

Early socialization and environmental enrichment of lactating piglets affects the caecal microbiota and metabolomic response after weaning

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Supplementary information

Supplementary tables

Supplementary Table S1. Phylum relative counts for suckling and weaned piglets (from highest to lowest considering the global average) and for conventional reared piglets (CTR) or with environmental enrichment (ENR). All the detected phyla are included.

	LACT				WEAN			
	CTR	ENR	SEM	adjPvalues	CTR	ENR	SEM	adjPvalues
<i>Firmicutes</i>	38.18	39.45	3.264	0.9626	57.37	43.31	4.182	0.6236
<i>Bacteroidetes</i>	43.69	42.74	3.080	0.9626	30.04	36.35	2.998	0.5997
<i>Proteobacteria</i>	6.32	6.52	0.889	0.9626	5.18	13.00	2.162	0.4410
<i>Spirochaetes</i>	1.96	4.85	1.064	0.9626	3.83	3.31	0.589	0.9941
<i>Fusobacteria</i>	6.80	2.63	1.953	0.7823	0.03	1.96	0.644	0.3571
<i>Planctomycetes</i>	0.34	0.77	0.201	0.9177	0.48	0.19	0.120	0.9228
<i>Cyanobacteria</i>	0.33	0.97	0.297	0.7823	0.32	0.09	0.082	0.5997
<i>Synergistetes</i>	0.47	0.78	0.193	0.9369	0.13	0.26	0.051	0.6236
<i>Verrucomicrobia</i>	0.68	0.29	0.235	0.9634	0.31	0.33	0.083	0.9990
<i>Actinobacteria</i>	0.26	0.11	0.094	0.9626	0.54	0.25	0.113	0.5997
<i>Tenericutes</i>	0.18	0.10	0.036	0.9177	0.67	0.17	0.180	0.4410
<i>Elusimicrobia</i>	0.44	0.14	0.129	0.9177	0.14	0.27	0.103	0.5997
<i>Lentisphaerae</i>	0.16	0.31	0.084	0.9626	0.01	0.07	0.020	0.4410
<i>Chlamydiae</i>	0.00	0.01	0.005	0.6237	0.45	0.01	0.127	0.1537
<i>Deferribacteres</i>	0.04	0.09	0.024	0.6237	0.07	0.23	0.066	0.5413
<i>Fibrobacteres</i>	0.02	0.09	0.038	0.9177	0.19	0.13	0.051	0.9990
<i>TM7</i>	0.01	0.11	0.041	0.4849	0.15	0.02	0.036	0.4390
<i>Euryarchaeota</i>	0.05	0.03	0.008	0.9626	0.07	0.04	0.015	0.5997
<i>WPS-2</i>	0.07	0.01	0.032	0.9177	0.01	0.01	0.003	0.5413

Supplementary Table S2. Genus relative abundance counts for suckling and weaned piglets (from highest to lowest considering the global average) and in conventional reared piglets (CTR) or with environmental enrichment (ENR). Only predominant genera (+1%) are included.

	LACT				WEAN			
	CTR	ENR	SEM	adjPvalues	CTR	ENR	SEM	adjPvalues
<i>Prevotella</i>	18.78	13.66	2.243	0.9573	11.93	10.06	2.015	0.9432
[<i>Prevotella</i>]	6.72	6.21	1.080	0.9573	5.28	12.57	1.650	0.4413
<i>Bacteroides</i>	5.90	8.12	1.507	0.9573	2.50	2.66	0.542	0.8931
<i>Phascolarctobacterium</i>	3.33	3.30	0.591	0.9842	4.77	3.23	0.430	0.4413
<i>Campylobacter</i>	1.26	1.10	0.422	0.9573	1.45	8.29	2.092	0.3842
<i>Fusobacterium</i>	6.81	2.63	1.954	0.7993	0.03	1.96	0.645	0.3842
<i>Oscillospira</i>	2.65	2.30	0.333	0.9842	2.57	2.80	0.302	0.9432
<i>Treponema</i>	0.81	2.98	0.863	0.9573	3.16	1.46	0.517	0.6459
<i>p-75-a5</i>	0.72	0.47	0.250	0.9573	3.38	2.47	0.868	0.9432
<i>Lactobacillus</i>	2.72	2.07	0.718	0.9573	0.43	0.82	0.325	0.5380
<i>Megasphaera</i>	2.67	1.86	0.685	0.9573	0.40	0.83	0.316	0.8931
<i>Sphaerochaeta</i>	1.15	1.88	0.466	0.9573	0.67	1.86	0.411	0.5266
<i>Roseburia</i>	0.21	0.17	0.076	0.9573	3.05	1.18	0.703	0.5380
<i>Anaerovibrio</i>	0.94	0.38	0.448	0.9573	1.80	0.94	0.286	0.5266
<i>CF231</i>	1.23	1.09	0.249	0.9573	0.60	0.69	0.164	0.8931
<i>Desulfovibrio</i>	0.73	0.68	0.108	0.9573	1.15	0.77	0.259	0.9432
<i>Butyricimonas</i>	0.59	1.14	0.308	0.9573	0.12	0.61	0.153	0.4746
<i>Ruminococcus</i>	0.33	0.30	0.085	0.9573	1.18	0.65	0.193	0.5226

Supplementary Table S3. PCA and OPLS-DA models parameters for ¹H-NMR serum profiles of weaned piglets

Enriched vs control P-value	n	PCA			OPLS-DA			
		Number of components	R ² _{x(cum)}	Q ² _(cum)	Number of components	R ² _{x(cum)}	R ² _{y(cum)}	Q ² _(cum)
Total spectre	7	1+1	0.64	0.31	1+1	0.62	0.57	0.28
≤ 0.20	7	2	0.85	0.76	1+1	0.83	0.57	0.33

≤ 0.18	7	2	0.88	0.81	1+1	0.87	0.52	0.28
≤0.16	7	2	0.89	0.83	1+1	0.88	0.52	0.29
≤0.14	7	2	0.88	0.81	1+1	0.86	0.61	0.32
≤0.12	7	2	0.90	0.81	1+1	0.86	0.66	0.34
≤0.10	7	2	0.90	0.81	1+1	0.86	0.66	0.34
≤0.08	7	2	0.90	0.81	2	0.89	0.68	0.47
≤0.06	7	3	0.95	0.83	2	0.91	0.68	0.53

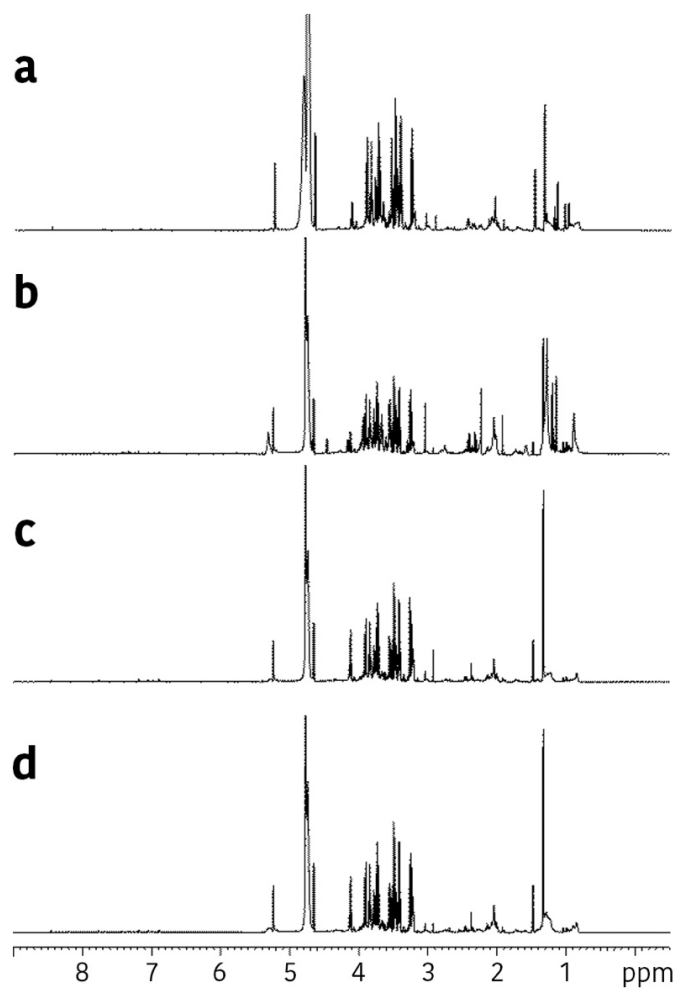
Supplementary Table S4. Brief description of the genes analysed.

Gene abbreviation	Gene full name	Functional group
OCLN	Occludin	Intestinal barrier
ZO1	Zonula occludens 1	Intestinal barrier
CLDN1	Claudin-1	Intestinal barrier
CLDN4	Claudin-4	Intestinal barrier
CLDN15	Claudin-15	Intestinal barrier
MUC2	Mucin 2	Intestinal barrier
MUC13	Mucin 13	Intestinal barrier
TFF3	Trefoil factor 3	Intestinal barrier
TLR2	Toll-like receptor 2	Pattern recognition receptors (PRRs)
TLR4	Toll-like receptor 4	Pattern recognition receptors (PRRs)
IL1β	Interleukin 1 beta	Immune response
IL6	Interleukin 6	Immune response
IL8	Interleukin 8	Immune response
IL10	Interleukin 10	Immune response
IL17A	Interleukin 17	Immune response
IL22	Interleukin 22	Immune response
IFN-γ	Interferon gamma	Immune response
TNF-α	Tumor necrosis factor alpha	Immune response
TGF-β1	Transforming growth factor beta 1	Immune response
CCL20	Chemokine (C-C motif) ligand 20	Immune response
CXCL2	Chemokine (C-X-C motif) ligand 2	Immune response
IFNGR1	Interferon gamma receptor 1	Immune response
REG3G	Regenerating-islet derived protein 3 gamma	Immune response

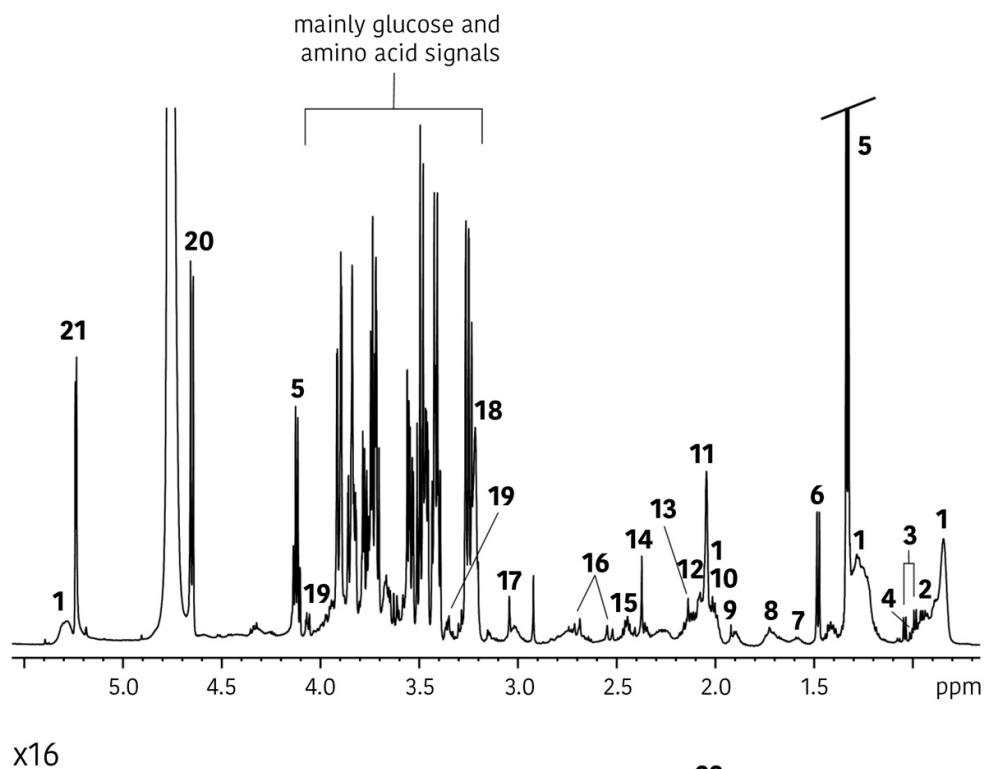
PPARGC1α	Peroxisome proliferative activated receptor gamma, coactivator 1 alpha	Immune response
FAXDC2	Fatty acid hydrolase domain containing 2	Immune response
GBP1	Guanylate binding protein 1	Immune response
HSP27	Heat shock protein 27	Intestinal homeostasis
HSP70	Heat shock protein 70	Intestinal homeostasis
GPX2	Glutathione peroxidase 2	Digestive enzyme / hormone
SOD2	Superoxide dismutase	Digestive enzyme / hormone
ALPI	Intestinal alkaline phosphatase	Digestive enzyme / hormone
SI	Sucrase-isomaltase	Digestive enzyme / hormone
DAO1	Diamine oxidase	Digestive enzyme / hormone
HNMT	Histamine N-methyltransferase	Digestive enzyme / hormone
ANPEP	Aminopeptidase-N	Digestive enzyme / hormone
IDO1	Indoleamine 2,3-dioxygenase	Digestive enzyme / hormone
GCG	Glucagon	Digestive enzyme / hormone
CCK	Cholecystokinin	Digestive enzyme / hormone
IGF1R	Insulin-like growth factor 1 receptor	Digestive enzyme / hormone
PYY	Peptide YY	Digestive enzyme / hormone
SLC5A1	Solute carrier family 5 (sodium/glucose cotransporter) member 1	Nutrient transport
SLC16A1	Monocarboxylate transporter 1	Nutrient transport
SLC7A8	Solute carrier family 7 (amino acid transporter light chain, L System) member 8	Nutrient transport
SLC15A1	Solute carrier family 15 (oligopeptide transporter) member 1	Nutrient transport
SLC13A1	Solute carrier family 13 (sodium/sulfate symporters) member 1	Nutrient transport
SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporter) member 2	Nutrient transport
MT1A	Metallothionein 1A	Nutrient transport
SLC30A1	Solute carrier family 30 (zinc transporter) member 1	Nutrient transport
SLC39A4	Solute carrier family 39 (zinc transporter) member 4	Nutrient transport
CRHR1	Corticotropin releasing hormone receptor 1	Stress indicators

NR3C1	Glucocorticoid receptor	Stress indicators
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	Stress indicators
ACTB	β -actin	Housekeeping
B2M	β 2-microglobulin	Housekeeping
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping
TBP	TATA-Box binding protein	Housekeeping

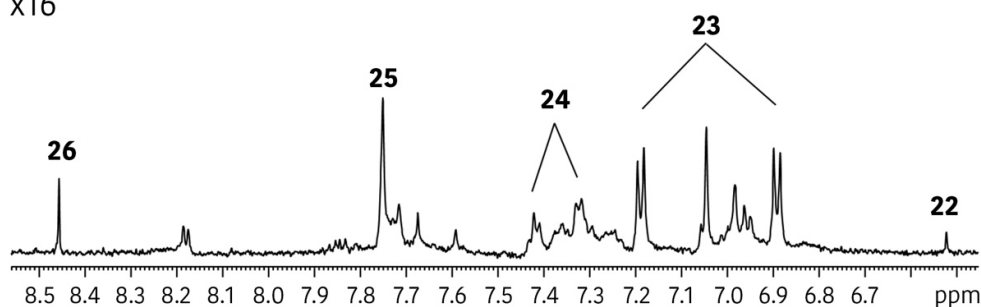
Supplementary figures



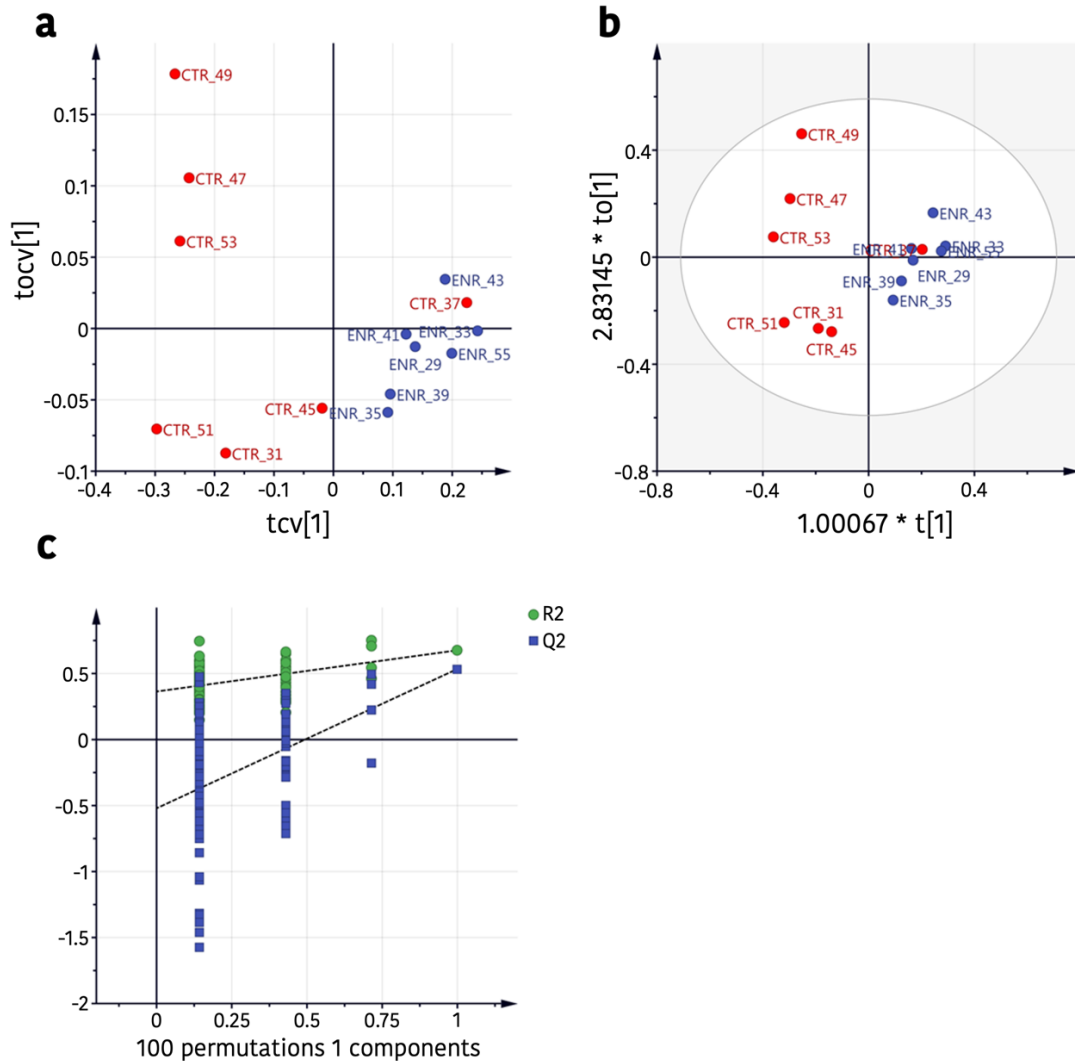
Supplementary Figure S1. Representative $^1\text{H-NMR}$ spectra of serum from piglets of different experimental treatment. (a) Lactating piglets control group; (b) lactating enriched piglets' group; (c) weaned piglets control group and (d) weaned enriched piglets' group. All spectra were acquired using a 600 MHz spectrometer.



x16



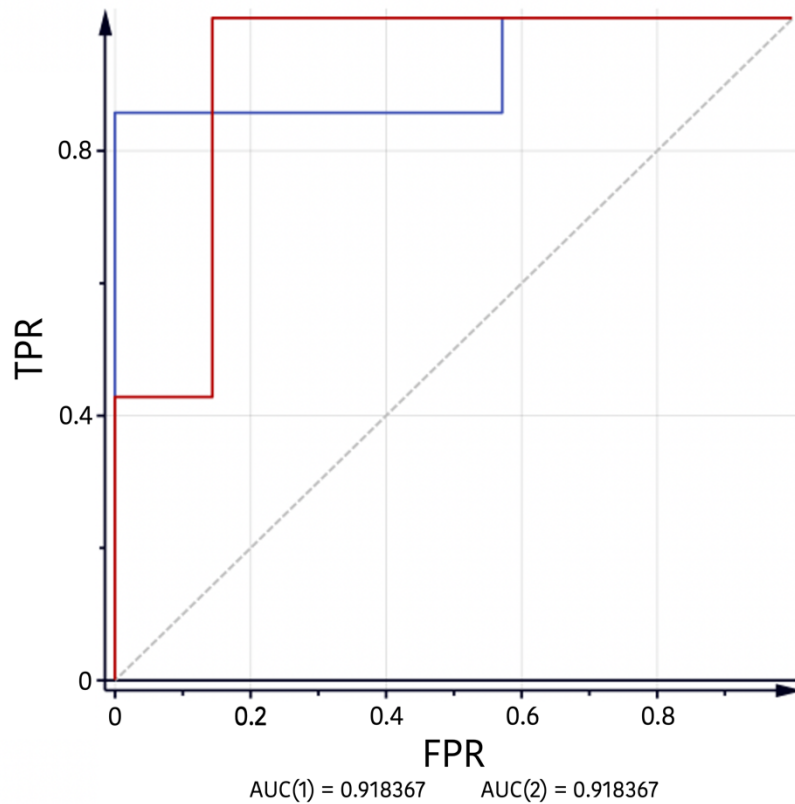
Supplementary Figure S2. Representative ^1H CPMG spectrum (600MHz) of serum from nursing piglet. Assignments: **1**, LDL/VLDL; **2**, leucine; **3**, valine; **4**, isoleucine; **5**, lactate; **6**, alanine; **7**, adipate; **8**, arginine; **9**, acetate; **10**, proline; **11**, N-acetyl glycoproteins; **12**, O-acetyl glycoproteins; **13**, glutamine/glutamate; **14**, pyruvate; **15**, glutamate; **16**, citrate; **17**, creatine; **18**, choline; **19**, myo-inositol; **20**, β -glucose (anomeric proton); **21**, α -glucose (anomeric proton); **22**, fumarate; **23**, tyrosine; **24**, phenylalanine; **25**, methyl histidine; **26**, formic acid.



Supplementary Figure S3. Validation of the OPLS-DA model between enriched and control piglets at after weaning period. OPLS-DA ($P \leq 0.06$) plot (**a**) derived from $^1\text{H-NMR}$ serum spectra of weaned enriched piglets (blue) and weaned control group (red). Cross validation plot (**b**) of the OPLS-DA ($P \leq 0.06$) model. 100 random permutation test plot (**c**) relative to OPLS-DA ($P \leq 0.06$) model for all samples including enriched and control piglets, where the vertical axis corresponds to R^2 (green circles) and Q^2 (blue squares) values for the model and the horizontal axis corresponds to the correlation coefficient between the original Y and the permuted Y .

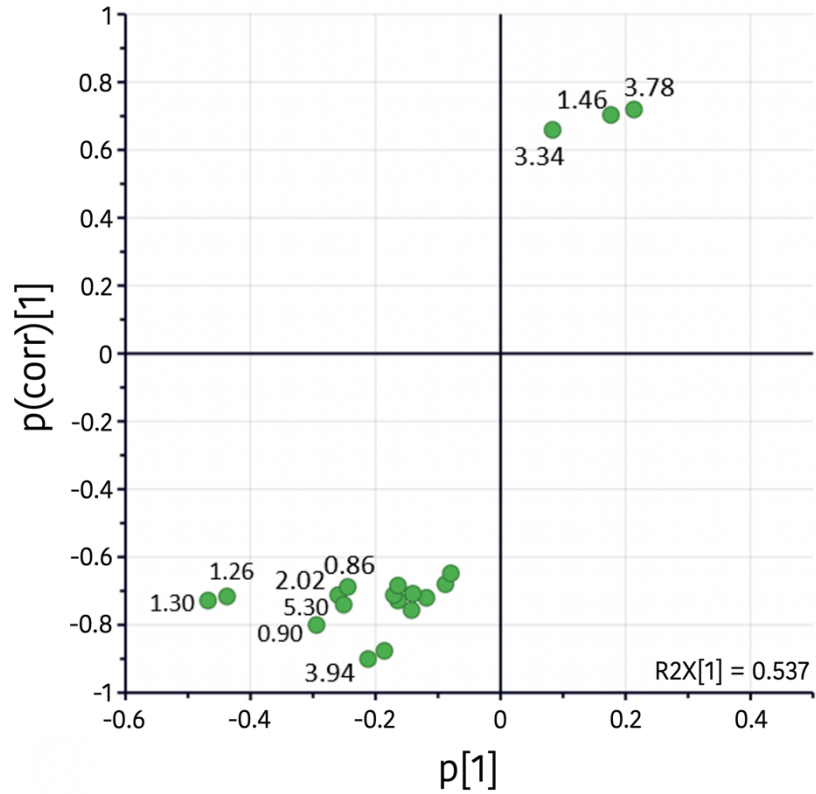
The OPLS-DA ($P \leq 0.06$) model constructed to discriminate between enriched and control piglets after weaning was confirmed by cross-validation, the score plot of the regular scores (**Supplementary Fig. S3a**) compared with the score plot of the CDs course (**Supplementary Fig. S3b**) were almost the same with very little shifting of the spots which is a strong indication that the OPLS-DA ($P \leq 0.06$) model is devoid of influential observations and it is very stable to the inclusion or exclusion of all the different observations. Furthermore, the plot of permutation test (100 times) (**Supplementary Fig. S3c**) performed for all samples including enriched piglets and

control piglets shows that the new parameters ($R^2=0.36$ and $Q^2= -0.52$) were lower than the original values indicating a lack of over-fitting.



Supplementary Figure S4. Receiver operating characteristic (ROC) plot for the OPLS-DA ($P \leq 0.06$) model at after weaning period. Weaned enriched piglets (blue) and weaned control group (red).

The operating characteristic (ROC) plot (**Supplementary Fig. S4**) for the OPLS-DA ($P \leq 0.06$) model displays the true positive classification rate (TPR) for enriched group classification (blue) or for control group classification (red) by the constructed model plotted against the corresponding false positive classification rate (FPR) at various threshold settings of the criterion parameter (YPredPS). Thus, for red curve, TPR (or Sensitivity) represents the probability that a test result will be positive when the enrichment practice is present and TNR (or Specificity) corresponds to the probability that a test result will be negative when the enrichment practice is not present. Every point on the ROC curves represents a pair sensitivity/specificity values corresponding to a particular decision threshold. For both curves the area under the curve (AUC) of the ROC plot has a value of 0.92 indicating high sensitivity and specificity and thus, a high prediction power of the model.



Supplementary Figure S5. S-plot corresponding to OPLS-DA ($P \leq 0.06$) model between enriched and control piglets at after weaning period. The covariance value for each variable included is represented on the horizontal axis in the model. The vertical axis represents the correlation values obtained with respect to the dependent variable. The points at the ends of the S-plot curve indicate higher contributions to the classification.