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1 # Dietary Proanthocyanidins Exert Localized Immunomodulatory Effects in
2 Porcine Pulmonary and Gastrointestinal Tissues during *Ascaris suum*-induced
3 Type 2 inflammation

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21 **Running title:** Proanthocyanidins and *Ascaris* in pigs

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27 # Abstract

28 Bioactive dietary components may considerably influence intestinal health and resistance to enteric
29 disease. Proanthocyanidins (PAC) are dietary polyphenols with putative health-promoting activity
30 that have been increasingly studied for their anti-inflammatory and immunomodulatory effects.
31 However, whether dietary PAC can regulate type-2 immune function and inflammation at mucosal
32 surfaces remains unclear. Here, we investigated whether diets supplemented with purified PAC
33 modulated pulmonary and intestinal mucosal immune responses during infection with the helminth
34 parasite *Ascaris suum* in pigs. *A. suum* infection induced a type 2-biased immune response in lung
35 and intestinal tissues, characterized by pulmonary granulocytosis, increased Th2/Th1 T cell ratios in
36 tracheal-bronchial lymph nodes, intestinal eosinophilia, and modulation of genes involved in
37 mucosal barrier function and immunity. We observed that PAC had only minor effects on
38 pulmonary immune responses, regardless of concurrent *A. suum* infection. However, RNA-
39 sequencing of intestinal tissues revealed that dietary PAC significantly enhanced transcriptional
40 responses related to immune function, antioxidant responses, and cellular stress activity, both in
41 uninfected and *A. suum*-infected animals. *A. suum* infection and dietary PAC both induced distinct
42 changes in gut microbiota composition, primarily in the jejunum and colon, respectively. Notably,
43 PAC substantially increased *Limosilactobacillus reuteri* abundance in the colon of both naïve and
44 *A. suum*-infected animals. Thus, dietary PAC may have distinct beneficial effects on intestinal
45 health during infection with mucosal pathogens, whilst having limited activity to modulate
46 naturally-induced type-2 pulmonary inflammation. Our results shed further light on the mechanisms
47 underlying the health-promoting properties of PAC-rich foods, and may aid in the design of novel
48 dietary supplements to regulate mucosal inflammatory responses in the gastrointestinal tract.

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53 # Introduction

54 Effective immune function is essential for maintenance of health and tissue homeostasis. The role
55 of diet in regulating immunity and inflammation at mucosal barrier surfaces has been well-
56 established, and immunomodulatory dietary components have therefore gained tremendous
57 attention in scientific research in recent years. Polyphenols, terpenoids, and carotenoids are
58 examples of three central groups of phytonutrients, which have been extensively studied for their
59 beneficial impact on health and disease[1-4]. Proanthocyanidins (PAC) are a type of polyphenol,
60 commonly found in a plant-based diet, which have characteristic chemical structures with known
61 anti-oxidant and anti-inflammatory properties[5].
62
63 Numerous studies have demonstrated that PAC play an important role in the regulation of immune
64 function and may offer therapeutic potential towards inflammatory intestinal diseases. PAC exert
65 strong antioxidant and anti-inflammatory effects in cellular models[6, 7], and are able to modulate
66 various physiological parameters when consumed as part of the diet, such as increasing mucus
67 production in human patients with ulcerative colitis, and alleviating inflammation in TNBS-induced
68 colitis by inhibiting NF-kB signaling pathways[8, 9]. Another consistent outcome of dietary PAC
69 supplementation is changes in gut microbiota (GM) composition, which has been observed in both
70 murine and porcine models, with some evidence also from human studies[10-14]. PAC have been
71 shown to increase the abundance of Lactobacilli and *Bifidobacterium* species, which are commonly
72 associated with a healthy gut environment, as well as increasing levels of faecal short chain fatty
73 acids (SCFA) such as propionic acid[11, 15, 16]. Thus, the immunomodulatory effects of PAC may be
74 caused by direct interactions with immune cells and/or indirect modulation of immune responses as
75 a result of PAC-induced alteration of the GM[17]. These bioactive effects on intestinal immune cells
76 or the GM may have significant implications for inflammation in the gut, as well as at distant sites,
77 such as the lungs, with increasing evidence suggesting a gut-lung axis and clear connection between
78 gut function, the microbiome, and lung homeostasis[18].
79
80 Parasitic worm (helminth) infections are widespread in humans and animals worldwide and cause
81 substantial morbidity[19, 20]. The characteristics of immune responses during helminth infection
82 include a strongly Th2 polarized immune response, characterized by eosinophilia, mastocytosis and
83 increased production of IL-4, IL-13 and other type-2 cytokines[21]. Thus, helminth infection models

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84 offer a valuable opportunity to assess how different dietary interventions can promote resistance to
85 parasitic infection, as well as modulating type-2 responses which play a significant role in
86 pathologies such as allergies. In mice, PAC may regulate allergen-induced type-2 inflammation in
87 the lungs by decreasing the expression of IL-4, IL-5 and IL-13[22]. However, the ability of PAC to
88 modulate pathogen-induced type-2 mucosal immune responses, such as those induced by tissue-
89 invasive helminths, has not been examined in detail. Such studies may shed light on the interactions
90 between PAC-rich diets and immunity to helminth infection, and other type-2 driven pathologies,
91 such as asthma and ulcerative colitis.
92
93 Pigs are a highly translatable large-animal model for humans, due to the anatomical and
94 immunological similarities between humans and swine. The porcine roundworm *Ascaris suum* is
95 widely prevalent in pig farms globally and closely related to *A. lumbricoides,* which is the most
96 common helminth in humans[23]. After infection, *A. suum* larvae have a complex migratory path,
97 which includes migration through the liver and lungs before returning to the small intestine[24]. At
98 each of these anatomical sites, the migratory larvae cause strong inflammatory reactions. Studies in
99 both the natural porcine host and murine models have shown that larvae elicit significant levels of
100 type-2 (e.g. IL-5), but also type-1/17 (IL-6 and TNFα) cytokines as they migrate in the liver, lungs
101 and gut[25, 26]. Furthermore, *A. suum* infection has been shown to increase the susceptibility to
102 bacterial lung infections in both mice and pigs[27, 28].
103
104 The life cycle of *A. suum,* with larvae relocating between the intestines, the liver and the lungs,
105 offers numerous sites of interaction with the immune system. In this respect, *A. suum* infections in
106 pigs have proved useful for assessing the effects of different dietary components on type-2 immune
107 function in both the gut and the respiratory tract. For example, studies in this model have shown
108 that dietary retinoic acid can enhance *A. suum*-induced pulmonary eosinophilia, whilst treatment
109 with probiotics, such as *Lactobacillus rhamnosus* GG, can suppress the prototypical type-2
110 response in lymph nodes draining the lungs during infection[29, 30]. However, studies on the effects of
111 concomitant PAC-supplementation and helminth infection are scant. Here, we explored the effect of
112 dietary PAC on host immune function in *A. suum*-infected pigs. We examined the impact of PAC
113 on systemic immune parameters, inflammatory and immune reactions at the mucosal barrier of both
114 the lung and the intestinal tract, and infection- and dietary-induced changes in the gut microbiota.

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115 Thus, the aim was to investigate how PAC consumption may modulate a naturally-induced type-2
116 mucosal response in multiple tissue sites.
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143 # Material and Methods

144

145 ## Proanthocyanidins and diets

146 The PAC used for this study were from a standardized grape seed extract (Bulk Powders,
147 Denmark). Based on LC-DAD-MS and LC-DAD-MS/MS analyses[31, 32], the PAC purity of the
148 extract was >95%. Further analysis of the PAC showed that they were composed of 99%
149 procyanidin oligomers and polymers, with a mean degree of polymerization of 4.2. The basal diet
150 (NAG, Denmark) was based on ground wheat and barley and was formulated to provide 16.2%
151 crude protein (**Supplementary Table 1**). Pigs received either the basal diet or the basal diet
152 supplemented with 1% PAC. Feed intake was adjusted for body weight throughout the experiment
153 and was calculated to provide the PAC-supplemented pigs with approximately 300 mg PAC/kg
154 BW. All pigs were weighed weekly and they were monitored and fed twice daily at 8:00 in the
155 morning and 15:00 in the afternoon, with access to water *ad libitum*.

156

157 ## Pig experiment

158 9-weeks old pigs were selected from a Specific Pathogen Free (SPF) Danish farm with no history of
159 helminth infection. On arrival, pigs were confirmed free of helminth-infection by fecal egg count
160 and negative by serology for *A. suum*. Pigs were vaccinated (p.o.) against *Lawsonia intracellularis*
161 4.5 weeks prior to the start of the experiment (ENTERISOL® ILEITIS VET., Boehringer
162 Ingelheim). A total 24 pigs (Duroc/Danish Landrace/Yorkshire; 12 castrated males and 12 females)
163 were randomly distributed into four treatment groups that were balanced for sex and initial
164 bodyweight. Bodyweights were recorded weekly (**Supplementary Figure 1**). Each of the four
165 groups were housed in two pens consisting of three pigs each. From day 1 of the experiment, 12
166 pigs were fed the basal diet and 12 the PAC-supplemented diet. At day 14, half the pigs in each
167 group were inoculated with 5000 embryonated *A. suum* eggs by gastric intubation (**Supplementary**
168 **Figure 2**). Pigs were euthanized at day 28 of the experiment, i.e. day 14 post-infection, (p.i.) by
169 captive bolt pistol stunning followed by exsanguination. Throughout the study, weekly blood and
170 fecal samples were taken. Blood was collected by venipuncture of the jugular vein and serum
171 separated and frozen at -80 °C. At necropsy, the entire small intestine was removed and processed
172 for *A. suum* larval counts using a modified agar-gel technique[33]. Worm burdens were assessed by
173 manual enumeration using a dissection microscope of blinded samples conserved in 70% ethanol.
174 Digesta samples were collected from the proximal colon and cooled on ice before transfer to – 80

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175 °C storage. Small pieces (1cm^3) of lung (right cranial lobe) and mid-jejunal tissue were preserved in
176 RNAlater. A further piece of jejunal tissue was also collected for histology using BiopSafe® Biopsy
177 Sample System (Merit Medical). Histology slides were stained with hematoxylin & eosin, and
178 eosinophils were enumerated by blinded microscopy.
179

180 **Broncho-Alveolar Lavage**

181 Broncho-alveolar lavage (BAL) was performed at necropsy by introducing 500 ml PBS into the
182 lungs to recover BAL cells from both lung lobes. The BAL fluid was filtered through 2-layer fine
183 gauze sheets into clean 50 mL centrifuge tubes, and stored at room temperature (RT) until further
184 processing. The recovered cell suspensions underwent a series of washing with HBSS and
185 centrifugation. To remove red blood cell (RBC) contamination the cell suspension was incubated
186 for 5 minutes at RT in RBC lysis buffer (Sigma-Aldrich). Finally, the cells were resuspended in 5
187 mL RPMI-1640 media supplemented with 10% foetal bovine serum and penicillin/streptomycin.
188 Cells were enumerated and either used for flow cytometry (see below) or plated out on 48-well
189 plates at a concentration of 1.2 x 10^5 cells/well and incubated overnight (37 °C, 5% CO_2). The next
190 day, cells were stimulated with either excretory/secretory (E/S) products from either *A. suum* or
191 *Trichuris suis*,
192 or lipopolysaccharide (LPS; 500 ng/mL) for 24 hours. Following stimulation, the supernatant was
193 collected and stored at – 20 °C before further analysis by ELISA.
194

195 **DNA extraction and 16S rRNA gene amplicon sequencing**

196 DNA from small and large intestine samples was extracted using Bead-Beat Micro AX Gravity Kit
197 (A&A Biotechnology, Gdynia, Poland) as per manufacturer's instructions. The DNA purity and
198 concentration were determined by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific,
199 USA) and Qubit™ 1x dsDNA high sensitivity kit on Varioskan Flash (Thermo Fisher Scientific,
200 USA), respectively.
201 A 16S rRNA gene amplicon library was constructed by amplifying the 16S rRNA gene with unique
202 molecular identifier (UMI) containing multiple forward and reverse primers (**Supplementary**
203 **Table 2**). PCR conditions for the amplification were as follows: 95°C for 5 min, 2 cycles of 95°C
204 for 20 s, 48°C for 30 s, 65°C for 10 s, 72°C for 45 s, and a final extension at 72°C for 4 min. A
205 second PCR step was then performed to barcode PCR amplicons with the following conditions:
206 95°C for 2 min followed by 33 cycles of 95°C for 20 s, 55°C for 20 s, 72°C for 40 s, and a final

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207 extension at 72°C for 4 min. After each PCR reaction, PCR amplicons were cleaned up using
208 SpeedBeads™ magnetic carboxylate (obtained from Sigma Aldrich). The size of barcoded PCR
209 products (approximately 1500 bp) was checked by 1.5% agarose gel electrophoresis.
210 A sequencing library from pooled barcoded PCR products were prepared by following the ligation
211 sequencing kit SQK-LSK110 (Oxford Nanopore Technologies, Oxford, UK) protocol. Next,
212 prepared library was sequenced by Oxford Nanopore GridIONX5 sequencing platform as described
213 in manufacturer's protocol (https://nanoporetech.com/products/gridion). Sequencing was run until
214 there was no longer active pores.
215

216 **Data analysis workflow for 16S rRNA gene sequencing**

217 Nanopore sequencing software GridION version 21.02.5 (https://nanoporetech.com) was used for
218 data collection. Base calling and demultiplexing of sequencing data were performed by ONT's
219 Guppy version 4.5.2 (https://nanoporetech.com). Nanofilt version 2.7.1[34] was then used for filtering
220 and trimming of demultiplexed sequences. Briefly, data were filtered on a minimum 1000 and
221 maximum 1600 reads with a minimum average read quality score of 8. After filtering, 15
222 nucleotides were trimmed from start and end of reads. Taxonomy assignment was achieved by
223 using parallel_assign_taxonomy_uclust.py script of Quantitative Insights into Microbial Ecology
224 (Qiime) 1 version 1.8.0[35]. Greengenes database version 13.8 was used as a reference database[36].
225 The reads classifications did not include UMI correction due to low coverage of UMI clusters.
226 Qiime 2 version 2020.6.0[37] was used to set rarefaction depth to 5000 reads per sample. Sample
227 reads below 5000 were removed from the analysis; a total of 42 samples were included for
228 microbiome analysis (n=19 for small intestine samples and n= 23 for large intestine samples).
229 Normalized data were then processed in RStudio version 1.3.1073 using R version 4.0.2 and R
230 packages phyloseq[38], vegan[39], tidyverse[40], ggpubr[41] , reshape2[42] and viridis[43].
231 Raw 16S rRNA sequence data is available at Sequence Read Archive (www.ncbi.nlm.nih.gov/sra/)
232 under accession number PRJNA753018.
233

234 **Measurement of gut microbial metabolites**

235 Short-chain fatty acids and DL-lactic acid were analyzed in colonic digesta samples by GC-MS as
236 previously described[44].

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237 **Flow-cytometry**

238 Tracheal-bronchial lymph nodes were collected from the bifurcature and stored on ice in FBS
239 supplemented RPMI until further processing. Single-cell suspensions were prepared by passing
240 lymph nodes through a 70 µm cell-strainer. Cells were washed and stained with the following
241 antibodies: mouse anti-pig CD3-FITC (clone BB23-8E6-8C8; BD Biosciences); mouse anti-pig
242 CD4-PE-Cy7 (clone 74-12-4; BD Biosciences); mouse anti-human T-bet-APC (clone 4B10;
243 BioLegend); mouse anti-human GATA3-PE (clone TWAJ; Invitrogen). BAL cells were collected as
244 described above, and stained with mouse anti-pig granulocytes-Alexa Fluor647 (clone 2B2; Bio-
245 Rad) and mouse anti-pig CD203a-FITC (clone PM18-7; Bio-Rad). Granulocytes were defined as
246 $2B2^{+}CD203a^{-}$. For all stainings isotype controls were included and gates were set using FMO
247 controls. Data was acquired on an Accuri C6 flow cytometer (BD Biosciences) and analyzed using
248 C6 software.

249

250 **Enzyme-linked immunosorbent assay**

251 IL-1β and TNFα concentrations in alveolar macrophage supernatant and CRP levels in serum were
252 analyzed using commercial ELISA kits (Duosets; R and D systems) according to the manufacturer's
253 instructions. Levels of IgM, IgA and IgG_1 in serum specific for *A. suum* antigen were measured as
254 previously described[45].

255

256 **RNA-sequencing**

257 RNA was extracted from lung and intestinal tissue following homogenization (gentleMACS,
258 Miltenyi Biotech) using miRNAeasy kits (Qiagen) according to the manufacturer's instructions.
259 RNA was subsequently used for library preparation and 150bp paired-end Illumina NovaSeq6000
260 sequencing (Novogene, Cambridge, UK). Sequence data was subsequently mapped to the Sus
261 Scrofa (ss11.1) genome and read counts generated which were used to determine DEG using
262 DEseq2. Pathway analysis was conducted using gene-set enrichment analysis (Broad Institute, MA,
263 USA). RNA sequence data from lung and intestinal tissues are deposited at the NCBI Gene
264 Expression Omnibus (Acession numbers: GSE174042 and GSE168840).

265

266 **Statistical analysis**

267 All statistical analysis was performed using GraphPad Prism 8, IBM SPSS Statistics 27 or R
268 packages. The data were analyzed using a mixed-model analysis, with diet and infection status as

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269 fixed factors and pen and pig as random factors, or with two-way ANOVA and t-tests, as indicated.
270 Where appropriate, time was included as an additional fixed factor to account for repeated
271 measures. One pig (in the *A. suum* + PAC group) was excluded from analysis as it displayed post-
272 mortem pathology indicative of ileitis and aberrant values on several immunological assays.
273 Shapiro-Wilk and Kolmogorov-Smirnov tests were used to tests for assumptions of normality in
274 analyses, and square-root transformations were used to approximate normal distributions when
275 appropriate. For gut microbiota α-diversity analysis, pairwise Wilcoxon Rank Sum Test from the R
276 package was used to obtain Benjamin–Horchberg corrected p-values. Statistical analysis for
277 distance-based redundancy analysis (db-RDA) was done by using permutational ANOVA in the R
278 package vegan. Volcano Plots were created using VolcaNoseR[46], and principal component analysis
279 was carried out using ClustVis[47].
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302

303

304 # Results

305

306 **Proanthocyanidins exert minor effects on systemic antibody and inflammatory biomarkers**

307 Acute infections with *A. suum* induce potent immune reactions in the lungs and intestine before
308 expulsion of the majority of the invading larvae starting from around day 18 p.i.[48]. We fed pigs
309 either a control diet or a diet supplemented with 1% PAC for 14 days, before half the pigs in each
310 group were infected with 5000 embryonated *A. suum* eggs. Mean larval burdens at day 14 p.i. were
311 not altered by PAC supplementation (2914 larvae ± 928 larvae (mean ± SD, $n = 6$) in control-fed
312 pigs and 3155 ± 1057 larvae (mean ± SD, $n = 5$) in PAC fed pigs). In order to examine whether
313 PAC influenced the development of the immune response to *A. suum,* we first examined serological
314 markers of infection in the different treatment groups. *A. suum* infection resulted in a significant
315 increase in serum IgM, IgA, IgG1 specific for *A. suum* antigenic extracts compared to un-infected
316 groups (**Figure 1A-C**). PAC supplementation increased the levels of all three antibody classes in
317 infected pigs, however the effect of diet was not statistically significant. To assess the effect of both
318 *A. suum* and dietary PAC on systemic inflammation, we quantified C-reactive protein (CRP) levels
319 in serum. CRP levels on day 0 p.i. (i.e. after 14 days of PAC supplementation) were significantly
320 lower in PAC fed pigs compared to controls (**Figure 1D**). However, CRP levels measured on day
321 14 p.i. (i.e. after 28 days of PAC supplementation) were no longer affected by diet, and infection
322 had no impact on CRP levels (**Figure 1E**). Thus, whilst dietary PAC appeared to exert transient
323 anti-inflammatory properties in uninfected pigs, PAC had little capacity to alter systemic antibody
324 production induced by infection.

325

326 **Impact of *Ascaris suum* infection and dietary proanthocyanidins on Th1, Th2 and**
327 **granulocytic responses in pulmonary and gut tissues**

328 *A. suum* infection induced significant cellular changes in the BAL fluid and tracheal-bronchial
329 lymph nodes (LN). In the LN, the proportion of $CD3^+$ T cells was significantly decreased when
330 comparing infected pigs to controls, similar to what has been observed in mice[25] (**Figure 2A**). The
331 proportions of $CD3^+CD4^+$, $CD3^+CD4^+$T-bet$^+$ (Th1) and $CD3^+CD4^+$GATA3$^+$ (Th2) T-cells were not
332 significantly different across treatment groups, however Th2/Th1 ratios clearly demonstrated a
333 strong Th2-polarized immune response as a result of *A. suum* infection (**Figure 2B-E**). Moreover,

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334 infection markedly induced granulocytosis in BAL fluid, and eosinophilia in jejunal tissues. PAC
335 did not alter granulocyte numbers in BAL, and whilst intestinal eosinophils were numerically higher
336 in infected pigs fed PAC compared to those fed the control diet this difference was not significant
337 (**Figure 2F-G**). Taken together, these findings indicate that *A. suum* induced a strong type-2 biased
338 cellular response in the lungs and intestinal tissues, which was not significantly altered by
339 concurrent PAC consumption.
340

341 **Concomitant *Ascaris suum* infection and dietary proanthocyanidins tend to enhance cytokine**
342 **secretion in alveolar macrophages stimulated *ex vivo***

343 To further assess the effects of infection and dietary PAC on the profile of lung immune cells,
344 alveolar macrophages were isolated from BAL of all pigs in each treatment group and stimulated *ex*
345 *vivo*. Cells were first stimulated with LPS to assess how diet and infection may influence secretion
346 of the pro-inflammatory cytokines TNFα and IL-1β. Infection status did not significantly influence
347 LPS-induced secretion of these cytokines. Whilst levels of LPS-induced TNFα and IL-1β were
348 lower in uninfected pigs fed PAC, the secretion of these cytokines tended to be higher in infected
349 pigs fed PAC, albeit not significantly so ($p = 0.15$ for interaction between diet and infection status;
350 **Figure 2H**). To explore if *ex vivo* inflammatory responses to parasite antigens were modulated by
351 PAC or infection, macrophages were first stimulated with *A. suum* E/S products, however no
352 cytokine secretion was observed (data not shown). We therefore also stimulated cells with E/S from
353 another porcine helminth, *T. suis,* which we have previously shown to be a stronger activator of
354 innate cytokine production[49]. Interestingly, alveolar macrophages isolated from infected pigs
355 secreted significantly higher levels of TNFα when activated with *T. suis* E/S products (**Figure 2I**).
356 Similarly to LPS-stimulated cells, macrophages isolated from infected pigs fed PAC secreted higher
357 levels of TNFα and IL-1β compared to macrophages isolated from any other treatment groups,
358 albeit not significantly (**Figure 2I**). These findings suggest that *A. suum* infection primed
359 macrophages to be more responsive to stimulation from heterologous parasite antigens (but not
360 LPS), whilst PAC has only minor systemic immuno-stimulatory effects on cytokine production
361 during infection.
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363 **Transcriptional profiling of gut and lung tissues reveals modulatory effects of**
364 **proanthocyanidins during *Ascaris suum* infection**
365 ***Jejunum transcriptional responses***
366 To explore in more detail if PAC may influence the immunological response to *A. suum* infection,
367 we conducted RNA-sequencing of jejunal and lung tissues. Both *A. suum* and PAC treatment
368 strongly modulated gene expression in the intestine as compared to controls (**Figure 3 and 4**).
369 Principal component analysis showed a clear clustering of biological replicates according to
370 infection status (**Figure 3A**). *A. suum* infection significantly downregulated the expression of genes
371 such as the aldehyde dehydrogenase-encoding *ALDH1B1*, and the sodium-channel encoding
372 *SCN8A*. Interestingly, three of the top-ten downregulated genes as a result of infection were related
373 to circadian rhythm (*PER3, PER2, NOCT*) (**Figure 3B-C**). Moreover, *A. suum* infection
374 significantly upregulated the expression of interleukins *IL4, IL9, IL10, IL21,* the eosinophil marker
375 *EPX,* as well as TCR related genes *CD28* and *CD80* in intestinal tissue. Furthermore, we noted
376 strong upregulation of genes involved in aryl hydrocarbon receptor (AHR)-signaling including
377 *ARNTL* as well as smooth muscle contraction (*P2RX1*), which may relate to the increased intestinal
378 motility observed during the immune reaction to *A. suum* larvae[50] (**Figure 3B-C**). Analysis of gene
379 pathways modulated by infection revealed that pathways related to peroxisome function, as well as
380 the metabolism of fatty acids and glycerolipids were significantly suppressed, suggesting a
381 profound modulation of nutrient metabolism due to larval colonization of the intestine (**Figure 3D**).
382 Unsurprisingly, the main up-regulated gene pathways were related to immune function, such as the
383 IL-2, IL-4, and T-cell receptor related pathways, as well as granulocyte and B-cell signaling
384 (**Figure 3D**). Thus, consistent with the pulmonary and intestinal eosinophilia, *A. suum* induced a
385 type-2 inflammatory reaction concomitant with pathophysiological responses related to the changed
386 mucosal environment induced by larval antigens.
387
388 In uninfected pigs, dietary PAC resulted in a distinct clustering of treatment groups based on diet as
389 assessed by principal component analysis (**Figure 4A**). Downregulated genes included the glucose
390 transporter *SLC2A7*, which has previously been shown to be inhibited by polyphenols in cellular
391 models[51], as well as *MT-2B*, encoding a metallothionein protein known to be associated with
392 intestinal inflammation and oxidative stress in mice[52]. Interestingly, we also noted downregulation
393 of *EGFR,* encoding the epidermal growth factor receptor (**Figure 4B-C**). Significantly upregulated
394 genes included *TXNRD1*, encoding thioredoxin reductase 1, a protein involved in suppression of

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395 reactive oxygen species (ROS), as well as genes involved in cellular endocytic processes (*RAB7A*)
396 and extracellular matrix remodeling (*COL6A5*) (**Figure 4C**). Consistent with this, gene pathways
397 related to metabolic processes, such as translation elongation and ribosome function, were
398 significantly enriched (**Figure 4D**). Interestingly, we noted that a number of pathways that were
399 related to immune function (and that were also induced by *A. suum*), were upregulated by PAC.
400 These included pathways related to granulocyte function and B-cell signaling, indicative of an
401 immune-stimulatory effect of PAC. Furthermore, we observed a significant upregulation of
402 pathways related to both detoxification of ROS and selenoamino acid metabolism, suggestive of
403 enhanced antioxidant responses in PAC-fed pigs. Notably, we also observed a strong
404 downregulation of pathways related to heat shock responses, which are normally induced by
405 cellular stressors and offer protection against tissue injury[53] (**Figure 4D**). Collectively, these data
406 suggest that dietary PAC have significant effects on intestinal metabolism and function as a
407 cytoprotective agent in the intestinal mucosa, by inducing antioxidant responses and regulating
408 responses to cellular stressors.
409
410 Given that PAC appeared to induce transcriptional pathways with functions in immunity and
411 inflammation, we next asked whether concurrent PAC consumption could modulate the intestinal
412 transcriptomic response to *A. suum* infection. We observed that within infected pigs, there was once
413 again a clear clustering according to diet based on principal component analysis (**Figure 5A**).
414 Inspection of genes differentially expressed in infected, PAC-fed pigs, relative to infected pigs fed
415 the control diet, revealed that expression of genes involved in intestinal nutrient metabolism were
416 increased, such as *ORAI2* and *AGTR1,* which both play a role in calcium uptake[54, 55]. Consistent
417 with the suppression of *EGRF* expression in uninfected pigs fed PAC, the expression of a number
418 of genes related to EGF signaling, including *BTC* and *AREG,* were downregulated. *AREG* encodes
419 amphiregulin, a cytokine involved in type-2 inflammation induced by a number of different
420 helminth species[56] (**Figure 5B**). In agreement with the data showing an enrichment of antioxidant
421 pathways in uninfected pigs fed PAC, we noted that PAC supplementation during infection also
422 resulted in the upregulation of the oxidative stress pathway, which included significant enrichment
423 of *SOD3, GPX3* and *NQO1* - genes encoding proteins with known anti-oxidant properties (**Figure
424 5C**). Down-regulated pathways in infected pigs fed PAC were mainly related to metabolic activity
425 such as cholesterol metabolism, but these were not significant following FDR adjustment (data not
426 shown). Collectively, these data show that PAC exert a significant influence on the intestinal

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427 transcriptional environment during enteric helminth infection mainly by promoting the transcription
428 of genes involved in regulating oxidative stress and nutrient metabolism.
429

430 ***Lung transcriptional responses***

431 Next, transcriptional profiling of the lungs by RNA-sequencing was performed to investigate the
432 effect of larval migration in the lungs and the potential impact of dietary PAC on gut-lung interplay.
433 In comparison to the intestine, the modulation of gene expression in the lungs was only modestly
434 modulated by both *A. suum* infection and/or PAC supplementation. Interestingly, as was the case in
435 the jejunum, *A. suum* infection regulated the expression of numerous genes related to circadian
436 rhythm. Notably, *PER1, PER2, PER3, NR1D1, NR1D2* and *DBP* were suppressed, whereas *NPAS2,*
437 *ARNTL* were significantly upregulated (**Figure 6A**). A number of studies have touched upon the
438 importance and complex interplay between circadian rhythm, immune regulation and parasite-host
439 interactions[57]. Of note, *ARNTL* was also significantly upregulated by PAC (**Figure 6B**). In
440 coherence with the above described granulocytosis in the lungs in BAL fluid, *A. suum* infection
441 upregulated the expression of *CCR3,* which is essential for eosinophil recruitment. Infected pigs fed
442 PAC had significantly higher expression levels of genes related to innate immune function (*CD209*
443 and *OAS2*), and connective tissue growth factor *(CTGF)* in lung tissues compared to infected pig
444 fed a control diet (**Figure 6C**). CTGF is involved in wound repair and tissue healing, suggesting a
445 protective effect of PAC during *A. suum* infection. Intriguingly, the expression of the oxidative
446 stress inducer *ALOX15* was significantly increased by *A. suum* infection, but was significantly
447 down-regulated in infected pigs fed a PAC diet, which supports previously described reports of
448 PAC acting as a lipoxygenase inhibitor[58] (**Figure 6A and C**). Thus, *A. suum* infection induced
449 marked transcriptional responses in the lungs but somewhat less than compared to intestinal tissues,
450 which may indicate that lung homeostasis is somewhat restored by day 14 p.i. when the migrating
451 larvae have returned to the intestine. Furthermore, dietary PAC induced smaller transcriptional
452 changes in the lung compared to the intestine but may ameliorate wound healing and antioxidant
453 status during infection.
454

455 ***Ascaris suum* infection and proanthocyanidins alter gut microbiota composition with limited**
456 **effect on short chain fatty acids**

457 Previous studies have indicated that immunomodulatory and anti-inflammatory effects of PAC may
458 derive from changes in the GM and associated metabolite production[17]. Furthermore, *A. suum* and

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459 other helminths can markedly change host GM composition[59]. Therefore, to explore whether the
460 observed transcriptomic changes induced by diet and infection were accompanied by GM changes,
461 we used 16S rRNA gene amplicon sequencing to characterize both the small and large intestinal
462 GM composition. We initially analyzed the GM composition in the jejunum, at the main site of
463 *Ascaris* infection. Neither *A. suum* nor dietary PAC altered α-diversity (data not shown). Changes in
464 β-diversity were apparent primarily as a result of *A. suum* infection ($p < 0.05$ by distance-based
465 redundancy analysis; **Figure 7A**), with differential abundance analysis on genus level indicating an
466 enrichment in *Lactobacillus* spp. in infected pigs (**Figure 7B**). Moreover, *A. suum* infection
467 decreased the abundance of *Facklamia* spp. ($p = 0.053$ by mixed model analysis; **Figure 7C**). In
468 contrast, PAC did not have a significant effect on the small intestinal GM, with no changes in β-
469 diversity between PAC-fed pigs and control pigs ($p > 0.05$ by distance-based redundancy analysis;
470 **Figure 7A**). However, we did note that, within *A. suum*-infected pigs, those animals fed PAC
471 tended to have a higher abundance of amplicon sequences corresponding to *Limosilactobacillus*
472 *reuteri* (**Figure 7D**). *L. reuteri* has been associated with beneficial probiotic and anti-inflammatory
473 effects, and plays a role in the prevention of microbial translocation and inhibits colonization of
474 pathogenic bacteria[60, 61].
475
476 In the colon, we found that PAC had the largest effect on the GM composition, consistent with the
477 notion that PAC are extensively metabolized by, and can modulate, the large intestine microbiome
478 ($p < 0.05$ for β-diversity comparison between PAC and control group by distance-based redundancy
479 analysis; **Figure 8A- B**). PAC tended to decrease the abundance of *Bifidobacterium* in both naïve
480 and infected pigs (**Figure 8C**). Notably, the abundance of sequences closely related to
481 *Bifidobacterium thermacidophilum* was significantly increased by *A. suum,* but concomitant PAC
482 supplementation significantly suppressed this effect (**Figure 8D**). The reduction of *Bifidobacterium*
483 in pigs fed PAC contrasts to a previous study in pigs showing that PAC increased the growth of this
484 taxa[15]. However, similar to the trend in the small intestine, PAC supplementation resulted in the
485 significant increase of *L. reuteri* abundance in the colon of both naïve and infected pigs (**Figure**
486 **8E**). Interestingly, *A. suum* infection increased the abundance of *Lactobacillus* spp. in the colon
487 whilst significantly decreasing the abundance of *Turicibacter* spp. (**Figure 8F and G**). However, β-
488 diversity was not different between *A. suum* and control groups in colon, indicating that the effects
489 of infection on GM composition were mostly limited to the predilection site (the small intestine).
490 Finally, we investigated if the colonic GM changes were accompanied by changes in the

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491 concentrations of SCFA in the distal colon. Neither PAC nor *A. suum* infection altered levels of
492 acetic acid, propionic acid, n-butyric acid or D-lactic acid (**Supplementary Figure 3**). However,
493 we observed that dietary PAC decreased the concentrations of the branched-chain fatty acids iso-
494 valeric acid ($p < 0.05$) and iso-butyric acid ($p = 0.0616$), which may relate to altered protein
495 metabolism or colonic transit time[62], and is consistent with our previous work on pigs fed a
496 polyphenol-enriched diet[63] (**Figure S1**). Taken together, these results indicate distinct effects of *A.*
497 *suum* infection and PAC on specific bacteria taxa in a site-specific manner.

498

499

500 # Discussion

501

502 The immuno-modulatory effects of PAC have been investigated in numerous studies but their mode
503 of action and impact on immune function is still not fully understood. Furthermore, only limited
504 knowledge has been attained on the effects of PAC on type-2 immune response, which plays a
505 central role during helminth infections and may be relevant for inflammatory disorders, such as
506 food allergies and ulcerative colitis. Therefore, we used here a model of *A. suum* infection in pigs,
507 which offers a unique opportunity to explore the modulation of parasite-induced inflammation in
508 multiple tissues by dietary components.

509

510 Initial assessment of the systemic effects of PAC and *Ascaris* infection were demonstrated by
511 monitoring serum antibody levels and the acute-phase protein CRP, a marker for systemic
512 inflammation. *A. suum* infection resulted in a significant increase in serum antibodies, which were
513 further enhanced by dietary PAC, albeit not significantly. Interestingly, significantly lower CRP
514 levels were observed after 14 days of PAC supplementation, although this effect subsided by the
515 end of the study. Thus, PAC had limited effects on parasite-induced antibody levels, and prolonged
516 PAC supplementation did not appear to persistently alter inflammatory markers in serum.

517

518 The gut-lung axis is gaining increasing interest in numerous research fields, and the migratory
519 characteristics of *A. suum* render the investigation of gut-lung interplay greatly relevant in this
520 model. Here, we showed that *A. suum* infection induced granulocytosis in the lungs, and a Th2
521 polarized immune response was clearly demonstrated by Th2/Th1 T-cell ratios. PAC and *A. suum* in

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522 isolation, upregulated a number of similar genes, notably genes related to the circadian rhythm,
523 such as *ARNTL*. Interestingly, a recent study showed that *ARNTL,* also known as *BMLA1,* was
524 fundamental for the time-of-the-day dependent expulsion efficiency of *Trichuris muris* infection in
525 mice[64]. Furthermore, a study conducted in pigs also demonstrated an association between *ARNTL*
526 and adult worm burden[65]. However, in contrast to murine studies, which have demonstrated a role
527 for PAC in suppressing allergic responses in the lungs, we did not find a modulatory effect of PAC
528 on the type-2 cellular response to *A. suum* infection. *Ex vivo* stimulation of lung macrophages by
529 LPS or helminth antigens indicated a tendency of higher cytokine secretion levels in macrophages
530 isolated from infected pigs fed PAC. Moreover, although we observed transcriptional changes in the
531 lungs of infected pigs that are reflective of type-2 inflammation, these did not appear to be
532 markedly altered by concurrent PAC intake. The exception was an indication of regulation of
533 several genes such as *CTGF,* and *ALOX15,* which could suggest that PAC may augment wound-
534 healing and anti-oxidant status in lung tissues during *A. suum* infection. Thus, in our model, dietary
535 PAC had limited capacity to regulate lung immune function during helminth infection, although
536 further studies to elucidate whether PAC may potentiate protection towards secondary airway
537 infection during *A. suum* infection may be relevant.
538
539 We next assessed the impact of *A. suum* infection and PAC at the predilection site of infection, the
540 small intestine. *A. suum* infection induced stereotypical intestinal eosinophilia, which was
541 equivalent in both dietary groups. We had previously shown that eosinophilia in the jejunum of *A.*
542 *suum*-infected pigs could be potentiated by a polyphenol-enriched diet containing 5 % grape
543 pomace[63]. Grape pomace may contain several phytonutrients and fibrous components such as
544 lignin, which could contribute to synergistic effects, whereas the PAC diet in the present study was
545 composed only of purified PAC oligomers from grape seed extract. This may explain the
546 discrepancy between these results. Transcriptomic analysis of intestinal tissues revealed that a
547 number of genes and pathways were regulated by both infection and PAC supplementation. As
548 expected, *A. suum* induced the upregulation of type 2 immune related genes and pathways, as well
549 as having an important impact on nutrient metabolism-related genes. Notably, PAC and *A. suum* in
550 isolation were both able to modulate transcriptional pathways related to immune function and
551 antioxidant activity. Interestingly, PAC increased the expression of protein-encoding genes with
552 cytoprotective functions against oxidative stress, suggesting a role in improving gut heath by
553 minimizing cellular stress during inflammation. The antioxidant effect of PAC could be caused by

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554 the absorption of PAC-derived metabolites, produced as a result of microbial metabolism. Although
555 PAC are known to remain relatively stable until they reach the large intestines, PAC molecules with
556 low mDP may also be absorbed in the small intestines[66, 67]. Furthermore, PAC and their metabolites
557 may exert direct interactions with the gut mucosa and thus epithelial cells, as described in numerous
558 cell-based studies. PAC may intervene as scavengers of free radicals due to the hydroxyl groups
559 present in their molecular structures, which can neutralize free radicals via electron delocalization[5].
560 Another mechanism of the protective effects of PAC, may be via the induction of cellular
561 antioxidant defenses by modulating Nuclear factor erythroid 2-related factor 2 (Nrf2)-related genes,
562 which play an important role in regulating cellular resistance to oxidants, such as ROS[68].
563
564 The localized effect of PAC and infection in the intestines was also demonstrated by their impact on
565 the GM. *A. suum* infection caused substantial changes in the GM composition, most notably in the
566 small intestine. This is the first report of alterations in the GM by *Ascaris* in the predilection site of
567 the jejunum, and we found a significantly decreased abundance of *Facklamia* spp. Furthermore, we
568 noted that *A. suum* increased the abundance of lactobacilli in the colon. Consistent with this, an
569 increased abundance of lactobacilli has also been associated with *Heligmosomoides polygyrus*
570 infection in mice[69, 70]. This may potentially result from the increased mucus secretion that is a
571 stereotypical feature of helminth infections which may provide a niche environment for lactobacilli
572 to thrive[71]. Interestingly, the abundance of *L. reuteri* was significantly increased by PAC
573 supplementation in both naïve and infected pigs, suggesting a prebiotic effect, which may have
574 functional implications, given the known role of *L. reuteri* in modifying inflammation. However,
575 PAC also significantly decreased the abundance of *Bifidobacterium* spp., including *B.*
576 *thermacidophilum,* suggesting a complex regulation of the GM. Notably, the suppressive effect of
577 PAC on *Bifidobacterium* spp. stands in contrast to a previous study showing the opposite effect in
578 pigs fed PAC derived from cocoa[15]. These apparently contradictory findings may potentially be
579 explained by the differing molecular structures of PAC derived from different sources, as well as
580 potential interactions with differing basal diets. Given that PAC appeared to change the GM
581 composition, a key question is whether the immunomodulatory effects of PAC in the intestine
582 derive from direct interactions with PAC and mucosal immune cells during intestinal transit, or
583 whether PAC-derived microbial metabolites are absorbed and exert systemic bioactivity, as has
584 been proposed in previous studies[11, 15]. Given that PAC-related transcriptional changes we observed
585 were localized mainly to the gut, and not the lung, this may support a hypothesis that the activities

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586 were derived from direct interactions between PAC and cells at the level of the gut mucosa,
587 consistent with a lack of an effect of PAC on SCFA levels. However, further studies are clearly
588 needed to unravel these mechanistic aspects.

589

590 In conclusion, pigs infected with *A. suum* offered a robust model to study the effect of PAC on
591 pathogens that induce a strong, type-2 biased mucosal immune response in pulmonary and intestinal
592 tissues. Both *A. suum* infection and PAC in isolation had similar immunomodulatory capacity,
593 notably by modulating gene pathways related to B-cell function. PAC also affected transcriptional
594 pathways related to oxidative stress by significantly increasing the expression levels of protein-
595 encoding genes with cytoprotective properties. However, the canonical markers of type-2
596 inflammation, such as eosinophilia and Th2 T-helper cells in the lungs, were not modulated by PAC
597 intake. The limited effects of dietary PAC observed in the lungs is in coherence with a previous
598 study demonstrating no effect of PAC on gene expression levels of various immune-related genes in
599 alveolar macrophages and tracheobronchial lymph nodes isolated from *A. suum* infected pigs, which
600 were dosed with PAC derived from cocoa[29]. Thus, in contrast to some murine studies suggesting
601 beneficial effects of dietary PAC on asthma, our results suggest a restricted ability of PAC to
602 influence the development of Th2 responses in the respiratory tract in pigs. However, the significant
603 modulatory effects of PAC on porcine intestinal gene expression suggest a primarily gut-localized
604 effect of PAC. Thus, PAC may play a role in maintaining gut health during enteric infection in pigs
605 and humans, and further studies to address the functional implications of this diet-infection
606 interaction are highly warranted.

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630

631 Conflict of interest

632 The authors declare no conflicts of interests regarding this study.

633

634 Ethical statement

635 All experiments involving animals were conducted in agreement with the Danish legislation and the
636 Danish Animal Experiments Inspectorate with the license number 2015-15-0201-0076.

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654 # Figure Legends

655

656 **Figure 1 - Dietary proanthocyanidins exert limited effects on systemic antibody levels and**
657 **inflammatory biomarkers**
658 Serum levels of **A)** IgM, **B)** IgG1 and **C)** IgA specific for *Ascaris suum* antigen on day -14 pre-
659 infection (i.e. day of arrival) and day 14 post-infection (p.i.) with *A. suum*. **D)** C-reactive protein
660 (CRP) levels at day 0 p.i. (i.e. after 14 days of proanthocyanidin (PAC) supplementation) and **E)**
661 day 14 p.i. (**Mixed model analysis or t test**, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 6$ pigs per
662 group, except $n = 5$ pigs in PAC+*A. suum* group, and n=12 pigs per group in Panel D prior to *A.*
663 *suum* infection).
664
665
666 **Figure 2 - Effects of *Ascaris suum* infection and dietary proanthocyanidins on cellular**
667 **responses in the lungs and intestine**
668 **A-E)** Proportions of $CD3^+$ cells, $CD4^+$ T cells, T-bet$^+$ Th1 cells , GATA3$^+$ Th2 cells , and Th1/Th2
669 ratios in lung lymph nodes (LN) on day 14 post-infection (p.i.). **F)** Lung granulocytosis in broncho-
670 alveolar lavage (BAL) fluid at day 14 p.i. **G)** Eosinophils in mid-jejunum tissues at day 14 p.i.**)**
671 TNFα and IL-1β secretion *ex vivo* in alveolar macrophages stimulated with lipopolysaccharide
672 (LPS) (**H**) or *Trichuris suis* antigens **(I).** PAC: proanthocyanidins (**Mixed model analysis,**
673 *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 5\text{-}6$ pigs per group).
674

675 **Figure 3 - Modulation of gene expression and transcriptional pathways in intestinal tissue by**
676 ***Ascaris suum* infection**
677 **A)** Clustering of *A.suum*-infected and control groups as demonstrated by principal component
678 analysis. **B)** Volcano plot showing differentially expressed genes resulting from *A. suum* infection

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679 **C)** Top ten up- and down-regulated genes identified as a result of *A. suum* infection. ($n = 6$ pigs per
680 group). **D)** Significantly up- and down-regulated pathways ($p < 0.01$; $Q < 0.1$) identified by gene-
681 set enrichment analysis as a result of *A. suum* infection.
682
683
684
685 **Figure 4 - Modulation of gene expression and transcriptional pathways in intestinal tissue by**
686 **dietary proanthocyanidins**
687 **A)** Clustering of the two dietary groups as demonstrated by principal component analysis in
688 uninfected pigs. **B)** Volcano plot showing differentially expressed genes resulting from dietary
689 proanthocyanidin (PAC) supplementation. **C)** Top ten up- and down-regulated genes identified as a
690 result of dietary PAC supplementation in uninfected pigs. ($n = 6$ pigs per group). **D)** Significantly
691 up- and down-regulated pathways ($p < 0.01$; $Q < 0.1$) identified by gene-set enrichment analysis
692 (GSEA) as a result of dietary PAC supplementation. Highlighted are the HSF1 Dependent
693 Transactivation and Detoxification of ROS pathways, showing enriched genes as identified by
694 GSEA analysis.
695
696 **Figure 5 - Modulation of gene expression and transcriptional pathways in intestinal tissue by**
697 **dietary proanthocyanidin supplementation in *Ascaris suum*-infected pigs**
698 **A)** Clustering of the dietary groups within *Ascaris suum*-infected pigs as a result of dietary
699 proanthocyanidins (PAC) as demonstrated by principal component analysis. **B)** Volcano plot
700 showing differentially expressed genes resulting from dietary PAC supplementation in *A. suum*-
701 infected pigs. **C)** Top ten up- and down-regulated genes identified as a result of dietary PAC
702 supplementation in *A. suum*-infected pigs. ($n = 6$ pigs in *A. suum* group, $n = 5$ pigs in PAC+*A. suum*
703 group).
704
705 **Figure 6 - Modulation of gene expression in lung tissue by *Ascaris suum* infection and dietary**
706 **proanthocyanidins**
707 Effects on lung gene expression as shown by principal component analysis, volcano plot of
708 differentially expressed genes and top ten up- and down-regulated genes identified as a result of **A)**
709 *Ascaris suum* infection in pigs fed the control diet, **B)** dietary proanthocyanidins (PAC) in naïve

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710 pigs and **C)** dietary PAC in *A. suum*-infected pigs. (*n* = 6 pigs per group, except *n* = 5 pigs in
711 PAC+*A. suum* group).
712
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716 **Figure 7 - Changes in gut microbiota composition in the small intestine due to dietary**
717 **proanthocyanidins and *Ascaris suum* infection**
718 **A)** Changes in β-diversity in the small intestine, as identified by distance-based redundancy analysis
719 where a significant effect of *Ascaris suum* compared to all other treatment groups was identified.
720 No effect on β-diversity was reported when comparing PAC to control-fed naïve pigs **B)** Relative
721 abundance at genus level in naive or *A. suum*-infected pigs fed a control diet or PAC-supplemented
722 diet. Relative abundance of **C)** *Facklamia* spp. and **D)** *Limosilactobacillus reuteri* in naive or *A.*
723 *suum*-infected pigs fed a control diet or PAC-supplemented diet, as identified by differential
724 abundance analysis and mixed-model analysis**.** (*n* = 3 pigs in control group, *n* = 6 pigs in *A. suum*
725 group, *n* = 6 pigs in PAC group, and *n* = 5 pigs in PAC+*A. suum* group).
726
727 **Figure 8 - Changes in gut microbiota composition in the proximal colon due to dietary**
728 **proanthocyanidins and *Ascaris suum* infection**
729 **A)** Changes in β-diversity in the proximal colon as identified by distance based redundancy
730 analysis, where an effect of proanthocyanidins (PAC) compared to control was identified.. **B)**
731 Relative abundance at genus level in naive or *Ascaris suum*-infected pigs fed a control diet or PAC-
732 supplemented diet. Relative abundance of **C)** *Bifidobacteria* spp., **D)** *B. thermacidophilum*, **E)**
733 *Limosilactobacillus reuteri,* **F)** *Lactobacillus* spp. and **G)** *Turicibacter* spp. in naive or *A. suum*-
734 infected pigs fed a control diet or PAC-supplemented diet, as identified by differential abundance
735 analysis and mixed-model analysis (**$^*p < 0.05$).** (*n* = 6 pigs per group, except *n* = 5 pigs in PAC+*A.*
736 *suum* group).
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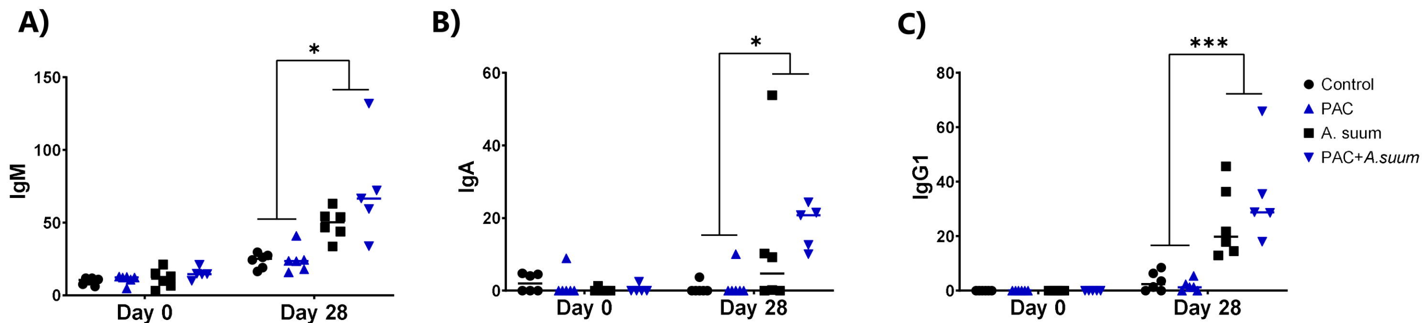
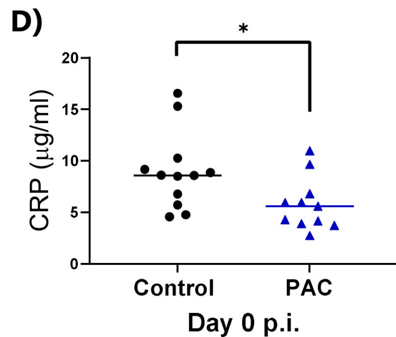
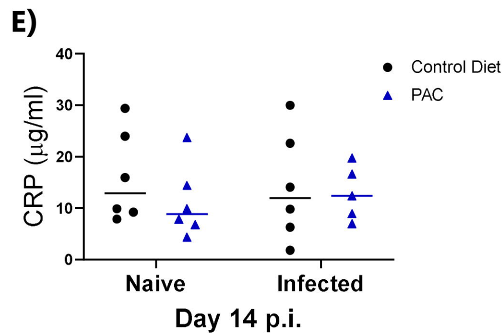
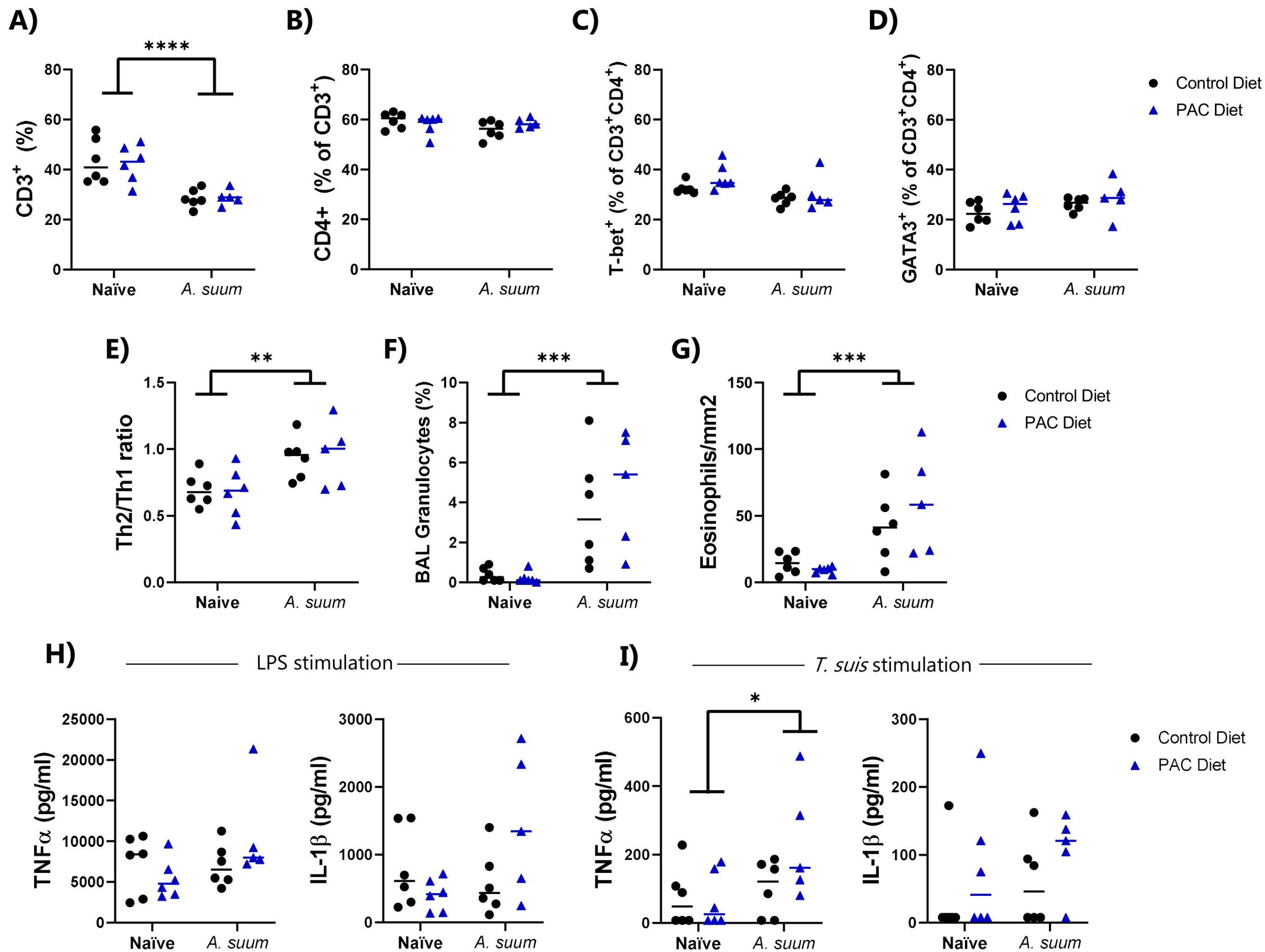

A)
B)
C)
IgM
IgA
IgG1
Day 0
Day 28
Control
PAC
A. suum
PAC+A.suum


D)
CRP (µg/ml)
Control
PAC
Day 0 p.i.


E)
CRP (µg/ml)
Control Diet
PAC
Naive
Infected
Day 14 p.i.


A)
B)
C)
D)
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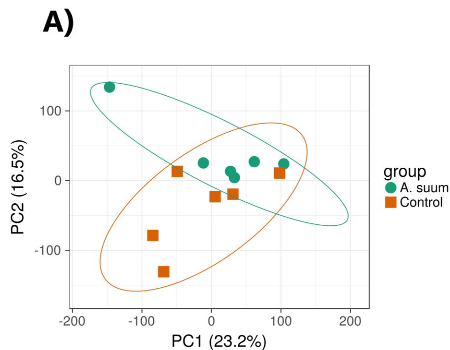
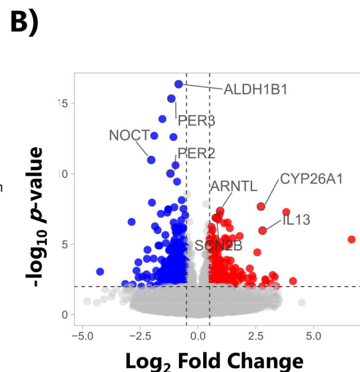
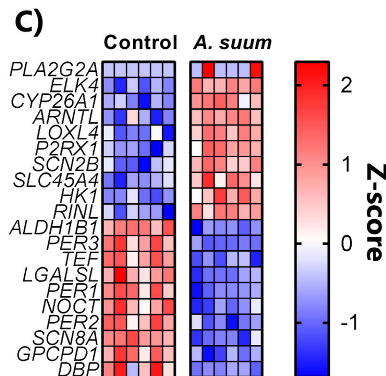
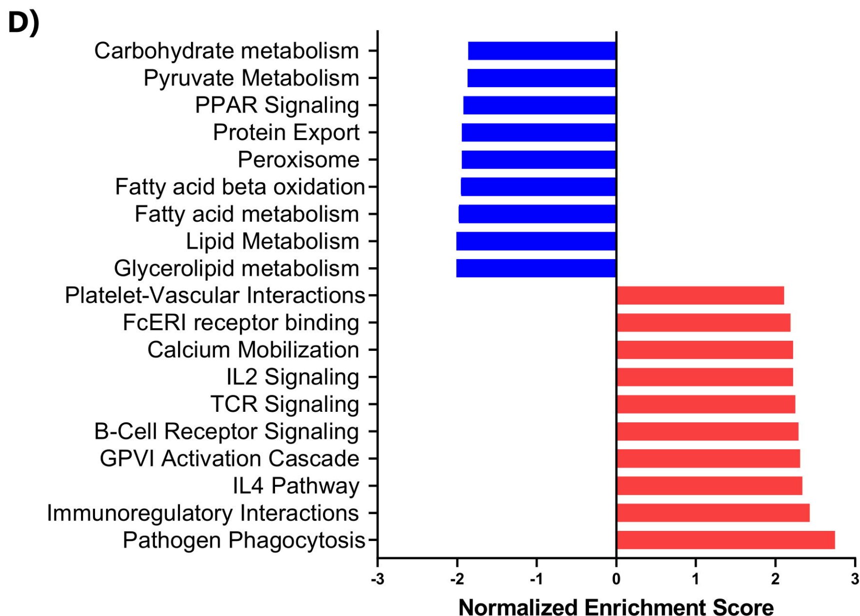
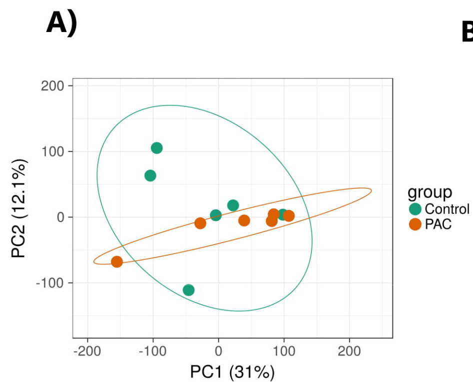
F)
G)
H)
I)
CD3+ (%)
CD4+ (% of CD3+)
T-bet+ (% of CD3+CD4+)
GATA3+ (% of CD3+CD4+)
Th2/Th1 ratio
BAL Granulocytes (%)
Eosinophils/mm2
LPS stimulation
T. suis stimulation
TNFα (pg/ml)
IL-1β (pg/ml)
Naïve
Naive
A. suum
Control Diet
PAC Diet
****
**
***
*

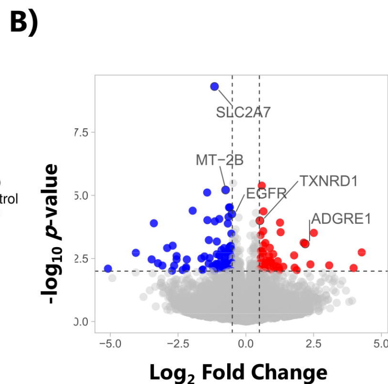

A)
group
A. suum
Control
PC1 (23.2%)
PC2 (16.5%)

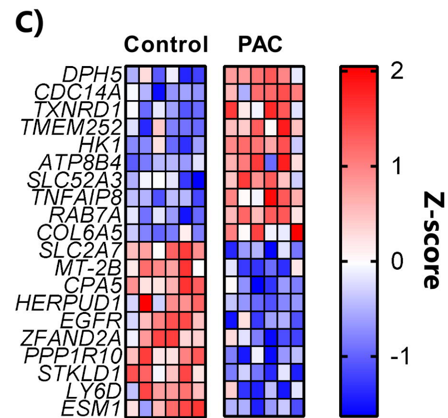

B)
ALDH1B1
PER3
NOCT
PER2
ARNTL
CYP26A1
IL13
SCN2B
-log10 p-value
Log2 Fold Change


C)
Control
A. suum
PLA2G2A
ELK4
CYP26A1
ARNTL
LOXL4
P2RX1
SCN2B
SLC45A4
HK1
RINL
ALDH1B1
PER3
TEF
LGALSL
PER1
NOCT
PER2
SCN8A
GPCPD1
DBP
Z-score

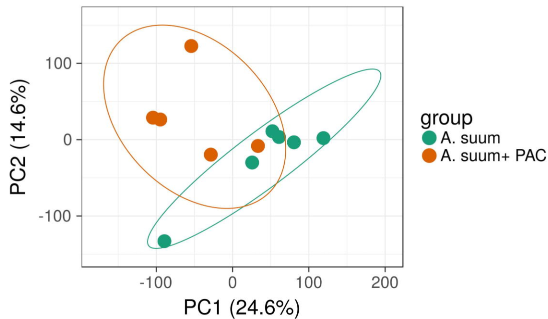

D)
Carbohydrate metabolism
Pyruvate Metabolism
PPAR Signaling
Protein Export
Peroxisome
Fatty acid beta oxidation
Fatty acid metabolism
Lipid Metabolism
Glycerolipid metabolism
Platelet-Vascular Interactions
FcERI receptor binding
Calcium Mobilization
IL2 Signaling
TCR Signaling
B-Cell Receptor Signaling
GPVI Activation Cascade
IL4 Pathway
Immunoregulatory Interactions
Pathogen Phagocytosis
Normalized Enrichment Score

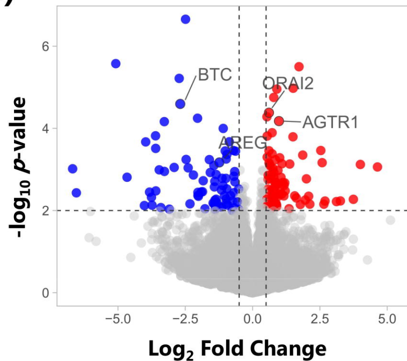

A)
PC2 (12.1%)
PC1 (31%)
group
Control
PAC

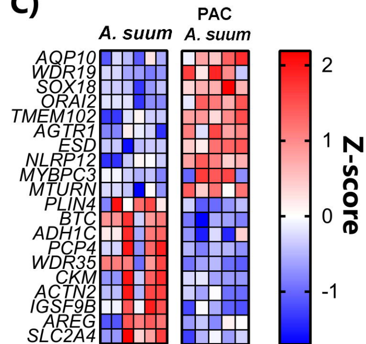

B)
SLC2A7
MT−2B
EGFR
TXNRD1
ADGRE1
$-\log_{10}$ p-value
Log_2 Fold Change

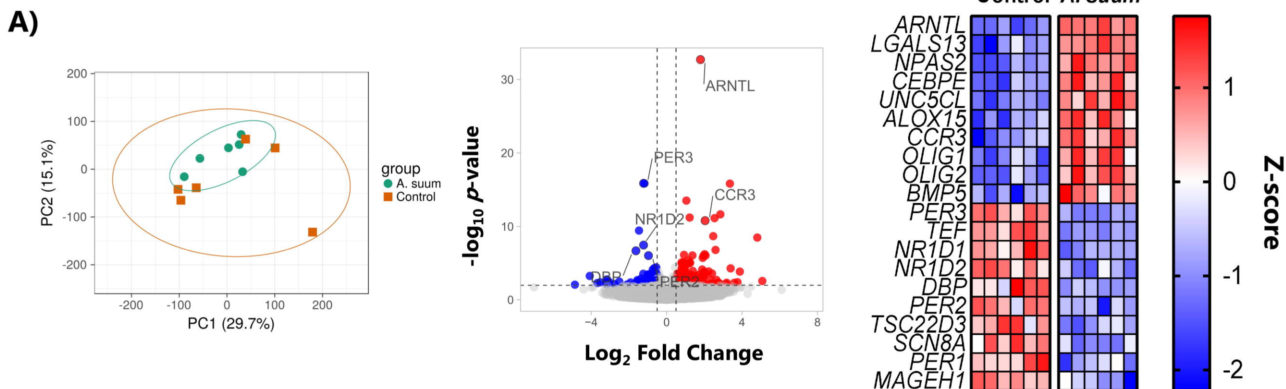

C)
Control
PAC
DPH5
CDC14A
TXNRD1
TMEM252
HK1
ATP8B4
SLC52A3
TNFAIP8
RAB7A
COL6A5
SLC2A7
MT-2B
CPA5
HERPUD1
EGFR
ZFAND2A
PPP1R10
STKLD1
LY6D
ESM1
Z-score

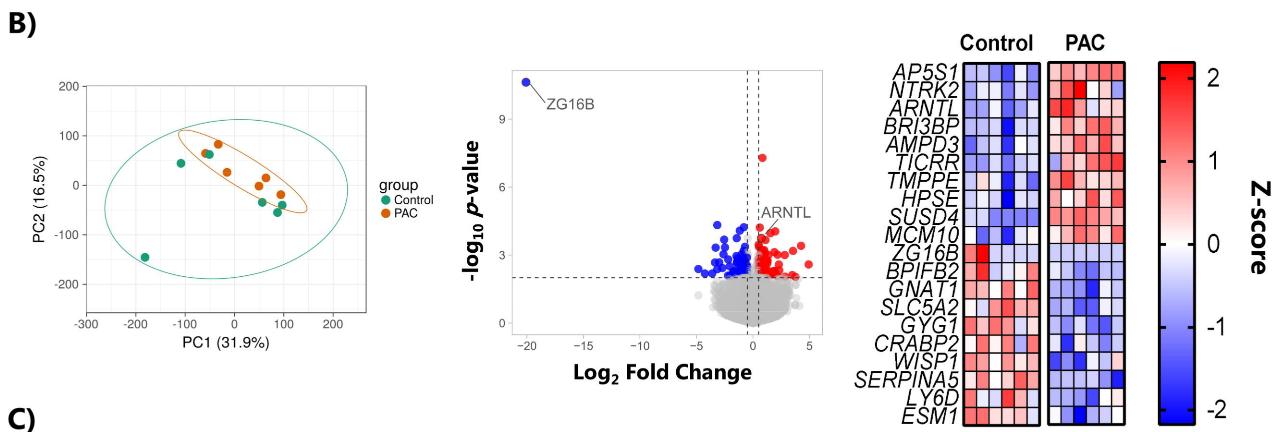
D)
Attenuation Phase
HSF1 Dependent Transactivation
Selenoamino acid Metabolism
Platelet-Vascular Interactions
Primary Immunodeficiency
Granulocytes Pathway
rRNA Processing
Detoxification of ROS
Retinoid Cycle in Rods
Pathogen Phagocytosis
Nonsense Mediated Decay
Cytoplasmic Ribosomal Proteins
Amino Acid Metabolism
Translation Initiation
Ribosome
B-Cell Receptor Signaling
Calcium Mobilization
Protein targeting to membrane
Translation Elongation
Normalized Enrichment Score
PAC
Control
HSPH1 HSPA4L BAG3 HSPA6 DNAJB1 HSPA8 HSPA5 HSPB2 HSPA1L HSPA12A TNFRSF21 HSPA2 HSPA13 DEDD2 HSPB1 FKBP4
PAC
Control
CAT CYCS NCF2 GPX7 PRDX5 TXN PRDX6 GPX1 GPX3 GPX6 NCF1 TXNRD1
Z-score

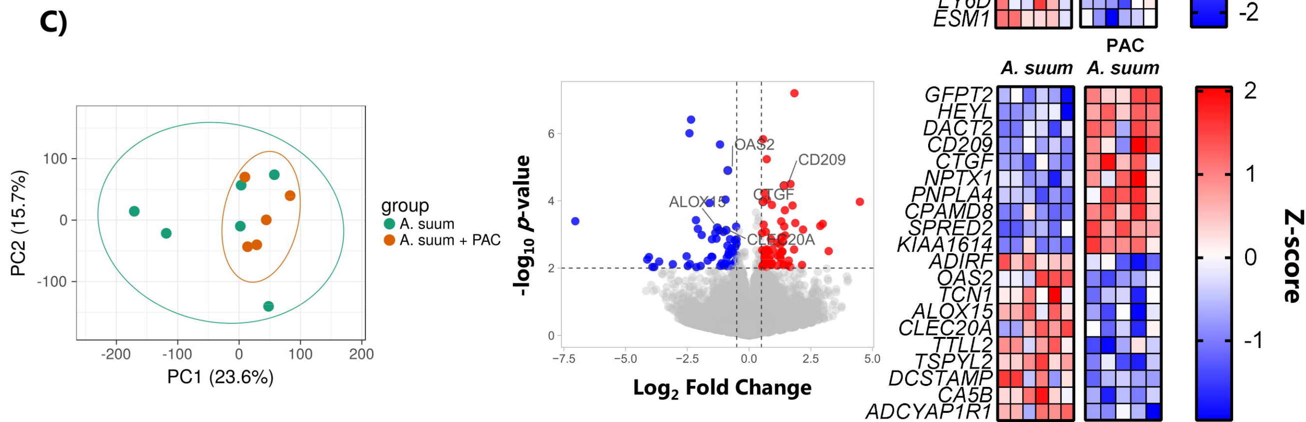

A)
PC2 (14.6%)
PC1 (24.6%)
group
A. suum
A. suum+ PAC


B)
-log10 p-value
Log2 Fold Change
BTC
ORAI2
AGTR1
AREG

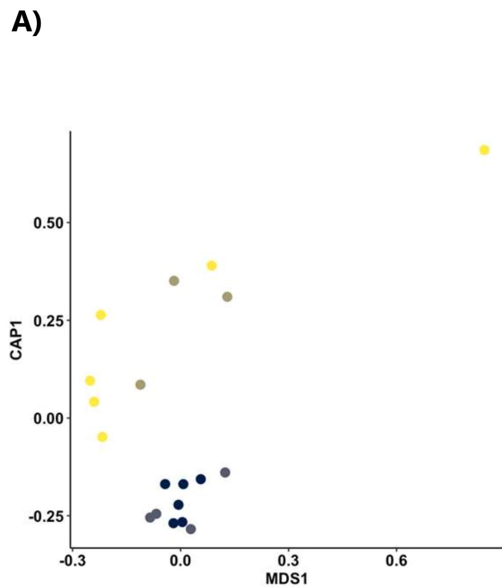

C)
A. suum
PAC
A. suum
AQP10
WDR19
SOX18
ORAI2
TMEM102
AGTR1
ESD
NLRP12
MYBPC3
MTURN
PLIN4
BTC
ADH1C
PCP4
WDR35
CKM
ACTN2
IGSF9B
AREG
SLC2A4
Z-score


A)
Control
A. suum
PC2 (15.1%)
PC1 (29.7%)
group
A. suum
Control
-log10 p-value
Log2 Fold Change
ARNTL
PER3
CCR3
NR1D2
DBP
PER2
ARNTL
LGALS13
NPAS2
CEBPE
UNC5CL
ALOX15
CCR3
OLIG1
OLIG2
BMP5
PER3
TEF
NR1D1
NR1D2
DBP
PER2
TSC22D3
SCN8A
PER1
MAGEH1
Z-score

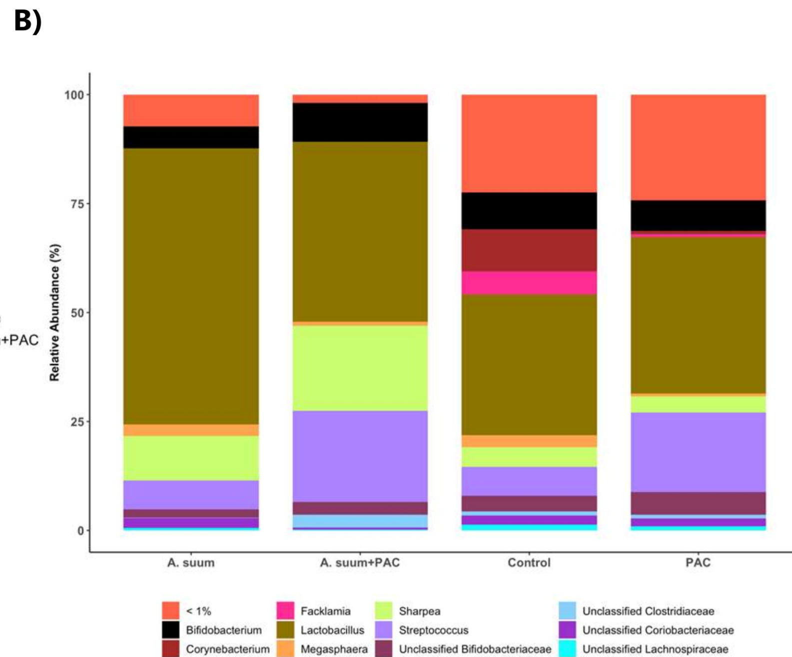

B)
Control
PAC
PC2 (16.5%)
PC1 (31.9%)
group
Control
PAC
-log10 p-value
Log2 Fold Change
ZG16B
ARNTL
AP5S1
NTRK2
ARNTL
BRI3BP
AMPD3
TICRR
TMPPE
HPSE
SUSD4
MCM10
ZG16B
BPIFB2
GNAT1
SLC5A2
GYG1
CRABP2
WISP1
SERPINA5
LY6D
ESM1
Z-score


C)
A. suum
PAC
A. suum
PC2 (15.7%)
PC1 (23.6%)
group
A. suum
A. suum + PAC
-log10 p-value
Log2 Fold Change
OAS2
CD209
ALOX15
CTGF
CLEC20A
GFPT2
HEYL
DACT2
CD209
CTGF
NPTX1
PNPLA4
CPAMD8
SPRED2
KIAA1614
ADIRF
OAS2
TCN1
ALOX15
CLEC20A
TTLL2
TSPYL2
DCSTAMP
CA5B
ADCYAP1R1
Z-score

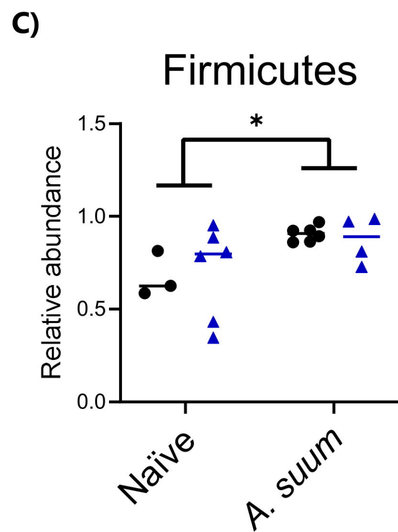
A)



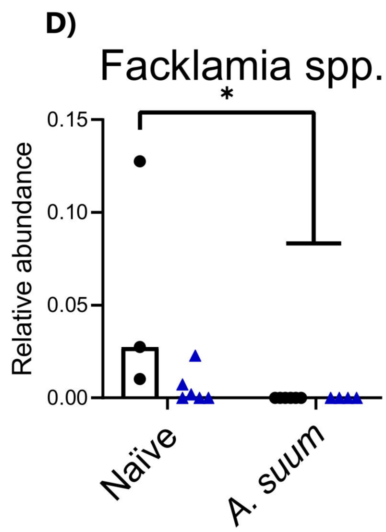
B)



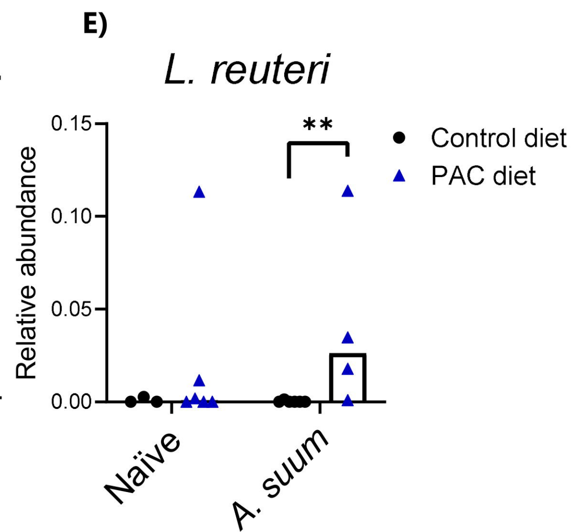
C)

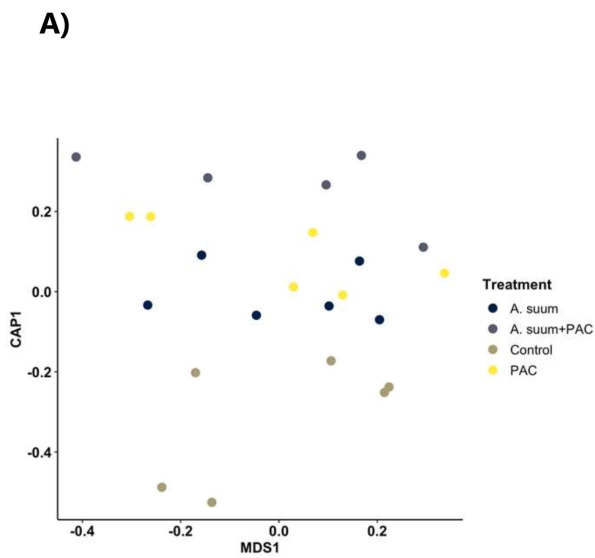


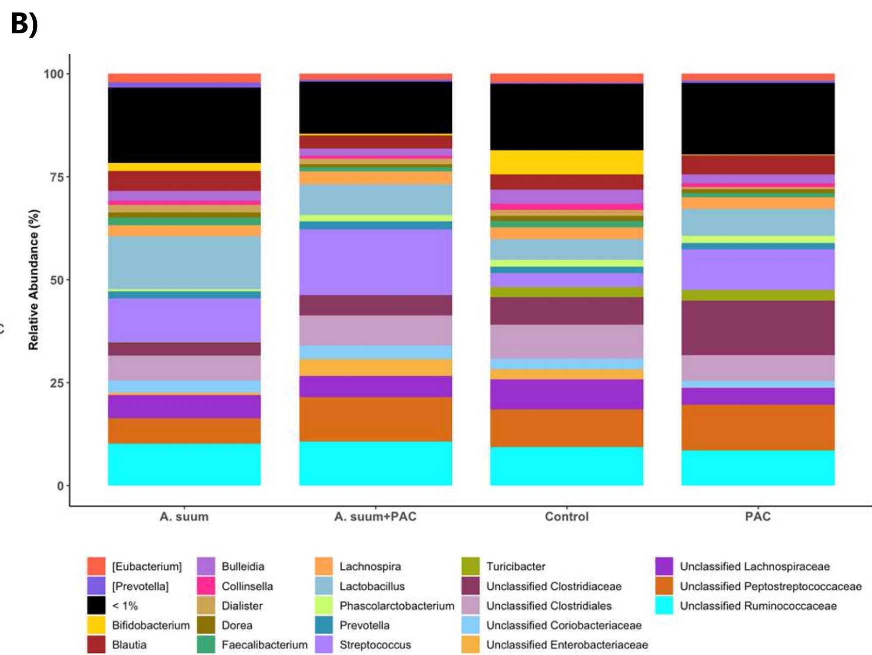
D)

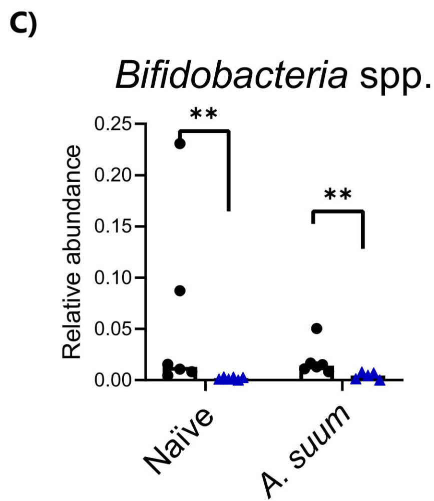


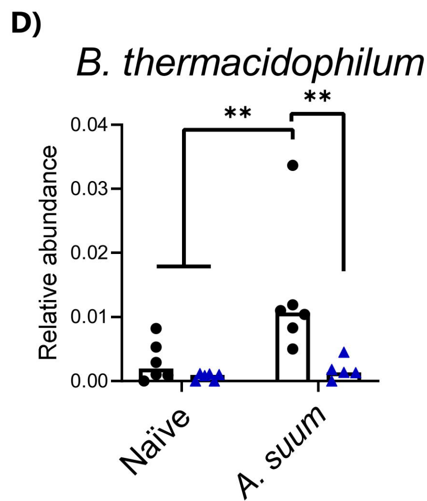
E)

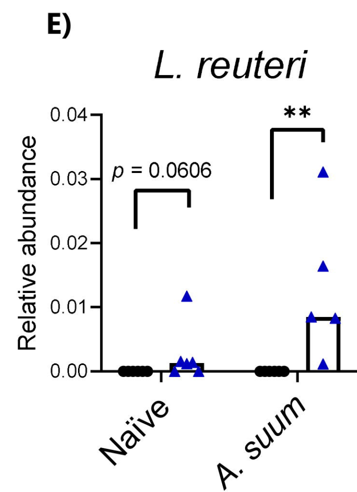


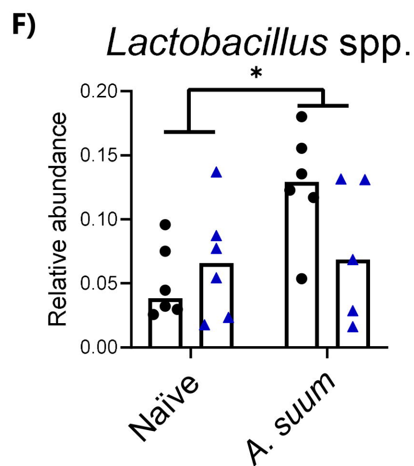

A)
CAP1
MDS1
0.2
0.0
-0.2
-0.4
-0.4
-0.2
0.0
0.2
Treatment
A. suum
A. suum+PAC
Control
PAC

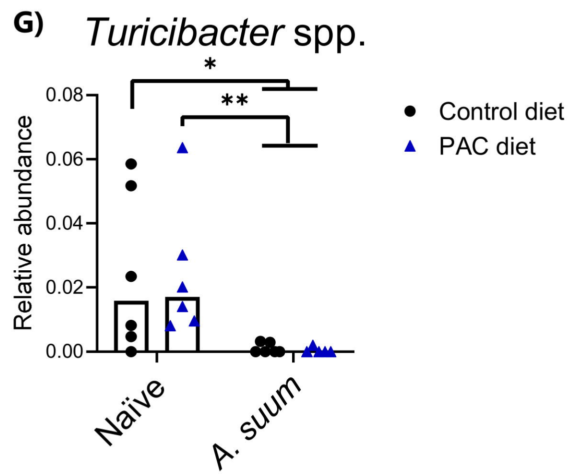

B)
Relative Abundance (%)
100
75
50
25
0
A. suum
A. suum+PAC
Control
PAC
[Eubacterium]
[Prevotella]
< 1%
Bifidobacterium
Blautia
Bulleidia
Collinsella
Dialister
Dorea
Faecalibacterium
Lachnospira
Lactobacillus
Phascolarctobacterium
Prevotella
Streptococcus
Turicibacter
Unclassified Clostridiaceae
Unclassified Clostridiales
Unclassified Coriobacteriaceae
Unclassified Enterobacteriaceae
Unclassified Lachnospiraceae
Unclassified Peptostreptococcaceae
Unclassified Ruminococcaceae


C)
Bifidobacteria spp.
Relative abundance
0.25
0.20
0.15
0.10
0.05
0.00
**
**
Naïve
A. suum


D)
B. thermacidophilum
Relative abundance
0.04
0.03
0.02
0.01
0.00
**
**
Naïve
A. suum


E)
L. reuteri
Relative abundance
0.04
0.03
0.02
0.01
0.00
p = 0.0606
**
Naïve
A. suum


F)
Lactobacillus spp.
Relative abundance
0.20
0.15
0.10
0.05
0.00
*
Naïve
A. suum


G)
Turicibacter spp.
Relative abundance
0.08
0.06
0.04
0.02
0.00
*
**
Naïve
A. suum
Control diet
PAC diet