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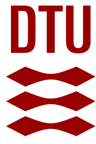

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Growth promotion in pigs by oxytetracycline coincides with down regulation of serum inflammatory parameters and of hibernation-associated protein HP-27

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1 **Growth promotion in pigs by oxytetracycline coincides with down regulation of**
2 **serum inflammatory parameters and of hibernation-associated protein HP-27**
3

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24

25 **List of abbreviations:** α1AG, Alpha 1-acid glycoprotein; AGP, antimicrobial growth

26 promoters; AHSG, α-2-HS-glycoprotein; ApoF, apolipoprotein F, CLU, clusterin; Hp,

27 haptoglobin; FRAP, ferric reducing anti-oxidant power; HP, hibernation associated

28 protein; HP-27, hibernation associated plasma protein; OTC, oxytetracycline; PON1,

29 paraoxonase type-1; SAA, serum amyloid A

30

31 **Keywords:** Proteomics; Immunometabolism; Oxytetracycline; Antimicrobial growth

32 promoters; hibernation-associated plasma protein-27.

33

34 Total number of words including figure and table legends: 5916

35

36 **Abstract**

37 The growth promoting effect of supplementing animal feed with antibiotics like
38 tetracycline has traditionally been attributed to their antibiotic character. However, more
39 evidence has been accumulated on their direct anti-inflammatory effect during the last
40 two decades. Here we used a pig model to explore the systemic molecular effect of feed
41 supplementation with sub therapeutic levels of oxytetracycline (OTC) by analysis of
42 serum proteome changes. Results showed that OTC promoted growth, coinciding with a
43 significant down regulation of different serum proteins related to inflammation,
44 oxidation and lipid metabolism, confirming the anti-inflammatory mechanism of OTC.
45 Interestingly, apart from the classic acute phase reactants also down regulation was seen
46 of a hibernation associated plasma protein (HP-27), which is to our knowledge the first
47 description in pigs. Although the exact function in non-hibernators is unclear, down
48 regulation of HP-27 could be consistent with increased appetite, which is possibly
49 linked to the anti-inflammatory action of OTC. Given that pigs are good models for
50 human medicine due to their genetic and physiologic resemblance, the present results
51 might also be used for rational intervention in human diseases in which inflammation
52 plays an important role such as obesity, type 2 diabetes and cardiovascular diseases.

53

54

55

56 **1 Introduction**

57 Immunometabolism is a relatively new field of study that investigates the interplay
58 between the metabolic state and the immune system. Relatively recently, it was realized
59 that obesity promotes inflammation, predisposing for a host of conditions such as
60 cardiovascular disease [1, 2]. Thought to be important in this process is the up
61 regulation of the metabolic or postprandial inflammation the intensity of which is
62 related to the energy value, glycemic index and lipid profile of food [3, 4]. Similar to
63 humans with unhealthy habits, intensively produced animals such as chicken and pigs
64 ingest large amounts of (high) energy feed, causing a state of inflammation too [5, 6].
65 The latter leads to a suboptimal feed utilization, muscle catabolism, decreased appetite,
66 and consequently diminished growth [4]. Interestingly, the growth retardation in
67 livestock used to be remedied by the use of the nowadays (in the EU) banned
68 antimicrobial growth promoters (AGP) as feed additives. This suggested that they act as
69 anti-inflammatory agents [5], although the conventional theory was that they worked by
70 an antibiotic mechanism [7]. The latter is quite unlikely, mainly because of the low, sub
71 therapeutic concentrations used, and a direct anti-inflammatory mechanism for growth
72 promotion by AGP was proposed [5]. Among the most effective AGP were macrolides,
73 shown to have also in humans direct anti-inflammatory effects on the immune system in
74 lung disease [8]. Cyclines were recently shown to have clear anti-inflammatory effects
75 on macrophages *in vitro* [9, 6], and in mice [10] and broilers [6], which in the latter was
76 paralleled by growth promotion. There are also studies which point at a similar
77 mechanism in pigs for cyclines [11].

78 Pigs are better experimental models for human research than chicken (or mice), due to
79 their genetic, anatomic and physiologic resemblance, in particular concerning the
80 gastrointestinal tract, and therefore more relevant for metabolic studies related to human

81 immunometabolism [12]. Interestingly, antibiotic use in children has recently been
82 associated with increased body weight gain [13].
83 In the present study, we aimed at investigating the mechanism behind growth promotion
84 induced by a cycline in pigs. The serum proteome of pigs supplemented in feed with
85 oxytetracycline (OTC) was compared with that of unsupplemented pigs by 2DE DIGE
86 followed by MALDI-TOF/TOF. In addition to complete serum, fractions of the same
87 sera enriched for less abundant proteins by three different methods were analyzed in the
88 same way. After validation, interpretation of results was performed through a
89 bioinformatics systems biology analysis of the regulated proteins. Results were
90 interpreted together with growth rates and clinical biochemistry parameters to define the
91 systemic effect of OTC supplementation in pigs.
92
93 **2 Materials and methods**
94
95 *Animals*
96 The experimental animal protocol was approved by the KU Leuven Ethics Committee
97 for Animal Experiments (protocol approval number P047/2008). Four week-old weaned
98 piglets (Piètrain x Hypor) were housed in one stable, containing six 2 m^2 pens, slatted
99 floors with bedding, separated by bars, containing five piglets each. Piglets were
100 weighed, and divided over the pens as equally as possible taking litter, weight, and sex
101 into account. Temperature was maintained at 26-27° C, and piglets were kept at a 16 h
102 light, 8 h dark cycle. Commercial starter and piglet meal diets (NV D2000 baby starter
103 and duo starter, Roeselare, Belgium) were offered to the animals from 28 to 47 d and
104 from 48 to 65 d of age, respectively, containing vitamins and minerals meeting or
105 exceeding National Research Council requirements. Feed and water were provided *ad*

106 *libitum* throughout the experiment. The experimental period lasted 37 days in which
107 control piglets (n=15) received only the commercial diet, whereas treated animals
108 (n=15) received a diet containing a commercial in-feed preparation of OTC (courtesy of
109 Huvepharma, Antwerp, Belgium) mixed in their feed with a final concentration of 200
110 ppm OTC. Piglets were weighed at days 0, 32 and 65 of the test period. During the
111 whole experiment the health status of the animals was monitored by visual and physical
112 examination. Two animals from control group and one animal from OTC group were
113 excluded from the study as they showed disease symptoms such as lameness, weight
114 loss and diarrhea. At test day 37, blood was collected by jugular venipuncture, and
115 allowed to clot for 1 hour at room temperature and was then centrifuged at 2000 x *g* for
116 15 min to obtain serum.
117
118 *Acute phase protein determination*
119 Serum levels of haptoglobin (Hp) and serum amyloid A (SAA) were used to determine
120 the general inflammatory status of the studied animals (n=27). Haptoglobin was
121 measured with a colorimetric commercial kit (Tridelta Development Ltd, Kildare,
122 Ireland) according to the manufacturer's instructions, and SAA was determined with a
123 competitive ELISA assay previously developed and validated [14].
124
125 *Sample material for proteomic analysis*
126 Four serum samples of each group were randomly selected from control and OTC-
127 supplemented piglets. All of those specimens were subjected to one-dimensional SDS-
128 PAGE or 2DE DIGE, both in non-fractionated form and after a depletion/enrichment
129 step. The latter methods were used for reduction of major serum protein levels to

130 facilitate uncovering differences in low abundant serum proteins. The following
131 approaches were used:
132
133 *Method 1: Albumin and IgG Depletion:* A commercial kit (ProteaPrep Albumin and IgG
134 Depletion Sample Prep Kit; Protea, Morgantown, WV, USA) was used which employs
135 an non-antibody based ligand-ligand interaction for capture of serum albumin and IgG,
136 thus suitable for its use in non-human species. A volume of 10 µL of serum was
137 subjected to this depletion with the ProteaPrep Kit following the manufacturer's
138 instructions. Besides the fall-through fraction containing the enriched material, also
139 bound proteins were collected by elution with 400 µL 0.1M glycine-HCl pH 2.5 (into
140 tubes containing Tris buffer for pH adjustment), for further testing. Fall-through and
141 bound proteins were kept at -20ºC until analysis.
142
143 *Method 2: Protein enrichment on hexapeptide resin:* the ProteoMiner protein
144 enrichment system (Bio-Rad, Hercules, CA, USA) is based on the use of a
145 combinatorial peptide binding library, which affinity-captures and amplifies the low-
146 abundance proteome [15]. A total amount of 10 mg (approx. 250 µL of crude serum) of
147 protein was treated according to the manufacturer's instructions. Both eluted and bound
148 fractions were kept for analysis. The depleted serum (the initially bound fraction of this
149 approach) was dialyzed against 100 mM ammonium acetate pH 7 in a PlusOne mini
150 1kDa cut-off dialysis kit (GE Healthcare, Munich, Germany), lyophilized and kept at -
151 20ºC. Samples were later dissolved in IPG sample buffer [16] before further analysis.
152
153 *Method 3: Triton X-114 cloud point separation:* This in-house protein depletion method
154 enriches more hydrophobic proteins. It consists of a phase separation with Triton X-114

155 where the detergent rich phase is collected after raising the temperature to above the
156 cloud point. The enriched fraction is then diluted at a temperature below the cloud point
157 and concentrated by a TCA-acetone precipitation step. Pellets were dissolved in
158 appropriate sample buffer and kept at -20ºC until analysis. Starting material for this
159 method was 400 µL of serum [17].
160 The protein concentration of fractions obtained by the above methods was determined
161 according to Bradford [18], and composition further analyzed as described below.
162
163 *Proteome analysis*
164 *One-dimensional electrophoresis*
165 A first screening and comparison of protein patterns of all different samples was
166 achieved by one-dimensional SDS-PAGE under reducing conditions on 140 x 140 x 1.5
167 mm gradient gels (10–15% T, 2.7% C) in a Hoefer SE 600 vertical electrophoresis
168 chamber (Hoefer scientific instruments, San Francisco, CA, USA). For serum samples,
169 0.5 µl serum were applied per lane and protein bands stained with colloidal Coomassie
170 Blue G-250 (Novex, Invitrogen, Carlsbad, CA, USA) [19]. For the less concentrated
171 fractions from depletion/enrichment experiments, 2.5 µg protein per lane were separated
172 in SDS-PAGE and subjected to silver staining [20]. Gels were scanned in a GE
173 Imagescanner III (GE Healthcare, Munich, Germany). All the images were digitalized
174 and analyzed by Image Studio Lite Software (Li-Cor Biosciences, Lincoln, NE, USA).
175
176 *2DE DIGE*
177 Differences in the serum proteome (or their enriched fractions) of control and OTC
178 piglets were evaluated by 2-DE DIGE. Samples were minimally labelled with CyDye
179 DIGE™ fluorescent dyes according to the manufacturer's instructions (GE Healthcare,

180 Munich, Germany). To exclude any effect of possible preferential labelling, dye swap
181 was performed. An IPG-DALT system was employed for 2DE, as described previously
182 [16]. This includes first-dimensional electrophoresis in IPG strips of 10 cm length
183 followed by SDS-PAGE as described above. Fluorescence images of the gels were
184 acquired on a Typhoon 9400 scanner (GE Healthcare, Munich, Germany). Image
185 analysis including spot detection, matching, normalization and quantification was
186 performed in four data sets. Spots of interest were identified after statistical analysis
187 (see below) and were excised from the gel after MS-compatible silver staining [16].
188
189 *MALDI-TOF/TOF analyses*
190 Sample preparation for mass spectrometry was performed as recently described [21].
191 Briefly, spots of interest were washed, destained, reduced and alkylated before in-gel
192 tryptic digestion (TrypsinGold, Mass Spectrometry Grade, Promega, Madison, WI,
193 USA). Extracted dried peptides were desalted and spotted prior to data acquisition on a
194 MALDI-TOF/TOF mass spectrometer (Ultraflex, Bruker Daltonics, Bremen, Germany)
195 in MS and MS/MS mode. Processed spectra were searched via an in-house Mascot
196 server (Matrix Science, Boston, MA) in the SwissProt/UniProt (Release 2014_06) or
197 EST_mammals (EST_120) database using the following search parameters: taxonomy:
198 sus scrofa; enzyme: trypsin/semi-trypsin, global modifications carbamidomethylation on
199 cysteine; variable modifications: Deamidated (NQ), Gln->pyro-Glu (N-term Q),
200 Oxidation (M); MS tolerance 100 ppm; MS/MS tolerance 1 Da; one missed cleavage
201 allowed. Identifications were considered statistically significant where $P < 0.05$.
202
203 *Interpretation of results through systems biology analysis*

204 Regulated proteins were listed as official gene names and subjected to systems biology
205 analysis using the “Set Distiller” module of GeneDecks website [22]. Because far more
206 human genes are annotated and more information in databases is available for humans
207 than for pigs, the human database was used for this functional analysis. Regulated
208 proteins were assigned to a specific pathway and retrieved when significant (P<0.05).
209
210 *Confirmatory methods*
211 Protein regulation results from 2DE DIGE analysis were validated by running the
212 following additional tests, all with unfractionated serum:
213
214 *Serum total IgG determination:* Total IgG was quantified by using a pig-specific ELISA
215 test (USCN Life Sciences, Hubei, China), following the manufacturer’s instructions.
216
217 *Alpha 1-acid glycoprotein (α1AG) measurement*: The relative abundance of α1AG was
218 determined by immunoblotting by using an in-house anti-bovine α1AG rabbit
219 polyclonal antibody [23] with cross-reactivity with pig. Diluted serum samples (1/10) as
220 well as pure bovine α1AGP (1µg) as positive control were separated on 4-12% SDS-
221 PAGE minigels and blotted onto PVDF membrane. α1AG was detected by a
222 biotinylated anti-α1AG polyclonal antibody and an HRP-Streptavidin conjugate (Sigma,
223 Saint-Louis, MO, USA) with the help of a chromogen (4-chloro-1-naphthol) [20].
224 Subsequent total protein staining of the membrane with Coomassie brilliant blue R-250
225 served as a loading control. Both images were evaluated by scanning on a GE
226 Imagescanner III (GE Healthcare, Munich, Germany). All the images were digitalized
227 and analyzed by Image Studio Lite Software (Li-Cor Biosciences, Lincoln, NE, USA).

228 For each lane, α1AG band intensity values were normalized relative to the total protein
229 staining.
230
231 *Biochemical measurement of paraoxonase type-1 (PON1) activity*: PON1 was
232 quantified by determining the serum arylesterase activity using p-nitrophenyl acetate as
233 substrate in an automated clinical chemistry analyzer (Olympus AU2700, Olympus
234 Diagnostica, Tokyo, Japan) using a method adapted for pig serum samples [24]. Intra-
235 assay and inter-assay CV ranges were 0.5% to 1.5% and 4.8% to 5.5%, respectively.
236 Serial dilution of 2 serum samples resulted in linear regression equations with
237 correlation coefficients close to 1 (r = 0.998 and 0.994). The assay detection limit was
238 0.15 U/L.
239
240 *Total serum antioxidant activity*: The total antioxidant capacity of the serum samples
241 studied was determined through the ferric reducing anti-oxidant power (FRAP) assay as
242 described elsewhere [25].
243
244 *Statistical analysis*
245 Statistical analysis of body weight measurements, acute phase protein determination and
246 validation results were performed by using the software program GraphPad Prism 5 for
247 Windows (GraphPad software, La Jolla, CA, USA). Differences in values of each
248 parameter described above were analyzed by two-tailed Student's t-tests, as
249 Kolmogorov-Smirnov test results indicated Gaussian distribution for all datasets. The
250 significance level was set at $P<0.05$ in all cases. DeCyder software Version 7.0 (GE
251 Healthcare, Munich, Germany) was used to identify differentially regulated spots
252 ($P<0.05$; Fold-change ± 1.3) by two-way ANOVA.

253

254 **3 Results**

255 *Effect of OTC supplementation on weight gain and serum levels of acute phase proteins*

256 Supplementation of animal feed with OTC at sub-therapeutic concentrations was

257 associated with significantly higher weight gain of supplemented animals at the end of

258 the experimental period compared to controls (Table 1A). OTC supplementation also

259 resulted in numerically lower levels of serum haptoglobin and significantly lower levels

260 of serum amyloid A (Table 1A).

261

262 *Enrichment of minor proteins*

263 Protein content and 1DE profile of initial serum samples were similar (CV% of protein

264 concentration was 13.05%; Fig.1A). Banding patterns of enriched samples were distinct

265 from serum, but typical for the respective method, and with little difference between

266 individual samples of each group (for Proteominer and ProteaPrep treatment, see Fig.1B

267 and 1C). Not enough material was obtained after Triton X-114 cloud point separation to

268 additionally screen individual samples in SDS-PAGE (Fig.1D). Recoveries of enriched

269 samples ranged between 0.1% of initial protein content for the Triton X-114 cloud point

270 separation to 6.9% for Proteominer and 38% for ProteaPrep depletion systems. No

271 differences in recovery were found between treatment groups.

272

273 *2DE DIGE*

274 Combining results from all 2DE analyses, differences in spot intensity between gels

275 from control and OTC-supplemented animals were identified in eighty-one matched

276 different spots ($P < 0.05$). The Proteominer method yielded only 5 differentially

277 regulated spots, all of which were identified as immunoglobulin heavy chains based on

278 earlier observations. These were not subjected to MS analysis, and left out in the further
279 analysis. For some of the regulated spots the amount of protein was insufficient to
280 confidently identify the present proteins by MALDI-TOF/TOF. In total, thirteen
281 different proteins were identified out of thirty-one spots (Table 2, Fig. 2, and
282 Supplemental Table 1). Among those were two faint spots identified as α-2-HS-
283 glycoprotein (AHSG) which were clearly smaller size breakdown products: in MS
284 analysis for spot #2259 only peptides of the amino-terminal part of the protein were
285 found, for spot #1376 only from the C-terminus (Fig. 2C). Analysis of the spots of the
286 intact AHSG chain (spots #328-331; Fig 2A) showed no differential regulation.
287 Clusterin (CLU) appears as two distinct spot chains in pig serum, but was in the present
288 study too faint for positive MS identification. In the Triton X-114 cloud point separation
289 method 2 CLU containing spots were identified (Fig. 2D), spot #715 showed only
290 peptides from the α-chain, spot #758 only from the β-chain. Both spot chains were
291 regulated in a similar way, but with different degree of significance.
292 From all spots a single protein was identified except spot #484 in which apart from
293 PON1 also peptides from α1AG were found.
294 Together, the two depletion methods identified three additional proteins not detected in
295 whole serum (hibernation associated plasma protein (HP-27), PON1, and apolipoprotein
296 F (ApoF)) as well as additional fragments of AHSG, α1AG, and Hp, with similar trends
297 but not always with the same statistical significance.
298
299 *Systems biology analysis*
300 Proteins confidently identified were listed and analyzed in GeneDecks to detect
301 significantly enriched pathways and GO biological functions (Table 3). Different

302 pathways and GO terms related with the innate immune response, antioxidant activity
303 and lipid metabolism were found highly significant.
304

305 *Additional analysis*

306 Results from the additional analyses performed to validate the proteomics results are
307 detailed in Table 1B.
308 OTC-supplemented pigs showed higher serum PON1 levels and total antioxidant
309 activity as measured by FRAP (not statistically significant), and significantly lower
310 levels of α1AG as detected by western blot. Additional evaluation of PON1 activity has
311 been undertaken because in spot #484 also peptides of α1AG had been found; activity
312 measurement showed numerically higher levels of PON1 in the supplemented group
313 (Table 1B).
314 Serum Hp concentrations in the samples analyzed by 2DE DIGE were significantly
315 lower in OTC-supplemented animals. No differences were found in the total IgG
316 content.
317

318 **4 Discussion**

319 In the present study we aimed at the identification of the systemic immune and
320 metabolic effects of OTC supplementation through the analysis of the pig serum
321 proteome in treated and untreated animals. We used serum as its composition is known
322 to reflect the physiological status of the individual [26], and proteomic analysis because
323 it allows for the analysis of multiple proteins. However, blood-derived samples are
324 difficult to analyze due to their high dynamic range (10-12 orders of magnitude) and
325 because of the presence of a low number of highly abundant proteins [27]. To allow
326 detection of concentration differences in low abundant serum proteins three different

327 enrichment techniques were used. Due to the different underlying principles, the
328 enrichment factor and resulting protein composition were highly dependent on the
329 respective methods. This is well in line with previous reports, even though they partly
330 used other prefractionation protocols [28]. All three methods in our experiments yielded
331 enough protein to perform 2DE DIGE analysis. The Proteominer method yielded only
332 spots of various immunoglobulin (Ig) chains. Similar differences concerning Ig chains
333 are regularly found in many proteomics and transcriptomics studies, with unknown
334 significance. Here, total immunoglobulin levels were not altered, as quantified by
335 immunoassay.

336 Animals treated with OTC showed significant growth promotion and reduction
337 in the acute phase protein SAA concentration compared to the control group. Proteomic
338 analysis showed that growth promotion by OTC was accompanied with serum proteome
339 changes largely consistent with down regulation of the inflammatory response. These
340 responses are seen with the classic acute phase proteins, as well as with the HDL
341 apolipoproteins CLU, ApoF and most likely also with PON1. Whereas the changes in
342 the former two are unambiguous, in the spot of PON1 also peptides of α1AG were
343 found, raising questions about the origin of the up regulation. Because quantification
344 based on MS was not possible, biochemical serum analysis of PON1 activity was
345 performed, and showed a numerical up regulation, whereas α1AG was significantly
346 down regulated in serum by immunological analysis. Furthermore, α1AG was also
347 down regulated in another spot (#515). Based on the above, we are fairly confident that
348 PON1 (and not α1AG) is responsible for the up regulation found.

349 Concerning the acute phase proteins, OTC-supplemented pigs showed down
350 regulated spots of Hp and α1AG. When analyzing all animals of the present experiment
351 (n=27) by colorimetric assay, Hp level decrease in serum was marked, but not

352 statistically significant. However, including only the animals from the 2DE DIGE
353 experiment (n=8), changes determined in this assay were significant, thus confirming
354 our proteomic results. A similar trend was observed when comparing the intensity of the
355 Hp alpha chain protein bands in Figure 1, which further supporting that OTC-treated
356 animals' plasma contained lower levels of Hp. Significantly lower levels of serum
357 α1AG were found in OTC-supplemented pigs by 2DE DIGE, and subsequently
358 confirmed by immuno-blotting. α1AG is in most species a positive acute phase protein.
359 Recent evidences suggest that α1AG is a negative acute phase protein in the pig during
360 infection, whereas it has been observed that obese pigs form certain breeds show
361 significantly higher serum levels of α1AG compared to lean counterparts [29]. In
362 humans, the levels of serum α1AG rise with obesity, which is considered as a sign of
363 overweight-associated low-grade inflammation [30]. Interestingly, levels of this protein
364 have also been identified to be strongly inversely correlated with growth rate in swine in
365 different studies, in the absence of confounding environmental, health and management
366 factors [29,31]. It is therefore possible that different genetic backgrounds and
367 inflammatory stimuli result in different responses of α1AG. In any case, the decrease in
368 α1AG levels in the present study paralleled those of SAA and Hp, and lower levels of
369 positive acute phase proteins are known to positively correlate with daily weight gain
370 and feed efficiency [32]. In contrast to Hp and α1AG, SAA was measurable only by
371 ELISA in serum and could not be identified by 2DE DIGE. This could be due to the
372 unique multimeric nature of pig SAA [33], causing it to disperse over the gel into
373 multiple small and hence undetectable spots. Based on the down regulation of the
374 positive acute phase proteins Hp, α1AG, and SAA up regulation of the negative acute
375 phase protein albumin was expected. No changes were observed in the main spot of
376 albumin either in 1DE or 2DE gels, however, some smaller fragments of albumin were

377 found in lower or higher concentration in OTC-supplemented animals, for which we
378 have no plausible explanation.

379 CLU, PON1 and ApoF are all HDL-related proteins which we found increased
380 in OTC-supplemented piglets. This also fits with an anti-inflammatory response because
381 HDL itself can be considered a negative acute phase reactant. However, this may not be
382 the only explanation, because no other HDL proteins were found to be regulated. CLU
383 (or apolipoprotein J) is a secreted chaperone with anti-apoptotic, antioxidant and anti-
384 inflammatory functions that maintains fluid-epithelial interface homeostasis, thereby
385 preventing the onset of inflammatory conditions [34]. Data relating CLU serum levels
386 during obesity and systemic inflammation are conflicting: some studies claim up-
387 regulation and a correlation with inflammatory markers [35] whereas in others either no
388 clear relationship was found [36] or a down regulation was observed [37, 38]. A
389 decrease in plasma CLU levels during weight reduction has been observed in humans
390 [36] and dogs [39], which has been proposed to be an effect of the hypocaloric diet
391 rather than being directly linked to weight loss [36]. Furthermore, CLU serum
392 concentration is associated with those of other adipokines in relation to insulin
393 sensitivity/hyperglycemia and lipid metabolism, but not to body fat mass and
394 inflammation [40]. Altogether, these data suggest that CLU might be an indicator of the
395 body response towards diet-induced oxidative stress. Indeed, there is recent molecular
396 evidence for the protective role of CLU against high-fat diet-induced insulin resistance
397 through the suppression of oxidative stress and inflammation [41]. The activities of
398 CLU and PON1 are closely related within the HDL particle, being responsible for the
399 protection of low density lipoproteins from oxidative modification [42]. PON1 is a
400 negative acute phase reactant in pigs [24]. PON1 is an antioxidant protein whose main
401 function appears to be the metabolization of toxic oxidized lipids of both low density

402 lipoprotein (LDL) particles as well as HDL particles [43]. Yet, this protein also exerts
403 anti-inflammatory functions, such as directly suppressing macrophage pro-inflammatory
404 responses [44]. Down regulation of PON1 levels is associated with exacerbation of
405 obesity-related inflammation and oxidative stress [45]. We determined the enzymatic
406 activity of PON1 in serum, and the total antioxidant activity, and both were numerically
407 lower in control animals. Although not significant probably due to the low sample size,
408 it suggested a link between inflammation and lipid oxidation, since the exhaustion of the
409 enzymatic activity of PON1 has been related with the systemic low-grade inflammation
410 produced by oxidative stress in coronary heart disease patients [46]. The third up-
411 regulated apolipoprotein, ApoF, is also an atheroprotective protein, and involved in
412 cholesterol transport between lipoproteins, and their levels are positively associated
413 with a favorable proteomic signature for HDL [47].

414 It is concluded that the above findings on the acute phase proteins and HDL
415 apolipoproteins can be correlated in a straight forward manner with OTC-
416 supplementation causing a lower inflammatory state associated with decreased
417 oxidative stress. This is much less straight forward with AHSG and HP-27. AHSG
418 (also known as fetuin-A) is a pleiotropic molecule with metabolic and both anti- and
419 pro-inflammatory roles [48]. Both AHSG under- and over regulation have been
420 described to correlate with inflammation, the first being related with the acute phase
421 reaction [49] and the latter with the development of obesity-related disorders [50].
422 Concerning AHSG, lower levels were found only with of two AHSG breakdown
423 products, a 34 kDa C-terminal fragment, and a 15 kDa N-terminal fragment, but not
424 with intact AHSG. The biological role of these smaller products is unclear.

425 Interestingly, in the OTC-supplemented pigs HP-27 was down regulated.
426 Hibernation-associated plasma proteins (HP) were first identified in chipmunks as a

427 group of proteins produced in the liver with fluctuating serum levels, depending on the
428 season. They appear at high concentration in the brain during hibernation [51].
429 However, they have been also identified in the plasma from non-hibernating mammals
430 such as squirrels [52] and cattle [53, 54]. To our knowledge, this is the first report of the
431 presence of HP proteins in pig serum. The exact function of HP proteins is unknown,
432 but structurally homologous proteins have been shown to contribute to energy
433 homeostasis, immunity, tumor cell apoptosis, and cell survival. It has been therefore
434 proposed that an increase in brain HP proteins during hibernation may protect
435 organisms from depressed metabolism and lethal diseases as observed in hibernators
436 [51]. The molecular mechanisms of the metabolic rate depression occurring during
437 hibernation include the suppression of energy-expensive cell functions, reprioritization
438 of ATP use, and enhanced expression of protective, anti-oxidative mechanisms [55].
439 This is accompanied by a lipid-based metabolism, transient insulin resistance and
440 hyperinsulinemia, but by none of the pathological features of metabolic syndrome [55].
441 In our case, the lower levels of HP-27 in OTC-supplemented animals might be
442 interpreted as indicative of a metabolic switch by OTC to an energy-saving, antioxidant
443 phenotype, similar to what happens in hibernators. Furthermore, pig HP-27 may depress
444 appetite, as has been suggested for bovine HPs injected intracerebroventricularly in
445 mice. Although the latter results have to be interpreted with caution [54], the lower HP-
446 27 serum levels found in the OTC group here could be consistent with the observed
447 increased appetite in OTC treated animals [5, 6]. Pro-inflammatory cytokines are
448 thought to be responsible for (in)appetence, but the present results may be indicative of
449 a new alternate pathway. However, it is entirely unclear whether or not inflammation
450 and HP-27 abundance are linked. Further research should be performed to confirm the
451 precise role of this relatively unknown protein.

In conclusion, in feed OTC supplementation of weaned piglets produced changes in the serum proteome largely consistent with an anti-inflammatory mechanism for OTC. These changes reflected a metabolic change to an energy-saving, growth-promoting phenotype associated with enhanced protection against lipid oxidation. The obtained results support the anti-inflammatory hypothesis as the main mechanism of action of AGP. The latter may also offer a plausible mechanism for the hitherto unexplained growth promotion by antibiotics observed in children [56, 13]. Finally, given that pigs are good models for humans for their great genetic and physiologic resemblance, the present results might also be relevant for human immunometabolic diseases such as obesity, type 2 diabetes and cardiovascular diseases [57].

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576

577 **Table 1. Animal data**

578 A. Weight gain and acute phase protein of pigs.

579 B. Validation of selected factors identified by proteomic analysis by other methods.

580

581 **Table 2. Proteins differentially regulated in OTC-treated pigs' serum and**

582 **identified by MALDI-TOF/TOF mass spectrometry**. Indicated by asterisk (*) is spot

583 #715, included (near significant) to complete the information about clusterin spot #758,

584 as both spots belong to different clusterin chains (α, and β respectively).

585

586 **Table 3.** GeneDecks pathway analysis of proteins differentially regulated in OTC-

587 treated animals.

588

589

590 **Legends to figures**

591

592 **Figure 1. SDS–PAGE protein profiles of serum samples before (A, Coomassie**

593 **staining) and after (B-D, silver-staining) depletion treatments.**

594 (A) Protein pattern of some whole serum samples from control and OTC-treated

595 animals is shown. In B and C the protein profile of fractions (bound and eluted)

596 obtained after Proteominer and ProteaPrep treatment is shown, respectively. Asterisks

597 indicate samples later analyzed by 2DE DIGE. In D the protein profile before (1) and

598 after (2,3) Triton X-114 cloud point separation of two control pig serum samples is

599 shown.

600

601 **Figure 2. 2DE of serum samples and depleted fractions**

602 Representative 2DE gel images of whole serum proteins (A) and after
603 enrichment/depletion treatment with Proteominer (B), ProteaPrep (C) and Triton X-114
604 cloud point separation (D). Protein spots significantly regulated after OTC treatment
605 (see Table 2) are indicated. Italic numbers (A; spots #328-331) represented unregulated
606 intact AHSG chains. Brackets indicate the position of the series of related CLU chains
607 (D).
608
609 **Supplemental table 1:** Peptide table of mass spectrometric protein identification (Carb:
610 Carbamidomethylation on cysteine, Ox: Oxidation on methionine)