C30:0)	HMDB0013341	1.33	0.23	0.5	0.32	2.57	0.94	1.56	0.71	18	41	0.082	0.17	10	0.020	T2	-48.24
acyl-akyl C32:1 (PC ae C32:1)	HMDB0013404	4.27	0.66	2.77	0.76	6.87	2.08	5.10	1.68	18	38	0.076	0.16	13	0.026	T2	-37.82

Phosphatidylcholine																	
acyl-akyl C38:4	HMDB0013420	8.27	1.16	7.2	0.93	11.8	2.75	10.58	2.62	18	30	0.060	0.17	7	0.014	T2	-29.89
(PC ae C38:4)																	
Phosphatidylcholine																	
acyl-akyl C40:4	HMDB0013442	1.21	0.19	1.17	0.14	1.71	0.38	1.69	0.36	18	32	0.064	0.17	5	0.010	T2	-29.22
(PC ae C40:4)																	
Phosphatidylcholine			0.40		0.40			~ - /		10	•	a a - a	-	_	0.010	-	
acyl-akyl C42:3	HMDB0013458	1.12	0.19	1.1	0.18	0.79	0.14	0.74	0.13	18	39	0.078	0.29	5	0.010	T1	42.25
(PC ae C42:3)																	
Phosphatidylcholine									~ ~ -	10	•	0.07	o 1 -		0.000	-	
acyl-akyl C42:4	HMDB0013448	0.24	0.04	0.25	0.03	0.34	0.08	0.37	0.07	18	30	0.06	0.17	4	0.008	12	-31.01
(PC ae C42:4)																	
Phosphatidylcholine	UN (DD0012451	0.64	0.05	0.(2	0.04	0.0	0.11	0.70	0.1	10	26	0.072	0.17	(0.012	T 2	10.74
acyl-akyl C42:5	HMDB0013451	0.64	0.05	0.63	0.04	0.8	0.11	0.79	0.1	18	36	0.072	0.17	6	0.012	12	-19.64
(PC ae C42.5)																	
Phosphalidylcholme	UMDD0012450	0.18	0.02	0.10	0.02	0.20	0.07	0.2	0.07	19	25	0.070	0.10	5	0.010	тγ	26.99
$(DC \approx C44.0)$	HMDB0013430	0.18	0.05	0.19	0.02	0.29	0.07	0.5	0.07	10	33	0.070	0.19	5	0.010	12	-30.00
(FC at C44.0)																	
eline C22:1	HMDB0013466	1 74	0.21	1 75	0.23	2 53	0.57	2 / 8	0.54	18	11	0.088	0.17	17	0.034	т?	-31.06
[SM (OH) C22.1]	11100013400	1./7	0.21	1.75	0.25	2.55	0.57	2.40	0.54	10		0.000	0.17	17	0.054	12	-51.00
Sphingomyeline																	
C16.0	HMDB0010169	92 64	113	97.67	10.05	121 72	23.06	130 53	22.98	18	34	0.068	0.17	6	0.012	т2	-23.89
(SM C16:0)		92.01	11.5	57.07	10.05	121.72	25.00	150.55	22.90	10	51	0.000	0.17	0	0.012	12	23.07
Sphingomyeline																	
C16:1	HMDB0029216	7.88	0.7	7.66	0.67	10.61	1.99	10.29	1.97	18	36	0.072	0.15	8	0.016	Т2	-25.75
(SM C16:1)	111111111111111111111111111111111111111	,	017	,	0107	10.01	1.,,,	10.29	,	10	20	0.072	0110	0	01010		20170
Sphingomveline																	
C18:0	HMDB0001348	12.54	1.24	12.77	1.27	16.46	3.19	16.58	3.14	18	40	0.080	0.17	19	0.038	T2	-23.85
(SM C18:0)											÷			-		_	

692 ¹Metabolite concentrations are reported in μM .

⁶⁹³²Human Metabolome Database (HMDB; http://www.hmdb.ca/; accessed March 2019) entry.

 3 Mean_R = mean of raw data computed after the quality control.

- $^{4}SD_{R}$ = standard deviation of raw data computed after the quality control.
- 5 Mean_r = mean of residuals computed after the quality control.

- $^{6}SD_{r}$ = standard deviation of residuals computed after the quality control.
- ⁶⁹⁸ ⁷Stability: "t" represents the number of times that the metabolite was selected in the Leave One Out procedure (LOO). N(t) indicates the number of
- 699 times that (t* \geq t). P_{st} represents an estimate of the probability that the selection of that metabolite is by chance rather than by a particular structure of
- 700 the dataset (Bovo et al., 2015, 2016).
- 701 ⁸Effect size: "t" represents the absolute value of the regression coefficient of the metabolite. N(t) indicates the number of times that ($|t^*| \ge |t|$). P_{si}
- 702 represents an estimate of the probability that sPLS-DA β estimate differs from 0 only by chance. Details are given in Bovo et al. (2015; 2016).
- ⁹Dir: based on β estimate, T1 indicates that the metabolite concentration is higher at day 12 than at day 21, whereas T2 indicates that the metabolite
- concentration is higher at day 21 than at day 12.
- 705 $10\Delta\%$ = relative time-specific difference in the metabolite quantification: the difference between the mean metabolite concentration at T1 and the mean
- 706 metabolite concentration at T2, divided by the mean metabolite concentration at T1 and expressed as percentage.