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

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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.

Abstract

The suckling phase is a critical period for the piglets due to their incomplete immune system development and their rapid growth rates. In this study, we analysed the metabolomic profiles of piglets over this period. Eighteen piglets (nine males and nine females) from three different litters were included in the study. Body weight was recorded at birth (T0), 12 (T1) and 21 (T2) days after birth. Plasma samples were collected at two critical time points of the suckling phase (T1 and T2) and about 180 metabolites of five different biochemical classes (glycerophospholipids, amino acids, biogenic amines, hexoses and acylcarnitines) were analyzed using a target metabolomics approach based on Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Metabolites whose 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 based on a Leave One Out (LOO) procedure. The level of twenty-three metabolites differed significantly ($P < 0.1$; both for stability and the effect size) between the two time points. Higher levels of six acylcarnitine (C14:1, C14:1-OH, C16-OH, C4, C5 and C5-OH), serine, threonine and tyrosine, and one phosphatidylcholine (PC ae C42:3) were observed at T1, whereas one biogenic amine (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 SM (OH) C22:1, SM C16:0, SM C16:1 and SM C18:0, were more abundant at T2. The Metabolite Set Enrichment Analysis and the Pathway Analysis modules suggested a perturbation of the "glycine and serine metabolism" and the "sphingolipid metabolism". Differences of these metabolites between these two time points might be related to the rapid growth and immunological maturation phases of the piglets in this period. Our results provided new information that could describe the biological changes of the piglets over the suckling period. The identified metabolites may be useful markers of the developmental processes occurring in the piglets over this critical pre-weaned phase.

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

1. Introduction

Metabolomics is an omics discipline that describes the metabolome which is defined as the catalog of the metabolites (i.e. small chemical compounds) present in a biological system, usually obtained by analysing different biofluids and tissue types (Psychogios et al., 2011; Hollywood et al., 2006). Blood is a tissue that serves as a liquid highway for the molecules that are secreted or discarded by different tissues and organs in the body, and blood serum and plasma are commonly evaluated to 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 biological mechanisms and physiological changes underlying adaptations and responses of the animals to different environmental and stressing conditions, treatments and developmental stages, including the interaction between these factors and different genetic backgrounds (Fontanesi, 2016; 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., 2016, Christensen et al., 2012), sexual dimorphisms (Bovo et al. 2015) and differences between breeds (Bovo et al. 2016) for the detection of novel biomarkers. Moreover, metabolomic studies were carried out in weaning and growing pigs to characterize the effects of different factors such as environment (Solberg et al., 2010, 2016; Dou et al., 2017), feeding (Luise et al., 2019a; Getty et al., 2015), malnutrition (Jiang et al., 2016), pathogen challenges (e.g. Sugiharto et al., 2014; Gong et al., 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;

Widdowson, 1971; Zhang et al., 1997). Large profit losses in the swine industry can be attributed to morbidity and mortality of piglets occurring during this period. Moreover, suckling piglets, for their similarity with human infants and their easy availability, have been also proposed as premier models for the study of pediatrics' metabolism, nutrition and toxicology (Odle et al., 2014). Owing to its relevance, the suckling phase has been extensively investigated in piglets, particularly to define basic nutritional needs (e.g. Gu and Li, 2003; Rezaei et al., 2013), the structural and functional modifications which occur in the GIT, including the development of the Gut-Associated Lymphoid Tissue (GALT) (e.g. Bailey et al., 2005; Lallès et al. 2007; Stokes 2017), the establishment of a microbial community (Chen et al., 2017; Motta et al., 2019) and the cross-talk between host and the microbiota that drives the settlement of the gut ecosystem that can affect the physiology of the piglets (Bailey et al., 2005; Mach et al., 2015; Motta et al., 2019; Schokker et al., 2014; Stokes, 2017). Despite these works have obtained a good characterization of this first part of the piglet life span, a detailed description of the physiological and biochemical changes over the piglet suckling period has not been completely achieved yet. The application of metabolomic approaches to investigate piglet's maturation in this phase under normal healthy condition remains scarce and limited to the description of plasma or serum amino acid profiles (Flynn et al., 2000; Yin et al., 2011).

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

2. Materials and methods

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

2.1. Animals and sampling

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

Piglets were then individually weighed at day 12 (T1) and at day 21 (T2) after birth. Average body weights were 4.16 ± 0.76 kg and 6.26 ± 1.2 kg, respectively. All animals remained healthy over 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 × *g* for 10 min to obtain plasma following the

128 procedure already described (Bovo et al., 2016). Plasma was snap-frozen in liquid nitrogen and stored
129 at -80 °C until further analysis.
130
131 **2.2. Analysis of plasma metabolites**
132 Targeted metabolic profiling of plasma samples was performed using the AbsoluteIDQ p180
133 Kit, (Biocrates Life Science AG, Innsbruck, Austria). The assay allows the simultaneous
134 quantification of 186 metabolites belonging to five biological classes, including amino acids (n. =
135 21), biogenic amines (n. 19), hexose (n. 1), carnitines (n. 40; divided in acylcarnitines,
136 hydroxylacylcarnitines and dicarboxylacylcarnitines), sphingomyelins (n. 15), phosphatidylcholines
137 (n. 76) and lysophosphatidylcholines (n. 14). Table S2 lists all analysed metabolites with full
138 biochemical names and abbreviations.
139 Preparation of the samples required 10 µL plasma, processed according to the manufacturer's
140 instructions (Biocrates Life Science AG). All samples were measured in a single assay together with
141 a mixed porcine plasma sample, obtained from 10 pigs and were analysed in triplicate as quality
142 control (QC).
143 The analytical platform included a Series 200 high-performance liquid chromatography
144 system (Perkin Elmer, Waltham, Massachusetts, USA) coupled with an API 4000 QTrap mass
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,

153 which is an integral part of the AbsoluteIDQ Kit (Biocrates Life Science AG). Results were expressed
154 in micromolar unit (μM).
155
156 **2.3. Statistical analyses**
157 Quality assessment of the metabolomic datasets obtained at the two time points was carried
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% ≠ NA, that means not obtained for
160 missing values) over QC samples and with less than 5% of missing values (NAs) were retained and
161 (ii) samples were considered as outliers and removed if the metabolite concentrations measured for
162 that sample lied 1.5 times the interquartile range below or above the corresponding median for 30%
163 of the data columns, i.e. metabolites. Full intra-plate CV% and excluded metabolites are reported in
164 Table S2**.**
165 Multilevel Partial Least Squares Discriminant Analysis (MLPLS-DA, Westerhuis et al., 2010)
166 was used to investigate differences between T1 and T2 plasma metabolomic profiles. This statistical
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
169 when dealing with small sample size, many irrelevant predictors and high co-linearity among them
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
172 metabolites related to the main response variable (time points), we applied the sparse version of this
173 technique (sPLS-DA; Chung and Keles, 2010). As described by Westerhuis et al. (2010), the
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

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

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

where, for the i^{th} piglet on the j^{th} time occasion, y was the concentration of a metabolite; β_0 was a fixed intercept term; T was a dummy variable distinguishing T1 and T2 data points; S was a 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 weight; $\beta_T, \beta_S, \beta_L, \beta_w$ were the related corresponding regression coefficient; β_{0i} was a random intercept term which was specific for the i^{th} piglet, and ε_{ij} was a random error term (Pinheiro and Bates, 2009).

Confounding effects were removed by computing the residuals of model (I) which represented the part of the metabolite concentration that cannot be explained by the confounding factors included in the model:

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

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 b_{0i}.

Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was applied on the between subject variation obtained from residuals of data (see equation II), by using as response variable a dummy variable distinguishing the two time points (T1 and T2). The sPLS-DA sparseness coefficient

200 eta (ranging from 0.1 to 0.9) and the number of hidden components K (ranging from 1 to 3) were
201 automatically selected by using an internal 10-fold cross-validation procedure.
202 Metabolites contributing with non-null coefficients to discriminant dimensions (i.e. $\beta \neq 0$)
203 were selected and validated by applying a permutation test coupled with a Leave One Out (LOO)
204 procedure as detailed in Bovo et al., 2015, 2016. The purpose of this approach was to evaluate both
205 the stability (P_{st}) and the effect size (P_{si}) of selected metabolites by testing the null hypothesis that
206 metabolite concentration is unrelated to piglet maturation. Metabolites having $P < 0.1$ for both
207 stability and effect size, simultaneously, were considered stable and significant (Bovo et al., 2016).
208 The relative time points-specific difference in the metabolite quantification (Δ%) was
209 calculated as the difference between the mean metabolite concentration at T1 and the mean metabolite
210 concentration at T2, divided by the mean metabolite concentration at T1 and expressed as percentage.
211 All analyses have been performed in R v. 3.3.1 (R Development Core Team, 2018) using the
212 "lme4" packages for the computation of metabolite residuals, the "spls" packages (function *cv.splsda*
213 and *splsda*) for the sPLS-DA analysis and the function "prcomp" for PCA analysis.
214
215 **2.4. Metabolomic enrichment and pathway analyses**
216 Metabolites identified using the statistical pipeline described above were mapped onto the
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
224 pathway-associated metabolite sets, by interrogating a total of 99 metabolite sets, and using only

those containing at least two compounds. The Pathway Analysis module exploits the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/; 80 metabolite sets interrogated) and The Small Molecule Pathway Database (SMPDB; http://smpdb.ca/; 99 metabolite sets interrogated) metabolic pathways with pathway enrichment and pathway topology methods. Pathway topology analysis estimates the node relative importance (or role) in the metabolite network. Here, it was applied with the human pathway libraries of KEGG and SMPDB using the relative betweenness centrality as measure of metabolite importance. Over-representation analyses were 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 $P < 0.05$, and characterized by at least two different input metabolites, were considered significantly over-represented.

3. Results

3.1. Metabolomic data

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

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

metabolomic profiles, we studied the between subject variation via PCA before fitting the sPLS-DA 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 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 (Figure S2). Values of the explained total variance for litter and sex were 21.78% and 14.81%, respectively. After removing these effects, the PCA on the new between subject variation did not evidence any residual effect.

3.3. Metabolomic profile differences between the two developmental neonatal phases

Differences in the plasma metabolic profiles between T1 and T2 were investigated by applying sPLS-DA on the within variance (after the removal of the confounding effects). This 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 sPLS-DA focuses on the within subject variation (variation similar among the subjects), the first component mainly describes the difference between the analysed classes due to a main acting factor (i.e. time points), whereas the other components describe the within subject variation that is different between the subjects by reflecting these differences at the level of the main acting factor (Westerhuis et al., 2010). Figure 1 demonstrates that the metabolomic profiles clearly cluster into two groups which represent T1 and T2. Therefore, the selected metabolites can discriminate the piglet profiles at the two different ages. Table S4 reports the stability, the effect size obtained with the multilevel sPLS-DA analysis and the relative time points-specific difference in the metabolite quantification (Δ%) for all quantified metabolites. According to the stability test, 23 metabolites (13.8%) had a $P_{st} < 0.1$ (threshold of significance based on the validation procedure). All the metabolic classes, except hexoses, were included in this stability test results list. Considering the effect size (significance test),

34 metabolites (~ 20.4%) had a $P_{si} < 0.1$ (Table S4). A total of 32 out of the 34 identified metabolites resulted significantly selected ($P_{si} < 0.05$), with two of them, PC ae C40:1 and PC ae C42:4, selected 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 T1, piglets had higher levels of: (i) six acylcarnitines including C14:1 (Δ% = 68.45%), C14:1-OH (Δ% = 41.18%), C16-OH (Δ% = 14.31%), C4 (Δ% =76.99%), C5 (Δ% = 130.01%) and C5-OH (C3-DC-M) (Δ% = 39.88%), (ii) three amino acids: serine (Δ% = 59.86%), threonine (Δ% = 60.75%) and tyrosine (Δ% = 28.07%), and (iii) one phosphatidylcholines: PC ae C42:3 (Δ% = 42.25%) compared to piglets at T2. At T2, piglets had higher levels of: (i) one biogenic amine: creatinine (Δ% = -20.20%), (ii) eight phosphatidylcholines including PC aa C30:2 (Δ% = -27.73%) , PC ae C30:0 (Δ% = -48.24%), PC ae C32:1 (Δ% =-37.82%), PC ae C38:4 (Δ% = -29.89%), PC ae C40:4 (Δ% = -29.22%), PC ae C42:4 (Δ% = -31.01%) , PC ae C42:5 (Δ% = -19.64%) and PC ae C44:6 (Δ% = -36.88), and (iii) four sphingomyelins including SM (OH) C22:1 (Δ% = -31.06%) , SM C16:0 (Δ% = -23.89%), SM C16:1 (Δ% = -25.75%) and SM C18:0 (Δ% = -23.85%) compared to piglets at T1.

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.

During the first two weeks of life, the intestine of the piglets starts to be colonized by lymphoid 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 develop an adult structure (Stokes et al., 2004). Furthermore, during the suckling period a continuous variation of the intestinal microbiota community is observed due to its un-stable colonization, potentially impacting the host metabolism (Chen et al., 2017). According to these considerations, the study clearly showed that the metabolomic profiles of the piglets at the two time points were different, mimicking, to some extent, the changes that occurred in these animals over the nine days that separated the two sampling points where animals grew on average more than two kg (about 50% of the average weight at T1).

The sMLPLS-DA allowed to disclose a total of 23 discriminant metabolites that can describe the physiological changes over this period. Day 12 showed higher concentrations of three amino acids (threonine, tyrosine and serine) and six acylcarnitines (C4, C5, C5-OH, C14:1, C14:1-OH and C16-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 growth and immune function of the piglets. It plays a critical role in the regulation of the intestinal mucosal integrity, as it is required for the production of mucins (Van Klinken et al., 1995), and is one of the major amino acids in plasma immunoglobulins (Smith and Greene, 1977). Since both mucin and immunoglobulin productions increase over the suckling period (Brown et al., 2006; Martin et al.,

2005), the lower threonine level at T2 could reflect, at least in part, the increased use of this amino 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 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 be due to the composition of the milk that is taken by the piglets, which can vary depending on the breed and parity of the sow, on her feeding and on environmental conditions (Picone et al., 2018; 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 are important for N-glycosylation in the endoplasmic reticulum and *O*-glycosylation of the Golgi apparatus (Blom et al., 2004).

Serine plays a central role in cell proliferation, as it is the predominant source of one-carbon 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) degradation of protein and phospholipid, (iii) biosynthesis from the glycolytic intermediate 3-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). The recent observation of the efficacy of serine supplementation in weaning pigs, to improve villus health (Zhou et al., 2018), would suggest that this non-essential amino acid can be nevertheless 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
377 development of the piglets at this stage (Le Floc'h et al., 2018; Rezaei et al., 2013). The common
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.
409 At day 21 the metabolic profile was discriminated by a higher concentration of nine
410 phosphatidylcholines and four sphingomyelins, indicating a clear influence of the age on lipid
411 metabolism. Pathway Enrichment analysis with the KEGG and SMPD libraries indicated that a lipid
412 related pathway, the sphingolipid metabolism, can capture the modified metabolism over the analysed
413 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

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 $CD4^+$ T cells (Molano et al., 2012). Piglets start to display $CD4^+$ T cells in the lamina *propria* between the second and fourth weeks after birth (Stokes et al., 2004). Based on these considerations, even if our 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 (referred to $CD4^+$ T cells) over the suckling period. Inspecting in details the results obtained for the 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 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 (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.

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

improve the usefulness of the suckling pig as an animal model for the study of pediatric metabolism. Further investigations are needed to clarify the connection of the observed metabolomic profiles with the host microbiota and the immune system development that, in turn, could be relevant to establish relationships with the post-weaning survival rates of the animals.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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672 **Figure 1.** Multilevel sPLS-DA (sMLPLS-DA) plot of the within subject variation of the piglet
673 metabolomic profiles at the two analysed time points (T1 and T2). Each point represents a piglet
674 profile at T1 (day 12) and at T2 (day 21). The same piglets are analyzed at T1 and T2 (paired structure
675 of the dataset).
676

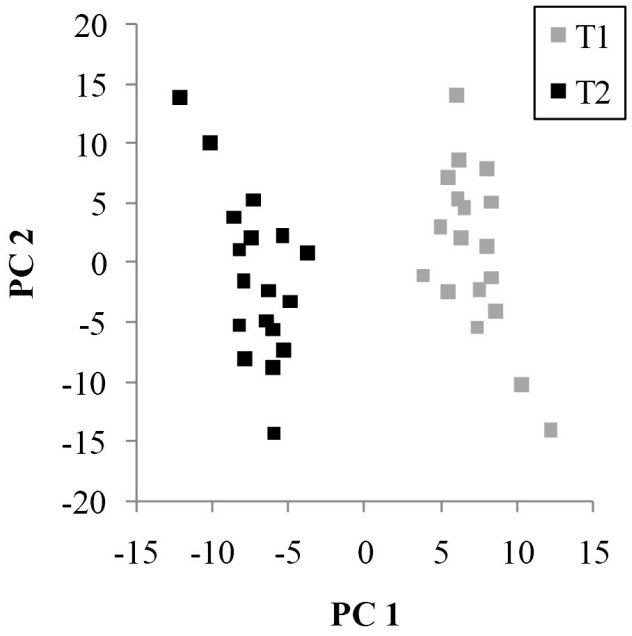

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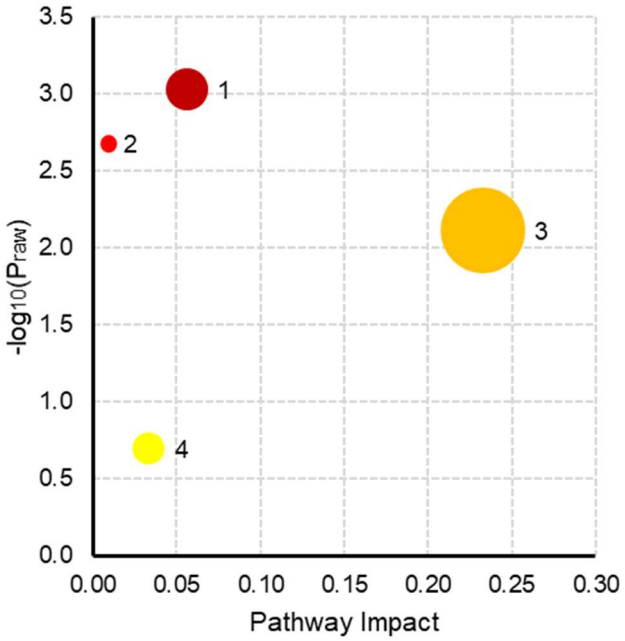
Figure 2. Metabolic pathway analysis of the 23 metabolites selected by the sMLPLS-DA. Analyses run over the human KEGG and human SMPDB databases. Each point represents a metabolic pathway. Size and color mirror the pathway impact and significance, respectively. The impact measures the importance (or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



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).

Metabolite (short name)[1]	HMDB[2]	T1				T2				Stability[7]			Effect Size[8]			Dir[9]	Δ%[10]
		$Mean_R$[3]	SD_R[4]	$Mean_r$[5]	SD_r[6]	$Mean_R$[3]	SD_R[4]	$Mean_r$[5]	SD_r[6]	t	N(t)	P_{st}	t	N(t)	P_{si}		
Tetradecenoylcarnitine (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
Hydroxytetradecenoylcarnitine (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
Hydroxyhexadecanoylcarnitine (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
Hydroxyvalerylcarnitine [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
Phosphatidylcholine 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 (PC ae 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
Phosphatidylcholine acyl-akyl C40:4 (PC ae 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
Phosphatidylcholine acyl-akyl C42:3 (PC ae 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
Phosphatidylcholine acyl-akyl C42:4 (PC ae 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	T2	-31.01
Phosphatidylcholine acyl-akyl C42:5 (PC ae 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	T2	-19.64
Phosphatidylcholine acyl-akyl C44:6 (PC ae C44:6)	HMDB0013450	0.18	0.03	0.19	0.02	0.29	0.07	0.3	0.07	18	35	0.070	0.19	5	0.010	T2	-36.88
Hydroxysphingomyeline C22:1 [SM (OH) C22:1]	HMDB0013466	1.74	0.21	1.75	0.23	2.53	0.57	2.48	0.54	18	44	0.088	0.17	17	0.034	T2	-31.06
Sphingomyeline C16:0 (SM C16:0)	HMDB0010169	92.64	11.3	97.67	10.05	121.72	23.06	130.53	22.98	18	34	0.068	0.17	6	0.012	T2	-23.89
Sphingomyeline C16:1 (SM 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	T2	-25.75
Sphingomyeline C18:0 (SM 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

[1]Metabolite concentrations are reported in μM.

[2]Human 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.

[7]Stability: "t" represents the number of times that the metabolite was selected in the Leave One Out procedure (LOO). N(t) indicates the number of 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 the dataset (Bovo et al., 2015, 2016).

[8]Effect size: "t" represents the absolute value of the regression coefficient of the metabolite. N(t) indicates the number of times that ($|t^*| \geqslant |t|$). P_{si} 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).

[9]Dir: 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.

[10]$\Delta\%$ = relative time-specific difference in the metabolite quantification: 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.