Divergent selection for feed efficiency in pigs altered the duodenum transcriptomic response to feed intake and its DNA methylation profiles

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1 RESEARCH ARTICLE

2 RUNNING HEAD: Transcriptomes and methylomes of the pig's duodenum

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4 duodenum transcriptomic response to feed intake and its
5 DNA methylation profiles

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14

15 ABSTRACT
16 Feed efficiency is a trait of interest in pigs as it contributes to lowering the ecological and economical
17 costs of pig production. A divergent genetic selection experiment from a Large White pig population was
18 performed for 10 generations, leading to pig lines with relative low- (LRFI) and high- (HRFI) residual feed
19 intake (RFI). Feeding behavior and metabolic differences have been previously reported between the
20 two lines. We hypothesized that part of these differences could be related to differential sensing and
21 absorption of nutrients in the proximal intestine. We investigated the duodenum transcriptome and
22 DNA methylation profiles comparing overnight fasting with ad libitum feeding in LRFI and HRFI pigs
23 (n=24). We identified 1,106 differentially expressed genes between the two lines, notably affecting
24 pathways of the transmembrane transport activity and related to mitosis or chromosome separation.
25 The LRFI line showed a greater transcriptomic response to feed intake than the HRFI line. Feed intake
26 affected genes from both anabolic and catabolic pathways in the pig duodenum, such as rRNA
27 production and autophagy. Several nutrient transporter and tight junction genes were differentially
28 expressed between lines and/or by short term feed intake. We also identified 409 differentially
29 methylated regions in the duodenum mucosa between the two lines, while this epigenetic mark was less
30 affected by feeding. Our findings highlighted that the genetic selection for feed efficiency in pigs
31 changed the transcriptome profiles of the duodenum, and notably its response to feed intake,
32 suggesting key roles for this proximal gut segment in mechanisms underlying feed efficiency.

33

34 NEW & NOTEWORTHY
35 The duodenum is a key organ for the hunger /satiety loop and nutrient sensing. We investigated how
36 the duodenum transcriptome and DNA methylation profiles are affected by feed intakes in pigs. We

37 observed thousands changes in gene expression levels between overnight-fasted and fed pigs in an high
38 feed efficiency pig lines, but almost none in the related low feed efficiency pig line.

39 **Keywords:** DNA methylation; duodenum; feed efficiency; postprandial transcriptome; transcriptomic
40 profiling.

41

42

INTRODUCTION

44 Provide a brief overview of the scope and relevance of the study, especially regarding previous
45 advancements in related fields.

46 In monogastric livestock, feed efficiency is the ability to convert the greater part of ingested feed into
47 body weight. It is a complex trait with many known influencing factors such as nutrition, metabolism,
48 genetics, microbiome, meteorological conditions, sanitary status, and gut microbiota. Feed efficiency is
49 a trait of great interest for livestock since farming feed cost is a large part of the production costs. Feed
50 also constitutes a large part of the environmental impacts of monogastric farming (1). In addition to
51 research into non-human-edible feed, improvement in the animal feed efficiency will reduce the
52 amount of feed needed to raise livestock, thus contributing to the reduction of its environmental
53 footprint (2–4).

54 Feed efficiency can be measured as Residual Feed Intake (RFI): the difference between the amount of
55 feed one animal is consuming and the amount predicted for its maintenance and production
56 requirements via multiple regression on several traits (average metabolic body weight, average daily
57 gain, and indicators of body composition). A negative RFI means that the animal is eating less (relatively
58 high efficiency), and a positive RFI means that the animal is eating more (relatively low efficiency) than
59 the population average for their specific growth rate and body composition. RFI is a heritable trait in
60 pigs (5). Differences in blood metabolome and brain, duodenum, liver, and muscle transcriptomes have
61 previously been observed in pigs with contrasted feed efficiency (6–10).

62 Gilbert et al. created a genetic divergent selection experiment from a Large White pig breed nucleus,
63 resulting in two pig lines of relative low (LRFI) and high (HRFI) residual feed intakes (5). After 10
64 generations of selection, pigs of the LRFI line eat around 100 grams per day less than pigs from the HRFI
65 to gain a similar amount of body weight, and are thus of greater feed efficiency. The response to
66 selection has led to multiple genetic and genomic changes between the lines (11). A previous
67 transcriptomic study has compared muscle, adipose tissues and liver transcriptome from generation 8 of
68 selection (12), with pathways involved in immune response, response to oxidative stress and protein
69 metabolism differentiating the two lines. These two lines also differ on their blood and muscle
70 metabolism (13, 14)—with a lower insulin level in the blood of LRFI pigs—and in their fecal microbiota
71 (15). The selection has led to distinct feeding behaviors between the lines: pigs from the LRFI line have a
72 lower daily feed intake, wait longer between two visits to the feeder, but eat more per visit, stay longer
73 in the feeder, and eat faster than pigs from the HRFI line (16).

74 The intestine is known to quickly adapt to various feeding challenges (17). The duodenum is one of the
75 key organs involved in nutrient sensing and satiety regulation, in relation with its proximal position in
76 the gut (18, 19). We hypothesized that divergent selection for feed efficiency might have altered the
77 duodenum physiological response to feed intake. The effect of feeding challenges on intestinal
78 transcriptomes has been investigated by several groups. Zhang et al. reported that pigs submitted to
79 different feeding frequencies, 1, 3 or 5 meals per day, have a different ileal and colonic mucosal
80 transcriptome (20), although they only investigated transcriptomes from overnight fasted pigs. Mazurais
81 et al. studied the impact of a 2-day fasting in the jejunum expression levels of targeted genes in piglets,
82 and noted 146 affected over the 954 studied genes (21). In mice, Yoshioka et al. have investigated the
83 effect of a high-fat and low-fat diet on the duodenal mucosa compared to fasted mice (22). They noted
84 only a modest effect on the transcriptome, but highlighted the downregulation of Slc5a1 after feed
85 intake. *Slc5a1* is the gene encoding for the sodium/glucose cotransporter 1 (SGLT1). It is also
86 documented that the gene *Slc15a1* encoding for the peptide transporter 1 (PEPT1) is downregulated by
87 feed intake in mice (23). More generally, the Solute Carrier (SLC) gene family encodes transmembrane
88 nutrient transporter, and is of great interest to better understand the digestive physiology (24–26).

89 DNA methylation is an epigenetic mark playing important roles in cellular differentiation and in the
90 regulation of gene expression (27, 28), including in the intestine (29). In mature mammalian tissues,
91 DNA methylation mostly occurs at CpG dinucleotides. Most CpG sites are methylated, except for CpG
92 sites in promoters and enhancers of expressed genes. These unmethylated regions are often relatively
93 enriched in CpG sites compared to the rest of the genome, constituting CpG island in an otherwise CpG
94 depleted genome (30). Promoter DNA methylation is generally negatively correlated with gene
95 expression, while gene body DNA methylation is rather positively correlated with gene expression.
96 Causality links between gene repression and promoter DNA methylation is debated; experimental
97 perturbation of DNA methylation (by knock out, drug, or epigenome editing) often induce
98 transcriptomic changes, but in development and diseases context (such as cancer), gene expression
99 changes mostly occurs before promoter DNA methylation changes (28). DNA methylation is essential
100 for proper intestinal epithelial cell differentiation (31), and DNA methylation profiles change through the
101 intestine's cellular (32) and developmental (33) maturations. In cattle, genomic predictions for feed
102 efficiency are correlated with DNA methylation differences at some imprinted loci (34). A 36h fasting
103 also resulted in changes in DNA methylation in adipose tissues in human (35).

104 We hypothesized that differences between LRFI and HRFI pigs in the intestinal responses to feed intake
105 might partially explain several of the physiological differences previously reported. We therefore
106 investigated the transcriptomic and DNA methylation profiles of the duodenum mucosa before or after
107 a meal in pigs from the 10th generation of LRFI and HRFI lines, in a total of 24 animals.

MATERIALS AND METHODS

Animal production and sampling

110 This experimentation was authorised by the French Minister of Higher Education, Research and
111 Innovation under the number APAFIS#21107-2018120415595562 v10, after examinations by the animal
112 experimentation ethic committee number 084.

113 Animals were raised using standard care in the INRAE pig experimental facility GENESI (36) up until the
114 day before the procedure. Animals were from two French Large White lines of pigs that have been
115 divergently selected on residual feed intakes for 10 generations (5). Pigs were from three litters in each
116 line, and of balanced sexes within litter when possible (one litter was represented by 3 males and one
117 female). At weaning (28 days old on average), animals were split into 2 pens of 12 animals, full-sibs and
118 sexes being equally distributed in the two pens (figure 1A). Pigs were slaughtered on the same day, at
119 the age of 61 days old (min 60 days, max 62 days). The day before sampling, feed access was removed at
120 5 p.m. in both pens. Animals had free access to water. At 8 a.m. the next day, feed access was
121 introduced back in one pen, but not in the other. Animals were slaughtered by electro-narcosis between
122 8.50 a.m. and 11.46 a.m., starting with animals left without feed access until 10.10 am where animals
123 with unlimited feed access were also sampled (figure 1B, supplementary table 1). The gastro-intestinal
124 tract was removed, and a 5 cm long section of the duodenum was sampled, opened longitudinally, and
125 the mucosa was collected by scratching the internal duodenal section with a glass microscope slide.
126 Samples were rinsed in PBS, and flash frozen in liquid nitrogen. Samples were preserved at -80°C until
127 extraction.

Sample extraction, library preparation, and sequencing

129 Frozen duodenum samples were reduced to a fine powder with the mixer-mill MM400 by rapid agitation
130 for 1 minute at 30Hz in a liquid-nitrogen cooled container with stainless steel beads. Fine powder
131 samples were conserved at -80°C until extraction. DNA and RNA were extracted from the powder using
132 NucleoSpin TriPrep mini kit columns (Macherey-Nagel), according to the manufacturer's main
133 instructions but with a few modifications. About 30-50 mg of powder were resuspended in 750 µL of
134 RP1 buffer with 7.5µL of ß-mercaptoethanol then homogenised with the mixer-mill MM400 by rapid
135 agitation for 2x2 minutes at 30Hz with stainless steel beads and incubated 10 minutes at room
136 temperatures. Lysates were loaded into columns in two steps. DNA elution was performed using 65 µL
137 of DNA elute solution. On-column digestion of residual DNA was performed for 30 minutes at room
138 temperature. RNA was eluted using 100 µL of RNase-free water.

139 RNA-seq libraries were prepared according to Illumina's protocols using the Illumina TruSeq Stranded
140 mRNA sample prep kit to analyse mRNA. Briefly, mRNA were selected using poly-T beads. RNA were
141 reverse transcribed to generate double stranded cDNA, then fragmented, and adaptors were ligated for
142 sequencing. Eleven cycles of PCR were applied to amplify libraries. Library quality was assessed using a
143 Fragment Analyser and libraries were quantified by qPCR using the Kapa Library Quantification Kit. RNA
144 sequencing was performed on an Illumina HiSeq3000 using a paired-end read length of 2 x 150 pb with
145 the Illumina HiSeq3000 Reagent Kits, at the GeT-PlaGe core facility, INRAE Toulouse (37).

146 Four DNA samples (one female and one male LRFI, one female and one male HRFI) were artificially
147 methylated using the M.Sss1 Enzyme (New England BioLabs) following the manufacturer instruction.
148 DNA libraries (4 fully methylated control, 4 input controls, and 24 samples) were prepared following
149 Bioo scientific's protocol using the Bioo scientific NEXTflex Methyl-seq Library Prep Kit for Illumina
150 Sequencing (part number NOVA-5118-02). Briefly, DNA was fragmented by sonication on a covaris M220
151 (400-500 bp), size selection was performed using AMPure XP beads and adaptors were ligated to be
152 sequenced. Library preparation failed for one sample (an LRFI fed sample). The 4 fully methylated
153 libraries and the 23 other samples were mixed at equimolarity when possible (62 ng of DNA per sample,
154 otherwise taking a maximal volume of 20 µL resulting in 5-21 ng of DNA for four samples), and a single

155   methyl-DNA precipitation (MeDP) was performed on 1 µg of the pool using Invitrogen's Methylminer kit
156   following the manufacturer instructions. After additions of the 4 inputs to the pool, 10 cycles of random
157   PCR were performed on the mixed library. Library quality was assessed using an Agilent Fragment
158   Analyser and the pool was quantified by qPCR using the Kapa Library Quantification Kit. MeDP
159   sequencing (MeDP-seq) was performed on two lanes of an Illumina NovaSeq 6000 using a paired-end
160   read length of 2 x 100 bp with the Illumina NovaSeq Reagent Kits S2, at the GeT-PlaGe core facility (37),
161   INRAE Toulouse.

162   RNA-seq and MeDP-seq libraries were demultiplexed, and the resulting fastq files are available at the
163   ENA database under the id PRJEB46060 / ERP130249.

RNA-seq bioinformatic processing

165   RNA-seq reads were processed using the nf-core/rnaseq pipeline (38) (version 3.0), using Salmon (39)
166   pseudo-alignment quantifications, Ensembl reference genome Sscrofa11.1, and the corresponding gene
167   annotation file from version 102 of Ensembl (40). Normalised counts were processed using {tximports}
168   (41) to generate transcript per million (TPM) values (supplementary table 2), and gene-length
169   normalised counts (supplementary table 3) were used to carry over differential gene expression analysis
170   with {limma} voom (42), using contrast matrices to test for different factors, and a false discovery rate
171   (FDR) threshold of 5%. The pig line (LRFI or HRFI), condition (fed or fasted) and sex (castrated male or
172   female) were used to build a model matrix. Contrast matrices were constructed to compare the two
173   lines, and to compare the feeding effect within each line. A total of 13,738 genes were analysed, others
174   having less than a total of 8 counts across our dataset. Gene ontology enrichment analysis was
175   performed using {clusterProfiler} (43), using Sus scrofa Gene Ontology. It should be noted that not all
176   genes annotated in the pig genome are associated with a Gene Ontology term, leading to slightly lower
177   gene numbers in gene lists used for the Gene Ontology Analyses. The set of genes used for differential
178   gene expression analysis was used as a reference set. Enriched Gene Ontology were simplified for
179   readability using the *simplify()* function, and simplified enriched ontologies were clustered using the
180   *pairwise_termsim()* function. Heatmaps were generated using {ComplexHeatmap} (44), and upset plots
181   were generated using the {UpSetR} package (45). The list of substrates for each SLC transporter was
182   obtained from the SLC tables at slc.bioparadigms.org (24). The list of tight junction genes was
183   downloaded from the AMIGO gene ontology website (46, 47), using the Gene Ontology term
184   GO:0070160.

MeDP-seq bioinformatic processing

186   As MeDP-seq data is conceptually similar to ChIP-seq data, MeDP-seq reads were processed using the
187   nf-core/chipseq pipeline (38) version 1.2.1 on Ensembl reference genome Sscrofa11.1 (40). CpG
188   methylation ratios were deduced from MeDP-seq coverages using BayMeth (48) and data from the
189   artificially methylated control samples. Genomic regions with anomalous coverage in input samples
190   were identified using the {greylistchip} bioconductor package (49) version 1.28.1 applied on each of the
191   four input samples separately, and the four greylist regions were merged together. Genomic regions
192   within our greylist were removed from the rest of the analysis.

193   To define lowly methylated regions (LMRs) we used the following steps: average DNA methylation ratio
194   was computed for each sample in 100 bp windows. We then kept the 100 bp windows for which the
195   DNA methylation ratio was under 40% in at least two samples within LRFI fed, LRFI fasted, HRFI fed, and
196   HRFI fasted pigs. Consecutive 100bp windows were merged, and only genomic regions of 500 bp or

197 more were kept. Unmethylated regions separated by 300 bp or less were merged. This resulted in the
198 definition of 60,509 LMRs covering 0.03% of the genome (mean size: 1089 bp, median: 700 pb,
199 minimum size: 500 bp, maximum size: 33,500 bp).

200 MeDP-seq coverage at LMRs was obtained for each sample using {EnrichedHeatmap} normalizeToMatrix
201 function (50). We used {DESeq2}50 to identify LMRs with differential coverage between the two LRFI
202 input control samples and the two HRFI input control samples. This resulted in the identification of
203 9,572 LMRs with differential coverage between inputs from the two lines at an unadjusted p-value
204 threshold of 5%. These regions were filtered as they might reflect structural genomic differences
205 between the two lines resulting in misestimations of their DNA methylation state by MeDP-seq.
206 Differentially methylated regions (DMRs) between the two lines were identified within the resulting
207 50,919 lowly methylated regions using {DESeq2} (51) with a linear model taking into account the pig line
208 (LRFI or HRFI) and condition (fed or fasted). Fed/fasted DMRs were called in each pig line independently
209 using the same approach. In addition to an FDR adjusted p-value below 0.05 and after manual
210 visualisations of some regions, we selected only regions with an absolute log2 fold change greater than
211 1 as differentially methylated regions in an attempt to further reduce false positives. DNA methylation
212 ratio at differentially methylated regions were visualised using {epistack} (52).

213 ## Data and script availability
214 RNA-seq and MeDP-seq reads are available through the ENA database, id PRJEB46060 / ERP130249.
215 Scripts used to process and analyse RNA-seq and MeDP-seq reads are available through a public gitlab
216 repository at the address: forgemia.inra.fr/genepi/analyses/rosepigs. Gene expression counts, lists of
217 differentially expressed genes, sample metadata tables, and DMR positions are available in this
218 repository (forgemia.inra.fr/genepi/analyses/rosepigs/-/tree/master/processed_files). Supplemental
219 material is available at URL:
220 https://entrepot.recherche.data.gouv.fr/dataset.xhtml?persistentId=doi:10.57745/V7FWEH DOI:
221 https://doi.org/10.57745/V7FWEH.

222 # RESULTS

223 ## The duodenum transcriptome is different between the high- and low- feed efficiency pig
224 lines
225 To compare pigs from the 10th generation of selection, 12 animals for each line were taken from 3
226 litters in the same breeding batch trying to balance sex ratios (figure 1A). After overnight fasting with
227 unrestricted water access, feed was reintroduced to 6 HRFI and 6 LRFI animals in a single pen (balancing
228 litters and sex, figure 1B). Duodenal mucosa samples were then collected, between 2 to 3 hours after
229 feed re-introduction, or between 12 to 14 hours of feed restriction. Duodenal transcriptomes were
230 obtained by RNA-sequencing after poly-A purification, enriching for messenger RNA. We compared 12
231 LRFI samples (6 fed and 6 fasted) with 12 HRFI samples (6 fed and 6 fasted), and detected 1,106 genes
232 differentially expressed between the LRFI and HRFI lines, independently of the feeding status of the
233 animals (figure 2A, supplementary table 4). In detail, 464 genes were identified as upregulated in the
234 LRFI line, and 642 genes were identified as up-regulated in the HRFI line.

235 Functional enrichment analysis revealed that genes upregulated in HRFI were notably involved in various
236 aspects of cell division, including spindle checkpoint signalling, chromosome segregation, DNA-
237 templated DNA replication, and immune response (figure 2B). Fewer processes were functionally

238 enriched in genes upregulated in the LRFI lines (figure 2C), including glycosphingolipid metabolic process
239 and transmembrane transporter activity.

240 ## The pig duodenum transcriptome response to feed intake is stronger in LRFI pigs than in
241 HRFI pigs

242 For each line, we compared duodenal transcriptomes in fasted or fed pigs. We detected 2,222
243 differentially expressed genes in the LRFI pig line, but only 61 differentially expressed genes in the HRFI
244 line (figure 3, A&B, supplementary table 4), leading to a total of 2,225 genes affected by feed intake in
245 one or both pig lines. Visualisation of expression profiles revealed that in the LRFI line, all 6 fasted pigs
246 were showing stark differences of expression as compared to the 6 fed pigs in the differentially
247 expressed genes, while 3 fasted HRFI pigs and 2 fed HRFI pigs did not have a transcriptomic signature
248 matching the feeding status signature of the LRFI line (figure 3A).

249 In the LRFI line, the 1,050 genes overexpressed in the fasted state were enriched in GO-BP categories
250 linked to autophagy, cell junction and plasma membrane receptors, Wnt signalling pathway, and protein
251 catabolic processes (figure 3C). The 1,172 genes overexpressed in the fed state in LRFI pigs were
252 enriched in GO-BP categories linked to protein folding, ribosome biogenesis and the sterol biosynthetic
253 process (figure 3D). In the HRFI line, the 41 genes overexpressed in the fasted state were enriched in
254 GO-BP categories linked to ubiquitin ligase complex, and with mitochondrial envelope to a lesser extent
255 (figure 3E). No GO-BP category was detected as enriched in the 20 genes overexpressed in the fed states
256 in HRFI pigs.

257 Among the genes impacted by the feeding status in one or both lines, 185 genes were also detected as
258 differentially expressed between the two lines (figure 3B). Visual inspection of these genes revealed that
259 they mostly respond to feed intake in the LRFI line and not in the HRFI line, resulting in differences in
260 average expression levels between the lines (figure 3F).

261 ## Duodenal expression of nutrient transporter and tight junction genes was altered by the
262 divergent selection on feed efficiency

263 The transmembrane transporter activity ontology term is overrepresented in genes with higher
264 expression in LRFI than in HRFI (figure 2C). The Solute Carrier (SLC) gene family encodes for
265 transmembrane transporters, including nutrient transporters expressed in the intestinal epithelium (24,
266 26). We therefore focused our analysis on the expression patterns of the 364 annotated SLC genes in the
267 pig genome in our experimental setup (figure 4A). In the duodenum mucosa, 28 SLC genes are
268 differentially expressed between the LRFI and HRFI lines: 17 are more expressed in the LRFI line, such as
269 the folate transporter *SLC25A32*, 11 are more expressed in the HRFI line such as the glucose and
270 galactose transporter *SLC2A10*. Thirty-five SLC genes are differentially expressed in the duodenum
271 between fasted and fed LRFI pigs between: 18 are more expressed in fasted pigs, such as
272 monosaccharides transporters *SLC5A1* and *SLC2A2* and the aspartate and glutamate transporter
273 *SLC25A13*, 17 are more expressed in fed pigs, such as the glutamate transporter *SLC17A8*. No SLC gene
274 was detected as differentially expressed by feed intake in the duodenum of HRFI pig.

275 The cell-cell junction organisation ontology term was enriched in genes with higher expression in fasted
276 LRFI pigs than in fed LRFI pigs (figure 3C). We therefore focused our analysis on the expression patterns
277 of the 77 annotated tight junction genes in our experimental setup (figure 4B). In the duodenum
278 mucosa, 5 tight junction genes are differentially expressed between the LRFI and HRFI lines, and all are

279   more expressed in the LRFI line: Claudin-2 (*CLDN2*), *CLDN4*, *CLDN15*, Partitioning defective 6 homolog
280   beta (PARD6B) and Protein Associated to Tight Junctions / InaD-like protein (*PATJ*). Fifteen tight junction
281   genes are differentially expressed by short term feed intake in the duodenum of LRFI pigs: 12 are more
282   expressed during short term fasting, such as Occludin (*OCLN*), Junctional adhesion molecule A (*F11R*) or
283   *PATJ*, and 3 are more expressed after feed intake: *CLDN5*, *CLDN11* and MICAL-Like 2 (*MICALL2*). No tight
284   junction gene was detected as differentially expressed by feed intake in the duodenum of HRFI pig.

285   Differences in DNA methylation profiles in the duodenum of LRFI and HRFI pigs
286   DNA methylation profiles were obtained by MeDP-seq on the same duodenum samples. Although
287   MeDP-seq primarily measures DNA methylation density, we converted it into DNA methylation ratio
288   using fully methylated control samples (see method) (48). For all samples, DNA methylation was lower
289   near the transcription start site (TSS) of expressed genes than at the TSS of un-expressed genes
290   (supplemental figure 1). We detected low DNA methylation regions (LMRs) in each experimental group
291   as putative regulatory regions. Amongst the 50,872 LMRs, 409 were differentially methylated between
292   LRFI and HRFI, 26 were detected as differentially methylated between fasted and fed LRFI pigs, and 3
293   were detected as differentially methylated between fasted and fed HRFI pigs (figure 5, supplementary
294   table 5, 6, and 7). We did not find global associations between DMRs and differential gene expression,
295   but some overlaps were intriguing. Of the 409 HRFI and LRFI DMRs, 20 were within 20kb of a
296   differentially expressed gene between LRFI and HRFI pigs (supplementary table 8), and the associations
297   between changes in DNA methylation and changes in gene expression were not always in the same
298   direction. Amongst those were *CLDN10* (hypomethylated on the first intron in HRFI, and more expressed
299   in HRFI than in LRFI, figure 6 A&B), *PHGDH* (Phosphoglycerate dehydrogenase, hypomethylated in an
300   internal exon and intron in HRFI, and more expressed in HRFI than in LRFI, figure 6 C&D), *ITLN2*
301   (Intelectin-2, hypomethylated in an internal intron in LRFI, and more expressed in HRFI than in LRFI,
302   figure 6 E&F), *FER* (Proto-oncogene tyrosine-protein kinase, hypomethylated in an internal intron in
303   HRFI, and more expressed in LRFI than in HRFI, figure 6 G&H), and the long non-coding RNA
304   *ENSSSCG00000044004* (hypomethylated at its promoter in HRFI, and more expressed in HRFI than in
305   LRFI, figure 6 I&J). Of the 29 DMRs between fed and fasted pig in one of the lines, 3 were within 20kb of
306   a differentially expressed gene between fasted and fed LRFI pigs (supplementary table 9). Amongst
307   those were *KDSR* (3-dehydrosphinganine reductase, hypomethylated in fed LRFI pigs compared to fasted
308   LRFI pigs, and less expressed in fed LRFI pigs compared to fasted LRFI pigs, figure 6 K&L). *KDSR3* was also
309   less expressed in fed HRFI pigs than in fasted HRFI pigs, but to a lower extent than in LRFI pigs.

310 **DISCUSSION**

311   Explain your interpretation of the data, especially compared with previously published material cited in
312   the References. Significance and limitations may also be present.

313   Here we demonstrated that the divergent selection on feed efficiency changed the duodenum mucosal
314   transcriptome in pigs, identifying 1,106 genes differentially expressed between the LRFI and HRFI lines.
315   Genes overexpressed in LRFI were enriched in gene ontologies relevant to glycosphingolipid metabolic
316   process, transmembrane transport, and exopeptidase and metallopeptidase activity. These functions do
317   not directly mirror results from the transcriptome of muscle, liver and adipose tissues (12), or in blood,
318   muscle and liver metabolism (13). Therefore, it is likely that genetic differences due to selection have led
319   to differences in gene expression that are distinct between tissues, and are not systemic. Functions
320   enriched in HRFI pigs were overwhelmingly related to mitosis related processes, such as chromosome

321 separation, DNA replication, and mitosis checkpoint. It is not known if it is due to issues with cell division
322 in the HRFI line that could be frequently failing, or if it simply reflects a higher cell division rate in the
323 duodenum mucosa of HRFI pigs. Histological examination of the duodenum from HRFI and LRFI pigs
324 might be very informative, especially if coupled with measures of cell divisions.

325 The LRFI line shows a very strong transcriptomic response to feed intake in its duodenum mucosa, with
326 2,222 differentially expressed genes. Gene expressed after fasting were enriched in catabolic functions
327 and autophagy, while genes expressed after feed intake were more anabolic, which was perhaps to be
328 expected. The large number of differentially expressed genes between fasted and fed LRFI pigs is in
329 contrast to the weak response observed in HRFI pigs, with only 85 differentially expressed genes. It is
330 not known if selection has increased the transcriptomic response in the LRFI line, suppressed the
331 transcriptomic response in the HRFI line, or both at the same time. In mice, only a modest
332 transcriptomic response to feed intake was observed by Yoshioka et al. (22), but it might be due to a
333 relatively poor sensitivity of the SAGE method used in the mice study. We observed that several nutrient
334 transporters had their expression level increased or decreased by feed intake in the LRFI line. We
335 notably confirmed in pigs the previous observation of the downregulation of *Slc5a1* in fasted mice (22).
336 *SLC5A1* encodes for the glucose transporter SGLT1. Different glucose absorption dynamics between LRFI
337 and HRFI lines might lead to different insulinemia, as it has been observed in the same pig lines in earlier
338 generations (13, 14). *SLC4A2* was more expressed in fed than in fasted LRFI pigs. It encodes an anion
339 transporter involved in balancing the cellular pH (53). It was also recently identified in a Epigenome
340 Wide Association Study (EWAS): DNA methylation profiles located at *SLC4A2* correlated with food
341 fussiness in children (54). We did not find any DMR near *SLC4A2* in our dataset. More generally, it is
342 tempting to hypothesise that the lack of transcriptomic response in the duodenum between fasted and
343 fed HRFI pigs might explain some feeding behaviours observed in the HRFI line, for example due to their
344 putative reduced nutrient sensing.

345 Genes from the cell-cell junction organisation ontology were over-represented in genes more expressed
346 in the fasted condition in the LRFI line. Cell-cell junction, tight junctions in particular, allow the intestine
347 epithelium to act as a barrier between the intestinal lumen and the rest of the organism (55). It may be
348 that LRFI pigs increase the sealing of tight junctions between meals, but HRFI pigs do not. Additional
349 analyses will be required to test this hypothesis, notably histological analyses of the duodenum mucosa
350 or by measuring duodenal permeability. The pathophysiological consequences of this regulation of tight
351 junction induced by genetic selection for feed efficiency should also be investigated, notably in relation
352 with the susceptibility to enteric infections (56, 57).

353 DNA methylation tends to be absent from active genomic regions (30). Using MeDP-seq, we detected
354 409 differentially methylated regions between LRFI and HRFI lines using conservative filtering steps. It is
355 likely that a finer DNA methylation measure such as whole genome bisulfite conversion followed by
356 sequencing would have allowed the identification of more differentially methylated regions.
357 Nonetheless, it can be concluded that the selection process for feed efficiency has resulted in distinct
358 duodenal DNA methylation profiles. We only detected 26 and 3 differentially methylated regions
359 between fasted and fed pigs of the LRFI and HRFI line, respectively. Thus, while short term feed intake
360 drastically changes the gene expression profiles of the duodenal mucosa, it barely affects the DNA
361 methylation profiles. Therefore, most—if not all—duodenum gene expression changes between fasted
362 and fed samples were not mediated by DNA methylation changes. It is likely that changes in DNA
363 methylation profiles need more time than changes in gene expression profiles. Nonetheless, it appears

364   that for DNA methylation profiles as for gene expressions, the LRFI line is more sensitive than the HRFI
365   line to meal intake.

366   While we could not correlate all the changes in DNA methylation profiles with overall changes in gene
367   expressions, we identify several examples of DMRs located near or within differentially expressed genes
368   (figure 6). This is the case for example for *CLDN10*, involved in tight junctions, *PHDGH*, involved in serine
369   biosynthesis, *ITLN2*, coding for a carbohydrate binding protein, *FER*, a tyrosine kinase regulating cell
370   junctions, *MUC13* (data not shown), a mucin gene, or *KDSR*, involved in the sphingolipid metabolism.
371   The associations between DNA methylation changes and gene expression changes could be either
372   positive or negative, which has been previously reported (58). With this dataset alone, we cannot
373   decipher causalities between the changes in DNA methylations and changes in gene expression levels.
374   Differences between the lines are likely due to genetic effects: selection for feed efficiency have resulted
375   in many genetic differences between the LRFI and HRFI pig lines (11, 59). These genetic differences may
376   alter DNA methylation profiles in cis (i.e. by destroying or creating a transcription factor site) or in trans
377   (i.e. by changing the expression level of a transcription factor or a signaling pathway resulting in
378   downstream DNA methylation changes). We observed no statistically significant difference in gene
379   expression levels between LRFI and HRFI pigs for the DNA methyltransferases *DNMT1*, *DNMT3A*, and
380   *DNMT3B*, nor for the Ten-eleven translocation methylcytosine dioxygenases (TET) *TET1*, *TET2* or *TET3*
381   that are involved in enzymatic DNA demethylation of cytosines (supplemental table 4). We did not
382   detect enough reads assigned to the *DNMT3L* genes to accurately assess its expression levels in our
383   samples. The Ubiquitin-like, containing PHD and RING finger domains, 1 (*UHRF1*) gene was globally more
384   expressed in the HRFI line than in the LRFI line. UHRF1 helps DNMT1 in replicating DNA methylation
385   during DNA replication (60). Further investigations are needed to assess if these changes of UHRF1
386   expression are of relevance. With our dataset alone, we cannot discriminate if the same genetic
387   differences are involved in changes in DNA methylation and gene expressions, or if the differences we
388   observed are simply coincidental. Genome wide association studies for gene expression levels and DNA
389   methylation levels have the potential to improve our understanding of the difference we describe in the
390   LRFI/HRFI pig lines, but would likely require an order of magnitude more samples to have enough
391   statistical power (61). The long non-coding RNA gene *ENSSSG00000044004* has a DMR right at a lowly
392   methylated region on its promoter, and more DNA methylation is associated with a reduction of its
393   expression. For this case, the correlation between DNA methylation changes and gene expression
394   changes likely reflects causality links. This uncharacterised long non-coding RNA lies between the
395   vasopressin and the oxytocin genes, for which the synteny is conserved in most vertebrates (62).

396   The pig is thought to be a good model of human digestive physiology (63–65). Compared to rodent
397   models, pigs have a diet, body size, and diurnal rhythm that resemble more the human ones. The
398   dataset produced here might prove useful to better understand the transcriptomic response to feed
399   intake of the human duodenum. Therefore, our dataset is publicly available, at the sequencing read
400   levels, in the form of gene expression tables, and in lists of differentially expressed genes. In addition,
401   better understanding of the biological mechanisms of feed efficiency may contribute to improvements
402   of feed efficiency in pig farming, leading to a more sustainable production.

403   DATA AVAILABILITY
404   RNA-seq and MeDP-seq reads are available through the ENA database, id [PRJEB46060](#) / ERP130249.
405   Scripts used to process and analyse RNA-seq and MeDP-seq reads are available through a public gitlab

406 repository at the address: forgemia.inra.fr/genepi/analyses/rosepigs. Gene expression counts, lists of
407 differentially expressed genes, sample metadata tables, and DMR positions are available in this
408 repository (forgemia.inra.fr/genepi/analyses/rosepigs/-/tree/master/processed_files).

SUPPLEMENTAL MATERIAL

410 Supplemental material is available at URL:
411 https://entrepot.recherche.data.gouv.fr/dataset.xhtml?persistentId=doi:10.57745/V7FWEH DOI:
412 https://doi.org/10.57745/V7FWEH.

413 **Supplementary table 1:** Sample metadata.

414 **Supplementary table 2:** Gene expression in Transcript per million, used for visualisations.

415 **Supplementary table 3:** Gene expression in pseudo-counts (length-scaled TPM), used differential
416 analysis.

417 **Supplementary table 4:** Results of the differential gene expression analysis.

418 **Supplementary table 5:** Genomic coordinates of the differentially methylated regions between lines.

419 **Supplementary table 6:** Genomic coordinates of the differentially methylated regions between fasted
420 and fed LRFI pigs.

421 **Supplementary table 7:** Genomic coordinates of the differentially methylated regions between fasted
422 and fed HRFI pigs.

423 **Supplementary table 8:** Line DMRs within 20kb of a differentially expressed gene between LRFI and
424 HRFI pigs.

425 **Supplementary table 9:** Fasted vs fed LRFI DMRs within 20kb of a differentially expressed gene
426 between fasted and fed LRFI pigs.

427 **Supplementary figure 1:** Low DNA methylation at the promoter of expressed genes. In each MeDP-seq
428 sample, we sorted genes according to expression levels from their corresponding RNA-seq sample, from
429 top (high expression) to bottom (low expression). Genes were binned in three groups of equal size : light
430 green (high expression), dark green (low to no expression), and purple (no expression). For each gene,
431 DNA methylation ratio is represented in the -2.5kb +2.5kb window surrounding their Transcription Start
432 Site (TSS). Bottom panels display the expression values in each bin of genes, as well as their average DNA
433 methylation ratio around TSS. **A.** LRFI samples in the fasted condition. **B.** LRFI samples in the fed
434 condition (one sample failed during library preparation). **C.** HRFI samples in the fasted condition. **D.** HRFI
435 samples in the fed condition.

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DISCLAIMERS

448 None.

AUTHOR CONTRIBUTIONS

450 Identify which authors participated in the research: Conceived and designed research, performed
451 experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript,
452 edited and revised manuscript, approved final version of manuscript. The information must be the same
453 as in the online submission site.

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FIGURE LEGENDS

Figure 1: Experimental setup. **A.** 24 pigs from two pig lines (12 LRFI and 12 HRFI) were used, distributed in 3 litters of 4 pigs for each line, with two males and two females in each litter (to the exception of litter E). Half of the pigs were fasted while the other half was fed before sampling. **B.** Fasting procedure. Pigs were slotted into two pens with ad libitum feeding. Feed was removed from the two pens overnight, and reintroduced in the morning into one of the pens 2 hours before sampling started in this pen.

Figure 2: Differential gene expression between the duodenum of LRFI and HRFI lines. **A.** 1,106 genes were detected differentially expressed between LRFI and HRFI, including 464 genes upregulated in LRFI

463   (top) and 642 genes upregulated on the HRFI line (bottom). Samples from fasted and fed pigs were
464   analyzed together for each line. **B & C.** Gene Ontology - Biological Process (GO-BP) enrichment analysis.
465   GO-BP terms statistically enriched in the 642 genes upregulated on the HRFI line (**B**) and the 464 genes
466   upregulated in LRFI line (**C**) are displayed as a tree using Jaccard similarity between each pair of GO-BP.

467   **Figure 3:** Duodenum transcriptomic response to feed intake. **A.** Gene expression heatmap of the 2,225
468   genes differentially expressed by feed intake in LRFI (2,222 genes), in HRFI (61 genes, including 58 genes
469   also affected by feed intake in LRFI). **B.** Upset diagram showing the overlap between differentially
470   expressed gene lists, between LRFI and HRFI (green), and due to feed intake in LRFI pigs (blue) or HRFI
471   pigs (red). **C, D & E.** Gene Ontology - Biological Process (GO-BP) enrichment analysis. GO-BP terms
472   statistically enriched in the 1,050 genes upregulated upon fasting in the LRFI line (**C**), the 1,172 genes
473   upregulated in fed pigs from the LRFI line (**D**), and the 41 genes upregulated in fed pigs from the HRFI
474   line (**E**) are displayed as a tree using Jaccard similarity between each pair of GO-BP. **F.** Gene expression
475   heatmap of the 185 genes differentially expressed both by line and by feed intake in LRFI and/or HRFI
476   pigs.

477   **Figure 4:** Gene expression heatmaps of the 59 SLC genes (**A**) or 18 tight junction genes (**B**) that are
478   differentially expressed either between the LRFI and HRFI lines, or by feed intake in each line.
479   Transporter names and known substrates are noted on the right of the heatmap. Three symbolic
480   columns indicate if each gene is significantly differentially expressed (marked with "*") or not (marked
481   with "·") between the two lines (Line column), or by feed intake in the LRFI line (LRFI column), or by feed
482   intake in the HRFI line (HRFI column). Side trees are coloured according to hierarchical clustering, and
483   are only used as visualisation guides.

484   **Figure 5:** Differentially methylated regions in the duodenum between LRFI and HRFI samples. **A.** Upset
485   plot of differentially methylated regions. Green: between LRFI and HRFI samples. Blue: between fasted
486   and fed samples in LRFI pigs. Red: between fasted and fed samples in HRFI pigs **B.** DNA methylation
487   stack profiles at differentially methylated regions between LRFI and HRFI samples. Top: log2 Fold Change
488   (FC) of MeDP-seq normalised coverage at DMR. The 409 LRFI/HRFI DMRs are sorted according to their
489   fold change. Then: each DMR is categorised into a hypermethylated in LRFI bin (hyperM, red) or
490   hypomethyated in LRFI bin (hypoM, blue). Then: Average DNA methylation ratio at ± 2.5kb of DMR
491   centers, in LRFI (blue) or HRFI (red) samples. Bottom: Distribution of fold changes in each bin, then
492   average DNA methylation ratios in each bin (hyperM in LRFI: red, hypoM in LRFI: blue).

493   **Figure 6:** Some differentially methylated regions at proximity differentially expressed genes. **A,C, E, G, I,**
494   **K:** DNA methylation profiles around differentially methylated regions. From top to bottom: gene
495   structure in black. Thick boxes are exons, thin lines are introns, arrows indicate gene direction. Grey box
496   indicates DMR location. Then, average DNA methylation ratio profiles ± 95% confidence interval per line
497   (**A, C, E, G, I,** blue: LRFI, red: HRFI) or per condition in each line (**K**, light blue: LRFI fasted, dark blue: LRFI
498   fed, light red: HRFI fasted, dark red: HRFI fed). Then genome coordinates of the corresponding region. B,
499   **D, F, H, J, L:** Gene expression values in LRFI fasted (light blue), LRFI fed (dark blue), HRFI fasted (light
500   red), HRFI fed (dark red) samples. Unit in transcript per million (TPM). Each dot is a sample, and boxplots
501   are added to ease visualisation. NS: non significant. *: FDR-adjusted p-values < 0.05. **: FDR-adjusted p-
502   values < 0.01. Adjusted p-values are from the differential expression analysis using limma voom.

A

		Line
		Family
		Sex
		Condition

Line
■ LRFI ■ HRFI

Family
■ A ■ B ■ C ■ D ■ E ■ F

Sex
■ F ■ M

Condition
■ fasted ■ fed

B

Ab libitum feeding

Feed removal

Fed

Slaughter and sampling

Fasted

17h   8h   12h

A Samples (24)

Line: HRFI, LRFI

Condition: fasted, fed

Sex: F, M

Family: A, B, C, D, E, F

Genes (1106)

Expression (Z-score)

B GO enrichment in HRFI genes

number of genes: 10, 20, 30, 40, 50

adjusted p value: 0.01, 0.04

endopeptidase inhibitor activity
extracellular region
humoral immune response
antibacterial humoral response
leukocyte activation
immune effector process
regulation of cell development
cell development
regionalization
cyclin-dependent protein serine/threonine kinase regulator...
cyclin-dependent protein kinase holoenzyme complex
regulation of DNA-templated DNA replication

immune response & cell development

DNA-templated DNA replication
cellular response to DNA damage stimulus
DNA metabolic process
ATP-dependent activity, acting on DNA
catalytic activity, acting on DNA
deoxyribonuclease activity

DNA replication & DNA metabolism

microtubule motor activity
microtubule-based movement
microtubule binding
ATP-dependent activity

microtubule

chromosome
chromosome organization
chromosome segregation
nuclear division
mitotic cell cycle
mitotic cell cycle process
cell cycle process
reproductive process
meiotic cell cycle process
supramolecular complex
spindle
cell division
microtubule cytoskeleton organization
microtubule-based process

mitosis

oocyte development
oocyte maturation
regulation of meiotic cell cycle
cellular process involved in reproduction in multicellular...
regulation of reproductive process

meiosis

regulation of mitotic sister chromatid separation
metaphase/anaphase transition of mitotic cell cycle
regulation of mitotic metaphase/anaphase transition
metaphase/anaphase transition of cell cycle
regulation of metaphase/anaphase transition of cell cycle
regulation of chromosome separation
regulation of chromosome segregation
spindle assembly checkpoint signaling
spindle checkpoint signaling
mitotic spindle checkpoint signaling
negative regulation of mitotic nuclear division
negative regulation of nuclear division

cell cycle

C GO enrichment in LRFI genes

exopeptidase activity
metallopeptidase activity
glycosphingolipid metabolic process

peptidase & metabolism

transporter activity
transmembrane transporter activity

transmembrane transporter

A Samples (24)

Line: HRFI, LRFI
Condition: fasted, fed
Sex: F, M
Family: A, B, C, D, E, F

Genes (2225)

Expression (Z-score): 2, 1, 0, -1, -2

Line
- HRFI
- LRFI

Condition
- fasted
- fed

Sex
- F
- M

Family
- A
- B
- C
- D
- E
- F

B

Number of differentially expressed genes

1984, 921, 2, 180, 54, 1, 4

Lines: 1106
Feed Intake in LRFI: 2222
Feed Intake in HRFI: 61

2000 1500 1000 500 0
Number of differentially expressed genes

C GO enrichment in genes overexpressed in fasted LRFI

number of genes: 10 20 30 40 50
adjusted p value: 0.01 0.02 0.03

- phagophore assembly site
- autophagosome
- transferase complex
- multivesicular body sorting pathway
- vacuolar transport
- endosome
- process utilizing autophagic mechanism
- autophagy
- anchoring junction
- cell-cell junction organization
- epithelium development
- plasma membrane signaling receptor complex
- receptor complex
- cell-cell signaling by wnt
- Wnt signaling pathway
- regulation of Wnt signaling pathway
- modification-dependent macromolecule catabolic process
- modification-dependent protein catabolic process
- ubiquitin-dependent protein catabolic process
- proteolysis involved in cellular protein catabolic process
- cellular protein catabolic process
- organonitrogen compound catabolic process
- proteolysis
- protein ubiquitination
- protein modification by small protein conjugation or removal

autophagy & endosome

cell-cell junction

receptor complex

Wnt signaling

catabolic process

D GO enrichment in genes overexpressed in fed LRFI

- ribonucleoprotein complex subunit organization
- ribonucleoprotein complex assembly
- protein-containing complex assembly
- protein folding
- sterol biosynthetic process
- rRNA metabolic process
- rRNA processing
- ribonucleoprotein complex biogenesis

protein synthesis

rRNA

E GO enrichment in genes overexpressed in fasted HRFI

- ubiquitin ligase complex
- cullin-RING ubiquitin ligase complex
- SCF ubiquitin ligase complex
- organelle envelope lumen
- mitochondrial intermembrane space

ubiquitin

mitochondria

F Samples (24)

Genes (185)

A

Samples (24)

	Known substrates	Transporter
* ·	folate	SLC25A32
* ·	Na+, Li+, Ca2+	SLC8B1
* ·	Zn, Fe, Mn, Cd	SLC39A14
* ·	aspartate, glutamate	SLC1A1
· *	Na+, Li+, H+, NH4+	SLC9A2
· *	neutral and basic amino acids	SLC3A1
· *	Gln, His, Ser, Ala, Asn	SLC38A7
* ·	sialic acid, other acidic sugars	SLC17A5
* ·	taurine	SLC6A6
· *		SLC18B1
· *		SLC30A1
· *	prostaglandin	SLCO2A1
· *	copper, cisplatin	SLC31A1
* ·	ADP, ATP	SLC25A5
* ·	cationic and large neutral amino acids	SLC7A1
* ·	glucose and galactose (urea and water)	SLC5A1
* ·	Na+ (H+)	SLC5A4
* ·	mannose, fructose, glucose	SLC5A9
* ·	biotin, pantothenate	SLC5A6
* ·		SLC25A43
* ·	glucose, galactose, fructose, mannose, glucosamine	SLC2A2
* ·	LCFA, VLCFA	SLC27A2
* ·		SLC35F2
* ·	myoinositol, chiro-inositol	SLC5A11
· *	copper, cisplatin	SLC31A2
· *	K+, Cl-	SLC12A6
· *		SLC37A3
· *	aspartate, glutamate	SLC25A13
· *	Mg2+ (Ba2+, Ni2+, Co2+, Fe2+, Mn2+)	SLC41A2
· *	choline	SLC44A1
* *		SLC35E3
* ·		SLC16A14
* ·	Gln, Asn, His, Ser	SLC38A5
* ·		SLC29A1
* ·		SLC43A3
* ·		SLC25A35
* ·		SLC12A8
* ·	glucose, galactose	SLC2A10
* ·	di- and tri-peptides, H+, histidine	SLC15A3
* ·		SLC10A4
* ·	GABA	SLC6A11
· *	large neutral amino acids, T3, T4, L-DOPA, BCH	SLC7A5
· *	Ala, Ser, Cys, Thr, Gln, Asn	SLC1A5
· *	ascorbic acid	SLC23A1
· *		SLC66A2
· *	T3, T4, rT3, estrone-3-sulfate, taurocholate	SLCO4A1
· *	branched chain amino acids, amino alcohols	SLC43A2
· *	glucose, fructose, galactose	SLC2A8
· *	dopamine	SLC6A3
· *		SLC9A3R2
· *	chloride bicarbonate	SLC4A2
· *	malate, phosphate, succinate, sulphate, thiosulphate	SLC25A10
* *	lactate, ketone bodies	SLC16A3
· *		SLC17A8
· *	glutamate	SLC16A6

Line LRFI HRFI
Feeding

B

Samples (24)

	CLDN18
· *	CASK
· *	RAP2A
* ·	PATJ
· *	CXADR
· *	TJP1
* ·	PARD6B
* ·	DLG3
· *	MARVELD2
· *	OCLN
· *	F11R
· *	TJP2
* ·	CLDN2
* ·	CLDN4
* ·	CLDN15
· *	MICALL2
· *	CLDN5
· *	CLDN11
* ·	CLDN10

Line LRFI HRFI
Feeding

Line
HRFI
LRFI

Condition
fasted
fed

Sex
F
M

Family
A
B
C
D
E
F

Expression (Z-score)
2
0
-1
-2