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1 **Gut and immune effects of bioactive milk factors in preterm pigs**
2 **exposed to prenatal inflammation**

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12

13 **Running title:** Bioactive milk diets in preterm neonates born with prenatal inflammation

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17 **Key words:** bovine colostrum, caseinoglycomacropeptide, lipopolysaccharide, osteopontin, prenatal
18 inflammation, preterm pigs

19 **Abstract**

20 Prenatal inflammation may predispose to preterm birth and postnatal inflammatory disorders, such as
21 necrotizing enterocolitis (NEC). Bioactive milk ingredients may help to support gut maturation in such
22 neonates, but mother's milk is often insufficient after preterm birth. We hypothesized that
23 supplementation with bioactive ingredients from bovine milk (osteopontin, OPN;
24 caseinoglycomacropeptide, CGMP; colostrum, COL) supports gut, immunity and NEC resistance in neonates
25 born preterm after gram-negative infection before birth. Using preterm pigs as a model for preterm infants,
26 fetal pigs were given intra-amniotic injections of lipopolysaccharide (LPS, 1 mg/fetus) and delivered three
27 days later (90% gestation). For five days, groups of LPS-exposed pigs were fed formula (FOR), bovine
28 colostrum (COL), or formula enriched with OPN or CGMP. LPS induced intra-amniotic inflammation,
29 postnatal systemic inflammation but limited effects on postnatal gut parameters and NEC. Relative to FOR,
30 COL feeding to LPS-exposed pigs showed less diarrhea, NEC severity, reduced gut IL-1 β and IL-8 levels,
31 greater gut goblet cell density and digestive enzyme activities, and blood helper T cell fraction. CGMP
32 improved neonatal arousal, gut lactase activities and reduced LPS-induced IL-8 secretion in intestinal
33 epithelial cells (IECs) *in vitro*. Finally, OPN tended to reduce diarrhea and stimulated IEC proliferation *in*
34 *vitro*. No effects on villus morphology, circulating cytokines or colonic microbiota were observed among
35 groups. In conclusion, bioactive milk ingredients exerted only modest effects on gut and systemic immune
36 parameters in preterm pigs exposed to prenatal inflammation. Short-term, prenatal exposure to
37 inflammation may render the gut less sensitive to immune-modulatory milk effects.

38 **New and noteworthy:** Prenatal inflammation is a risk factor for preterm birth and postnatal complications
39 including infections. However from clinical studies, it is difficult to separate the effects of only prenatal
40 inflammation from preterm birth. Using caesarean-delivered preterm pigs with prenatal inflammation, we
41 documented some beneficial gut effects of bioactive milk diets, relative to formula, but prenatal
42 inflammation appeared to decrease the sensitivity of enteral feeding. Special treatments and diets may be
43 required for this neonatal population.

44 **Introduction**

45 Preterm births (<37 weeks of gestation) represents 15 million infants every year (10% of all
46 pregnancies), and is the most important cause of neonatal mortality (5, 6). Prenatal infection and
47 inflammation, including chorioamnionitis (CA), inflammation of the fetal membranes, is a main
48 predisposing factor for preterm birth (40-70% cases) (14, 16, 25). The incidence of CA is inversely related to
49 gestational age at birth, and many CA cases are related to infection with the low-virulent bacteria,
50 *Ureaplasma* (15, 59). CA may be associated with increased risks of neonatal early-onset sepsis (EOS) (2, 24),
51 necrotizing enterocolitis (NEC) (1, 3), and neurodevelopmental disorders (58), but it remains elusive if these
52 effects are direct or indirect. In addition, it is unclear how the first milk diets (mother's own milk, standard
53 formula, enriched formula) (11) may modify the gut and systemic responses to prenatal inflammation. For
54 preterm infants with CA, sub-optimal neonatal feeding may induce a second inflammatory insult to further
55 increase the risk of infection and NEC (53). Our recent study on formula-fed preterm pigs has shown that
56 CA induced by 3 days of intra-amniotic (IA) exposure to LPS exerted strong fetal gut responses and
57 postnatal systemic inflammation but did not increase NEC sensitivity on day 5 (33). Thus, CA may
58 predispose to EOS in preterm infants while the effects on NEC are more unclear. Sheep studies have shown
59 CA-induced fetal inflammatory responses in the gut, lung and brain at birth (26, 50), but neither of these
60 earlier studies allowed postnatal recovery and recording of inflammation and NEC sensitivity after enteral
61 feeding. Possibly, an optimized milk diet for preterm neonates may not only protect the immature gut
62 against NEC, but also reverse negative impacts of CA on the postnatal systemic immunity and inflammation
63 in multiple organ systems.

64 In preterm pigs delivered by caesarean section after unaffected pregnancies, feeding bovine
65 colostrum (COL) reduces NEC incidence, improves gut maturation, and down-regulates expression of
66 intestinal genes related to inflammation, relative to infant formula (20, 37, 48, 49). Initial preterm infant
67 studies also show that COL decreases diarrhea (51) and the time to full enteral feeding (22). Similar to

68 mother's milk, COL contains multiple bioactive ingredients including anti-bacterial, anti-inflammatory
69 proteins and peptides (immunoglobulins, lactoferrin, transforming growth factor β , casein, osteopontin
70 (OPN)) (10). Bioactive components in COL may provide a better option or supplement than conventional
71 infant formula for preterm infants, and may help to reverse gut inflammation induced by prenatal
72 inflammation in infants born after CA. Among the milk bioactive components, OPN is an important protein
73 with higher levels in COL than in bovine mature milk and human milk (29). It exerts anti-inflammatory
74 properties (38) and anti-bacterial activities as well as protects the epithelial barriers via interactions with
75 macrophages and neutrophils (23, 54, 60). OPN-enriched formula fed to newborn infants shows systemic
76 immune-modulatory effects with lower plasma levels of TNF- α and a higher frequency of blood T cells than
77 regular infant formula (29, 55). Caseinoglycomacropeptide (CGMP) is another promising milk peptide
78 derived from kappa-casein with multiple immune-modulatory effects. For instance, CGMP decreases
79 inflammatory gene expressions in porcine intestinal epithelial cells *in vitro* (18), downregulates the
80 secretion of pro-inflammatory cytokines IFN- γ and TNF- α on rat splenocytes (41). CGMP is also considered a
81 prebiotic component, via its decoy functions against pathogenic *E.coli* adhesion to the gut mucosa (8, 17),
82 and via its ability to promote the growth of *Bifidobacteria* and *Lactococcus* spp. (7, 42).

83 Based on the already reported immune-modulatory effects of COL, OPN and CGMP, and the effects
84 of prenatal inflammation on postnatal gut and systemic inflammation (33), we hypothesized that feeding
85 COL, or formula (FOR) enriched with OPN or CGMP, would be superior to FOR feeding alone in protecting
86 against NEC and systemic inflammation in preterm neonates born after prenatal inflammation. Preterm
87 pigs were given an IA dose of LPS, as previously described (33), and were fed one of the above four diets for
88 five days. The study primary outcome was NEC incidence and secondary outcomes included gut functions
89 (absorption, enzyme activities, permeability), gut inflammation (inflammatory cytokines), and systemic
90 immune endpoints (hematology and neutrophil functions).

91 **Material and Method**

92 **Preterm pig experiments**

93 All animal procedures were approved by the Danish National Committee of Animal Experimentation
94 licence number 2014-15-0201-00418, which is in line with directive 2010/63/EU from the European
95 Parliament. The schematic overview of the animal experimental design was depicted in Fig. 1. Eight
96 pregnant sows (Large White x Danish Landrace x Duroc) were operated by laparotomy at d 103 of gestation
97 (term at 117±2 days of gestation), and each fetus received either an IA dose of 1 mg LPS (LPS, n = 141, from
98 *E.coli* 055:B5, Sigma Aldrich, Copenhagen, Denmark) or control (CON, n = 47, saline or no injection) in an
99 area close to the mouth, as previously described (33). Post-surgical monitoring of the sows included
100 frequent clinical evaluation and temperature measurement until the time of planned delivery to record any
101 potential signs abortion or infection. Preterm pigs were then delivered by caesarean section at d 106 (89-92%
102 gestation age). In experiment 1, to confirm the postnatal gut and systemic effects of prenatal LPS, a
103 proportion of the surviving pigs at birth with and without IA LPS injection (25 CON and 26 LPS pigs) were
104 fed formula until euthanasia on postnatal d 5. In experiment 2, to test the effects of bioactive milk
105 components in pigs born with prenatal inflammation, 44 caesarean delivered piglets with IA LPS injection
106 from five of the above eight pregnant sows were block-randomized, according to birth weight and gender,
107 into four groups fed with four different diets: formula (FOR, n=11), bovine colostrum (COL, n=10), casein-
108 glycomacropeptide-enriched formula (CGMP, n=11) and osteopontin-enriched formula (OPN, n=12). Pigs
109 that died before birth or during the first 24 h after birth were excluded from the data analyses.

110 After birth, each piglet was transferred to a pre-heated individual incubator (37-38°C) with
111 supplemental oxygen (0.5-2 L/min, for the first 24 h). If necessary, resuscitation was performed by physical
112 stimulation and positive airway ventilation until stable respiration. Each pig was then inserted a vascular
113 catheter (4F, Portex, Kent, UK) via the umbilical artery for blood sampling and parenteral nutrition (PN) and
114 an orogastric catheter (6F, Portex, Kent, UK) for enteral nutrition (EN), as described previously (21). During

115 the first 24 h, piglets also received maternal plasma (16 mL/kg) via the umbilical catheter for provision of
116 passive immunity. All piglets were nourished by PN (Kabiven, 3210 kJ/L, modified to meet piglet nutrient
117 requirement (44, 47), gradually decreased amount, 96-48 mL/kg/d), and by EN (gradually increased amount
118 of 24-120 mL/kg/d) until postnatal d 5, when they were euthanized for tissue collection. Four enteral diets
119 were designed isoenergetically (Table 1). Infant formula consisted of whey protein concentrate Lacprodan
120 80D (Arla Food Ingredients, Viby, Denmark), Seravit-SHS, Liquidgen MCT-SHS, Calogen LCT and Fantomalt
121 (Nutricia, Birkerød, Denmark). For enriched formulas, the levels of supplemented CGMP and OPN were 30
122 g/L and 2.2 g/L, respectively. Bovine colostrum powder (Colodan, Biofiber Damino, Gesten, Denmark) was a
123 commercial product previously used in clinical trials of preterm infants (22). Clinical conditions and faecal
124 characteristics were evaluated twice daily as previously described (33). The time from birth until first signs
125 of eyelid opening, standing and walking were recorded. At euthanasia, organs were weighed and tissues
126 were snap-frozen and stored at -80°C or fixed in paraformaldehyde 4% for later analyses. Five regions of
127 the gastrointestinal tract including stomach, proximal, middle and distal small intestines and colon were
128 evaluated for macroscopic NEC-like lesions by a scoring system from 1 to 6, as previously described (21). A
129 pig with a score of at least 3 in any the five gastrointestinal regions was defined as NEC.

130 **Hematology and systemic immune analyses**

131 At birth, amniotic fluid was collected for manual leukocyte counting to evaluate the levels of intra-
132 amniotic inflammation, as previously described (33). Arterial blood (d 1, 3 and 5) was collected for
133 hematology by an automatic cell counter (Advia 2120i Hematology System Siemens, Germany) and
134 systemic immune analyses including blood T cell phenotyping and blood neutrophil phagocytosis function.
135 For blood T cell characterization, blood erythrocytes were lysed (1 × BD FACS Lysing solution, BD
136 Biosciences, Lyngby, Denmark) and the remaining leukocytes were permeabilized
137 (Fixation/Permeabilization Concentrate, eBioscience, ThermoFisher, Roskilde, Denmark) for 30 min at 4°C in
138 the dark and washed with permeabilization buffer (eBioscience). The cells were incubated 15 min in the

139 dark at 4°C with porcine serum (Thermofisher) for Fc receptor blocking, and then stained with a mixture of
140 PerCP-Cy5.5 conjugated anti-pig CD3 antibody (IgG2a isotype, BD Biosciences, Lyngby, Denmark), FITC-
141 conjugated anti-pig CD4 antibody (IgG2b isotype, BioRad, Copenhagen, Denmark), PE-conjugated mouse
142 anti-pig CD8 antibody (IgG2a isotype, Biorad) and APC-conjugated anti-mouse/rat Foxp3 antibody (IgG2a
143 isotype, eBioscience). Samples were then analyzed by BD Accuri C6 flow cytometer (BD Biosciences). PerCP-
144 Cy5.5-conjugated mouse IgG2a negative control antibody (BD Bioscience), APC-conjugated rat IgG2a
145 negative control antibody (eBioscience), PE-conjugated mouse IgG2a negative control antibody and FITC-
146 conjugated mouse IgG2b negative control antibody were used as isotype controls. T cell subsets were
147 analyzed including helper T cells (Th, CD3⁺CD4⁺CD8⁻ lymphocytes), cytotoxic T cells (Tc, CD3⁺CD4⁻CD8⁺
148 lymphocytes) and regulatory T cells (Treg, CD3⁺CD4⁺Foxp3⁺ lymphocytes). Blood neutrophil phagocytosis
149 function was analyzed by flow cytometry using the pHrodo Red E.coli (560/585 nm) Bioparticles
150 Phagocytosis Kit for Flow cytometry (Thermofisher) as previously described (32). Phagocytic rate was
151 assessed as percentage of neutrophils exerting phagocytosis (pHrodo⁺ neutrophils), and phagocytic capacity
152 was assessed as the fluorescent intensity of pHrodo⁺ in the pHrodo⁺ neutrophil population.

153 **Serum biochemistry, tissue cytokines and brush border enzyme activities**

154 At euthanasia, serum samples were collected for biochemical analyses by Advia 1800 Chemistry
155 System (Siemens, Germany). Distal small intestinal tissues were homogenized and analyzed for IL-1β, IL-8,
156 IL-10 and I-FABP by ELISA (R&D Systems, Abingdon, UK). In addition, the small intestinal homogenates
157 (proximal, middle and distal regions) were also analyzed for brush border enzyme activities including
158 peptidases (aminopeptidase N (ApN), aminopeptidase A (ApA) and dipeptidylpeptidase IV (DPPIV)) and
159 disaccharidases (sucrase, maltase and lactase) by spectrophotometry, as previously described (45, 46).

160 **Gut histology and goblet cell density**

161 Fixed tissues from the small intestinal regions (proximal, middle and distal) were embedded in
162 paraffin, sectioned and stained with hematoxylin and eosin for measurement of villus height and crypt

163 depth by ImageJ (version 1.50i, NIH, USA). Mucin-containing goblet cells in the distal small intestine and
164 colon were stained with Alcian blue and Periodic acid-Shiff (AB-PAS) and quantified, as previously described
165 (28).

166 **Gut microbiota composition**

167 Colon contents collected at euthanasia were used to extract total cellular DNA using PowerSoil DNA
168 Isolation Kit (MoBio Laboratories, CA, US) (27, 40). The bacterial V3 region of 16S rRNA was chosen for
169 NextSeq paired-end 150bp amplicon analysis using primer compatible with Nextera Index Kit (Illumina, CA
170 USA): NXt_388_F: 5'-TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAGACWCCTA CGGGWGGCAG CAG-3' and
171 NXt_518_R: 5'-GTCTCGTGGGC TCGGAGATGTG TATAAGAGAC AGATTACCGC GGCTGCTGG-3' (Integrated
172 DNA Technologies; Leuven, Belgium).

173 ***In vitro* cell culture, proliferation and cytokine secretion**

174 IPEC-J2, an intestinal epithelial cell line (IEC), isolated from jejunum of a neonatal, unsuckled piglet
175 (ACC 701, DSMZ, Braunschweig, Germany) was cultured in Advanced Dulbecco's Modified Eagle Medium
176 /Ham's F-12 (DMEM/F-12) supplemented with heat inactivated 5% fetal bovine serum, 1% GlutaMAX and
177 0.2% penicillin-streptomycin (all from Gibco, New York, USA), at 37 °C and 5% CO₂. All experiments were
178 performed in 3-4 independent replicates at cell passages 5-15.

179 For proliferation assay, the cells were seeded at 5×10^4 cells/mL in a 96-well plate, cultured for 24 h,
180 and then serum starved for another 24 h. Thereafter, the cells were stimulated with bovine OPN
181 (Lacprodan OPN-10, Arla Foods Ingredients, Viby, Denmark) or CGMP (Lacprodan CGMP-20, Arla Foods
182 Ingredients) at various doses of 0.01-1 g/L (sterile filtered at 0.22 μ m) for 24 h, and then incubated with the
183 CellTiter 96 AQueous One Solution Reagent (Promega, Nacka, Sweden) for 4 h at 37 °C and 5% CO₂ before
184 measurement of the absorbance at 490 nm. Cell proliferation was then quantified relative to controls (set
185 as 100 %), which was cultured in the same serum-free medium.

186 For cytokine secretion assays, the cells were seeded at 1×10^5 cells/ml in a 24-well plate, cultured
187 until reaching 70-80% confluence, and serum starved for 24h. The cells were then stimulated with 0.05 g/L
188 CGMP or OPN with or without LPS (1 μ g/mL, 0127:B8; InvivoGen, Toulouse, France) for 24 h. Supernatants
189 were collected for analysis of IL-8 (specific porcine ELISA DuoSet kits, R&D Systems, Abingdon, UK).

190 **Statistical analysis**

191 All statistical analyses were performed using R, version 3.4.3. *In vitro* data including cell proliferation
192 and secreted cytokines were analysed by linear models with protein concentration and passage as fixed
193 factors (proliferation) or with treatment (with or without CGMP/OPN treatment) and cell passage as fixed
194 factors (cytokines), using lmer function. Incidences of diarrhea and NEC and first stand were compared
195 using Fisher exact tests. NEC severity score was analysed by non-parametric Mann-Whitney test. All other
196 continuous data from the animal experiment (except gut microbiota) were analysed by linear mixed models
197 with diet as a fixed factor, and litter as a random factor using lmer function. The post hoc Dunnett tests
198 were used to compare interventions with the negative control (FOR). Data transformation (log or sqrt) was
199 performed if necessary. Residuals and fitted values were used to evaluate normal distribution and variance
200 homogeneity. Data are shown as mean \pm SEM, and P values < 0.05 were regarded as statistically significant
201 and P values < 0.1 were considered as tendencies of being significant.

202 For microbiome analysis, the raw sequencing reads were merged and trimmed, and chimeric reads
203 were removed, resulting in zero-radius Operational Taxonomic Units (zOTUs) with UNOISE implemented in
204 Vsearch (12, 13, 43). The green genes (13.8) 16s rRNA gene database was used as reference for annotation.
205 Qiime pipeline (v1.9.1) (9) together with R packages vegan, ggpubr and ggplot2 were used for data analysis.
206 All the samples were rarefied to the minimum sampling depth (32719 counts) for alpha diversity index
207 calculation. The raw OTU table was normalized with cumulative sum scaling (39) to calculate unweighted
208 and weighted Unifrac distance, and adonis from R package (vegan)(36) was performed to test differences

209 among treatments after removing the litter effect. Specific taxa comparisons were analysed by ANCOM
210 with FDR correction (30). P values < 0.05 were considered as statistically significant.

211 **Results**

212 **Effects of fetal LPS exposure on postnatal gut outcomes and systemic inflammation in preterm pigs**

213 In experiment 1 testing fetal and postnatal effects of IA LPS, the incidence of fetal death was higher
214 in LPS vs. CON pigs (45/141 vs. 4/47, $P < 0.01$). Total leukocyte levels in the amniotic fluid were highly
215 elevated in LPS vs. CON pigs ($P < 0.001$, Fig. 2A), indicating LPS-induced IA inflammation. LPS pigs took
216 longer time to stand for the first time, approximately 49 h in LPS vs. 31 h in CON pigs ($P < 0.05$, Fig. 2B). At
217 euthanasia on day 5, circulating parameters showed responses to LPS with a tendency to increased IL-1 β
218 and glucose levels ($P = 0.09$ and 0.07 , respectively), increased iron level ($P < 0.01$) and decreased levels of
219 albumin and alanine aminotransferase activities (ALT), relative to CON pigs ($P < 0.05$ and 0.01 , respectively,
220 Fig. 2C-G). In contrast, gut parameters on day 5 showed no clear LPS effect on the measured inflammatory
221 endpoints or NEC incidence (35-48%, Fig. 2H). Lactase activity across three regions of the small intestine did
222 not differ between LPS and CON pigs (Fig. 2I), but it showed tendency to be lower in the middle small
223 intestine of LPS vs. CON pigs ($P = 0.07$).

224 **Clinical evaluations and physical activity in LPS-exposed preterm pigs**

225 There was a tendency for more CGMP pigs to be on their feet within the first two days, relative to the
226 FOR group ($P = 0.06$, Fig. 3A). During the study, diarrhea occurrence was dominant in FOR pigs (91%), but
227 lower in the remaining groups (40% in COL pigs, 4/10, $P < 0.05$; 55% in CGMP pigs, 6/11, $P = 0.15$; 58% in
228 OPN pigs, 7/12, $P = 0.15$, Fig. 3B). NEC incidence in the whole gastrointestinal tract was not different among
229 the groups but the NEC severity score tended to be lower in COL vs. FOR pigs ($P = 0.08$, Fig. 3C-D).

230 **Intestinal morphology, brush border enzyme and nutrient absorption in LPS-exposed preterm pigs**

231 Gut morphology data showed similar villus height (Fig. 4A) and crypt depth (Fig. 4B) across all the
232 three regions of the small intestine. FOR pigs had the lowest lactase activities across three intestinal regions,
233 relative to the other three groups (Fig. 4C). Comparing lactase activity in each specific small intestinal

234 region, COL pigs had 1.5 to 2-fold higher level than FOR pigs in proximal, middle and distal regions ($P < 0.01$,
235 $P < 0.001$ and $P = 0.07$, respectively), and CGMP pigs had higher levels than FOR pigs only in middle region
236 ($P < 0.01$, Fig. 4C). Plasma galactose levels following administration of a bolus of galactose solution, used as
237 a hexose absorption marker, showed numerically higher values in CGMP vs. FOR pigs (Fig. 4D).

238 **Gut cytokines, proteins and goblet cell density in LPS-exposed preterm pigs**

239 The pro-inflammatory cytokines IL-1 β and IL-8 and the anti-inflammatory cytokine IL-10 were
240 evaluated in the distal small intestinal tissues. IL-1 β levels tended to be lower in COL vs. FOR pigs ($P = 0.06$,
241 Fig. 5A), and IL-8 levels in COL and CGMP were lower than in FOR pigs ($P < 0.001$ and $P = 0.09$, respectively,
242 Fig.5B). No differences in IL-10 levels were observed (Fig. 5C). I-FABP, a gut maturation marker, showed a
243 tendency to be elevated in the distal small intestine of COL pigs (but not OPN nor CGMP pigs), relative to
244 FOR pigs ($P = 0.06$, Fig. 5D). Both colon and distal small intestinal mucin-containing goblet cell densities
245 were significantly higher in COL pigs than FOR pigs ($P < 0.05$, Fig. 5E-F) with intermediate values in OPN and
246 CGMP pigs.

247 **Blood biochemistry and systemic immunity in LPS-exposed preterm pigs**

248 Comparing serum biochemical parameters at euthanasia on day 5, COL pigs had higher levels of
249 cholesterol, phosphate, urea nitrogen, Mg and K than FOR pigs (all $P < 0.05$, Table 2). CGMP-enriched
250 formula-fed pigs had 2.4-fold higher levels of urea nitrogen ($P < 0.001$), and tended to have increased
251 serum levels of P ($P = 0.07$) and Na ($P = 0.08$), relative to FOR pigs (Table 2). Systemic parameters on d 5
252 modulated by LPS including albumin, ALT (Table 2), glucose and IL-1 β (Fig. 6A-B) were not different among
253 the feeding groups while serum Fe decreased in COL pigs (relative to FOR pigs) to the basal levels found in
254 pigs without LPS exposure ($P < 0.05$, Fig. 6C). Total blood leukocyte, neutrophil, lymphocyte and monocyte
255 counts on d 5 were not different among the groups (Table 2).

256 On day 3, COL pigs had higher frequencies of blood helper T cells (CD3⁺CD4⁺CD8⁻ lymphocytes) than
257 FOR pigs ($P < 0.05$), whereas expression levels of CD4 on blood helper T cells tended to be lower in COL and

258 OPN pigs than in FOR pigs ($P = 0.07$ and 0.06 , respectively, Fig. 6D-E). *In vitro* blood neutrophil phagocytic
259 capacity against *E.coli* on day 5 showed tendency to be lower in COL vs. FOR pigs ($P = 0.07$) while OPN and
260 CGMP pigs showed similar levels to that in FOR pigs (Fig. 6F).

261 **Colon microbiota composition in LPS-exposed preterm pigs**

262 High quality reads were merged and aligned to 1098 zOTUs, then classified into 55 bacterial groups
263 at genus level (28 identified genera and 27 unambiguously identified at upper levels). The numbers of
264 observed OTU (alpha diversity) were similar among the groups (Fig. 7A). *Enterococcus*, *Streptococcus*,
265 *Klebsiella*, *Clostridium* and spp. and members of Enterobacteriaceae, Clostridiaceae were dominant groups
266 in the colon content without significant differences in abundance of any bacterial groups among the four
267 treatments (Fig. 7B). Using PCoA analysis for both unweighted and weighted Unifrac distance matrix, no
268 difference was detected among the four groups (Fig. 7C-D). No specific bacterial groups were significantly
269 affected by COL, OPN or CGMP intervention, relative to FOR.

270 ***In vitro* cell studies**

271 We used porcine IPEC-J2 cell line to test the *in vitro* effects of CGMP and OPN on IEC proliferation
272 and related cytokine secretion. Among tested protein/peptide concentrations (0, 0.01, 0.1 and 1 g/L),
273 CGMP did not stimulate IEC proliferation. In contrast, OPN increased cell proliferation in a dose-dependent
274 manner with the highest proliferation of approximately 150% at 1 g/L (Fig. 8A).

275 For the cytokine secretion assay, cell stimulation with CGMP or OPN alone slightly down-regulated
276 the levels of IL-8, relative to control cells cultured in the same serum-free medium ($P = 0.06$ and $P < 0.05$,
277 respectively; Fig. 8B). LPS stimulation led to a 1.7-fold increase in IL-8 levels ($P < 0.001$; Fig. 8B). Co-
278 stimulation with LPS and CGMP, but not LPS and OPN, decreased IL-8 secretion, relative to LPS stimulation
279 alone ($P < 0.01$; Fig. 8B).

280 Discussion

281 Clinical studies indicate that prenatal inflammation may have a variety of effects on the preterm
282 neonate and its organs, depending on the nature, timing and length of fetal inflammatory exposure (52).
283 Studies in fetal lambs also support this conclusion (26, 34, 57), but these studies did not allow rearing of
284 preterm lambs exposed to prenatal inflammation, to investigate clinical effects after birth and postnatal
285 feeding, such as NEC. In our previous preterm pig study (33), we induced prenatal inflammation by an intra-
286 amniotic LPS injection three days before preterm birth and found that IA LPS led to strong fetal gut immune
287 responses and increased postnatal diarrhea and symptoms of systemic inflammation when preterm pigs
288 were fed infant formula. However, the postnatal NEC outcomes were not affected by IA LPS. In this study,
289 we first confirmed the most important effects of IA LPS in formula-fed pigs on postnatal day 5, including
290 increased systemic inflammation and decreased gut enzyme activities in LPS vs. CON pigs, with no impact
291 on NEC incidence. Then we aimed to dampen these detrimental effects of IA LPS by feeding bioactive milk
292 diets. We found that colostrum feeding reduced diarrhea and NEC severity, lowered levels of gut
293 inflammatory cytokines, and increased gut enzyme activities and goblet cell density. Enrichment of formula
294 with CGMP and OPN exerted some beneficial effects (reduced diarrhea and gut cytokines, increased gut
295 lactase activity) and the results were supported by proliferative and anti-inflammatory properties of CGMP
296 and OPN *in vitro*. However, we conclude that in the presence of prenatal inflammation, the *in vivo* effects
297 of these interventions were modest, relative to our previous studies on preterm pigs not subjected to
298 prenatal inflammation (31, 48). Possibly, exposure to inflammation before birth makes the preterm
299 newborn neonate less sensitive to the immune-modulatory effects of bioactive milk diets after birth.

300 For those preterm infants with insufficient or delayed intake of mother's own milk, intact bovine
301 colostrum may be a better alternative than processed infant formulas as it contains high levels of multiple
302 bioactive components, including IgG, lactoferrin and OPN (10). In recent years, multiple preterm pig studies
303 have showed positive effects of bovine colostrum to improve gut function and maturation, as well as to

304 decrease gut inflammatory responses (20, 48). In addition, bovine colostrum also reduced the time to full
305 enteral feeding and increased protein intake in preliminary studies in preterm infants, relative to infant
306 formula (22). This preliminary infant study did not allow us to evaluate if the postnatal effects of colostrum
307 feeding may depend on the inflammatory status at birth and previous animal studies with colostrum have
308 not addressed this research question. Now we demonstrate that in preterm pigs exposed to IA
309 inflammation, bovine colostrum feeding reduces or has tendency to reduce diarrhea, NEC severity and gut
310 inflammatory cytokine levels, reflecting an anti-inflammatory activity. For some of the parameters such as
311 NEC severity and gut IL1- β , the tendency may have become significant if the study sample size was greater.
312 Nevertheless, the effects of colostrum in this current study were much less pronounced than a previous
313 study using similar products and feeding regimes but without prenatal inflammation (e.g. clear colostrum
314 protective effects on NEC lesions in all regions, gut nutrient absorption, permeability, mucosal morphology
315 and tissue cytokines) (48). This suggests that adverse inflammatory conditions before birth may lead to a
316 less responsive gut status to immune-modulatory diets after birth. An immune-modulatory diet, like
317 colostrum, may be most effective in a state where the gut has not already been challenged with pro-
318 inflammatory stimuli before birth.

319 In addition to gut effects, some systemic immune parameters were also modulated by colostrum
320 feeding, including the increased percentage of blood T-helper cells, as well as decreased blood neutrophil
321 phagocytosis function, relative to formula feeding. Increased percentage of blood T-helper cells were
322 associated with the immune maturation over the first two weeks of life in preterm pigs (40), suggesting
323 systemic immune maturational effects of bovine colostrum in the current study. In contrast, decreased
324 neutrophil phagocytic capacity may relate to the numerical increase in blood neutrophil counts in COL vs.
325 FOR pigs, as newly recruited neutrophils from the bone marrow to the circulation may have a relatively less
326 mature function. It remains to be elucidated however, if COL pigs have an improved systemic immune
327 competence and infection resistance, relative to FOR pigs. Further, colostrum feeding in LPS-exposed pigs
328 reversed levels of several parameters including serum iron and gut lactase activity to levels found in

329 formula-fed pigs without prenatal LPS challenge. These effects may be derived from both anti-inflammatory
330 and nutritional properties of colostrum (relative to formula). The different nutritional composition between
331 colostrum and formula may also explain differences in serum levels of BUN, P, Mg and K.

332 CGMP and OPN have been reported to exert immune-modulatory functions both *in vivo* and *in vitro*
333 (18, 29, 41, 55). In the current study, we showed proliferative effects of OPN and anti-inflammatory
334 functions of CGMP in IECs *in vitro*, coupled with some moderate improvements of gut functions in preterm
335 pigs (e.g. less diarrhea and inflammatory cytokines and higher lactase activity). For CGMP, a previous *in*
336 *vitro* study using an IEC cell line demonstrated that CGMP decreased inflammatory gene expressions
337 including *IL1B*, *IL8* and *TNFA* (18). On the other hand, the *in vivo* beneficial trends of OPN in this study was
338 very modest (only weak tendency of reduced diarrhea), in contrast with a previous study showing
339 significant effects of OPN to reduce NEC severity and gut inflammation in preterm pigs without prenatal
340 inflammation (31). Strong innate immune modulations *in vitro* and modest *in vivo* effects support our
341 speculation that effects of bioactive milk proteins after birth is less pronounced if the newborn has already
342 been exposed to prenatal inflammation. Similar to colostrum, CGMP- and OPN- supplemented formula
343 diets may be more effective to prevent feeding-induced inflammatory reactions than to act as a therapy
344 when gut inflammation is already present due to prenatal inflammatory stimuli.

345 Interestingly, none of the diet interventions led to changes in the gut microbiota composition or
346 diversity, relative to infant formula. Both CGMP and OPN have previously been reported to modulate
347 microbiota composition and play a role in maintaining gut bacterial homeostasis (19, 35). In preterm pigs
348 without prenatal inflammation, colostrum feeding also reduced the abundance of *Enterococcus* spp. (56), a
349 bacterial genus that is often associated with increased gut inflammation (4). As indicated in a previous pig
350 study (33), fetal LPS-induced gut inflammation may alter bacterial gut colonization during early life, thereby
351 affecting the host-microbiota interactions and responses to nutrition. Similar abundance of *Enterococcus*

352 spp. in all four groups in this study also supports that the effects of colostrum, CGMP and OPN
353 supplementation were relatively mild.

354 In conclusion, milk diets containing bioactive components such as bovine colostrum, OPN- or CGMP-
355 enriched formulas exerted modest protective effects on the immature gut in preterm pigs exposed to
356 prenatal inflammation. Possibly, prenatal inflammation inhibits the protective effects of milk bioactive diets.
357 Potentially, the postnatal effects of prenatal inflammation are highly dependent on the nature, timing and
358 length of the inflammatory insult. Thus, preterm neonates exposed to prenatal inflammation may require
359 careful evaluation and highly individualized clinical care and diets. Further investigations are required to
360 define the optimal nutritional regimen for preterm infants subjected to prenatal inflammation, especially
361 when mother's own milk is lacking or absent.

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370 interest.

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Table 1. Nutritional compositions in the four diets used in preterm pigs

	Formula	CGMP-Formula	OPN-Formula	Colostrum
Protein (g/L)	40.6	70.6	42.8	67.6
Carbohydrate (g/L)	45.0	34.9	45	26.8
Lipid (g/L)	41.3	31.5	41.3	36.8
Energy (kJ/L)	3017.0	3016.9	3054.7	3004.7

Table 2. Blood biochemistry and hematology on d 5 in preterm pigs born with prenatal inflammation

	FOR (n=11)	COL (n=10)	CGMP (n=11)	OPN (n=12)
Total protein (g/L)	30.0±0.8	31.8±1.2	31.4±1.6	29.9±0.9
Albumin (g/L)	11.1±0.4	11.9±0.6	12.2±0.7	11.1±0.4
ALT (U/L)	18.6±0.6	20.4±0.7	23.7±3.6	18.3±0.9
Cholesterol (mmol/L)	2.4±0.1	3.0±0.2*	2.4±0.2	2.6±0.1
Iron (µmol/L)	6.2±0.8	4.5±1.4*	3.6±0.4	4.0±0.7
Phosphate (mmol/L)	1.5±0.1	1.9±0.1**	1.7±0.1 [#]	1.6±0.1
Urea nitrogen (mmol/L)	3.5±0.6	11.4±0.7***	8.4±0.9***	4.2±0.8
Mg ⁺⁺ (mmol/L)	0.80±0.03	1.05±0.05***	0.84±0.04	0.84±0.03
Na ⁺ (mmol/L)	148.0±0.7	147.1±1.1	152.2±2.8 [#]	149.3±1.9
K ⁺ (mmol/L)	3.4±0.1	4.2±0.1***	3.6±0.1	3.6±0.2
Total leukocytes	4.3±0.5	6.8±1.3	4.2±0.6	3.5±0.5
Neutrophils	2.7±0.5	4.2±1.3	2.4±0.4	2.0±0.4
Lymphocytes	1.5±0.1	2.3±0.3	1.7±0.2	1.3±0.1
Monocytes	0.10±0.02	0.10±0.02	0.07±0.01	0.10±0.02

552 *, P < 0.05; **, P < 0.01; ***, P < 0.001, relative to FOR. [#] P = 0.07-0.08, relative to FOR. Values (mean ± SEM)

553 in three diet treatment groups were compared with values in FOR pigs (control). ALT: Alanine

554 aminotransferase.

555 **Figure legends**

556 **Fig. 1.** Schematic overview of the animal experimental design. Fetal pigs from eight litters received either
557 LPS injection or saline/no injection (CON) at day 103 of gestation (3 days before delivery by caesarean
558 section). In experiment 1, a proportion of LPS and CON pigs were reared until postnatal day 5 to
559 characterize effects of IA LPS on postnatal outcomes. In experiment 2, IA LPS-exposed pigs were
560 randomized into 4 groups fed with 4 different diets until postnatal day 5. Exp, experiment; IA, intra-
561 amniotic; LPS, lipopolysaccharide.

562 **Fig. 2.** Effects of intra-amniotic (IA) LPS on gut and systemic parameters in formula-fed preterm pigs at
563 postnatal day 5. (A-B) Amniotic fluid leukocyte levels and time from birth to first stand. (C-G) Circulating
564 levels of IL-1 β , glucose, iron, albumin, and alanine aminotransferase (ALT). (H-I) NEC incidence and small
565 intestinal lactase activity. n = 32-36 in each group for (A) and 25-26 in each group for (B-I). Values are mean
566 \pm SEM. *, P < 0.05. CON and LPS: control and LPS-exposed pigs.

567 **Fig. 3.** Physical activities and clinical evaluations in IA LPS-exposed preterm pigs following five days of
568 postnatal feeding with various bioactive diets. (A) Number of pigs with first stand after the first 2 days after
569 birth, (B) diarrhea incidence during study, (C) NEC incidence, and (D) average NEC severity score across the
570 gastrointestinal tract. All treatment groups were used to compare to FOR group (control). n = 10-12 in each
571 treatment group. Values are shown by mean \pm SEM. *, P < 0.05. IA: intra-amniotic.

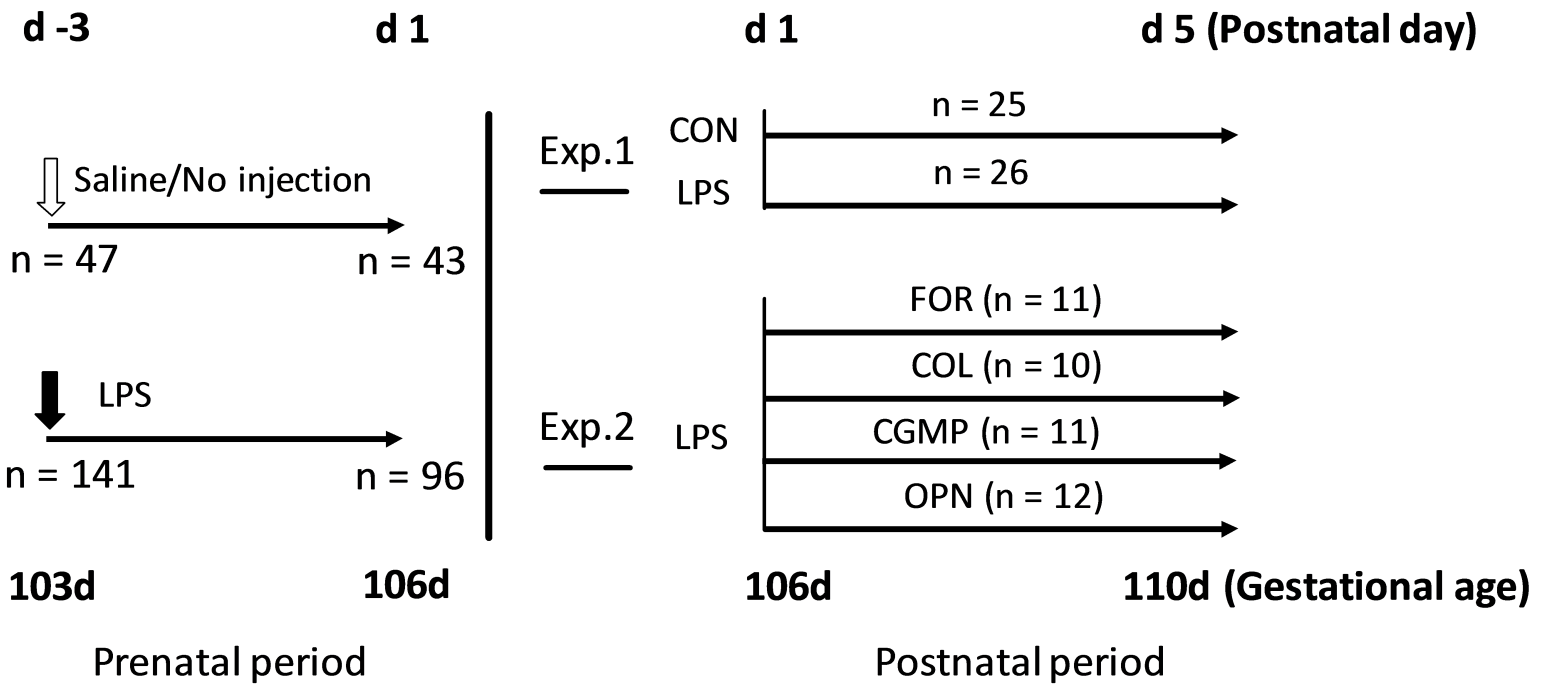
572 **Fig. 4.** Gut morphology, brush border enzymatic activities and nutrient absorption in IA LPS-exposed
573 preterm pigs following five days of postnatal feeding with various bioactive diets. (A-C) Villus height, crypt
574 depth and lactase activities across the three small intestinal regions. (D) Plasma galactose concentration
575 following a galactose test. All treatment groups were compared with FOR group (control). n = 10-12 in each
576 group for (A-C) and n = 8-10 in each group for (D). Values are mean \pm SEM. *, ** and *** P < 0.05, 0.01 and
577 0.001. IA: intra-amniotic. Prox: proximal, Mid: middle, Dist: distal intestine.

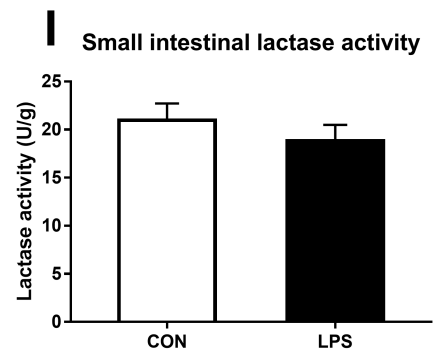
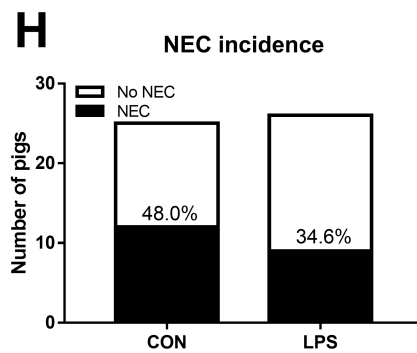
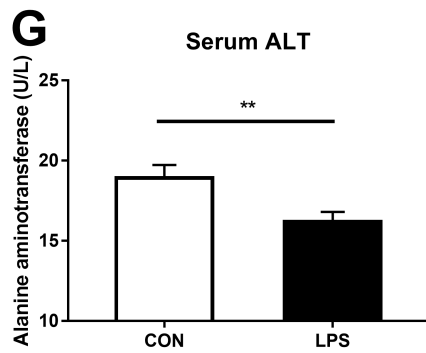
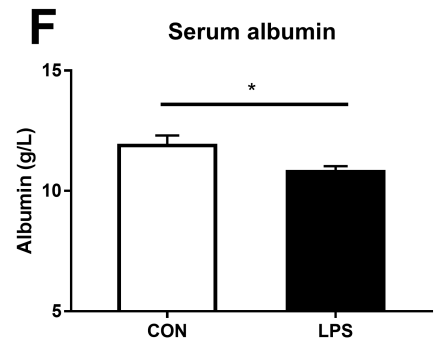
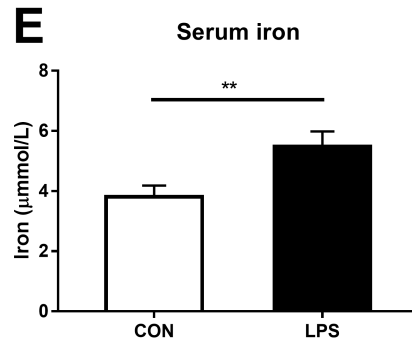
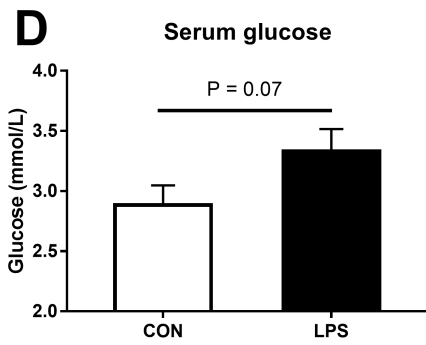
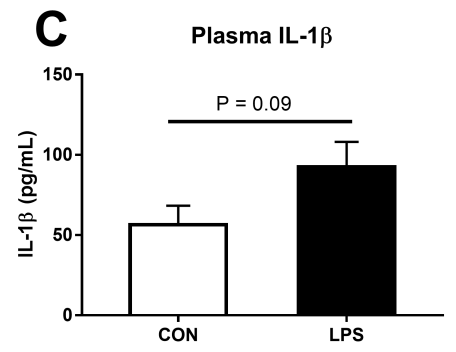
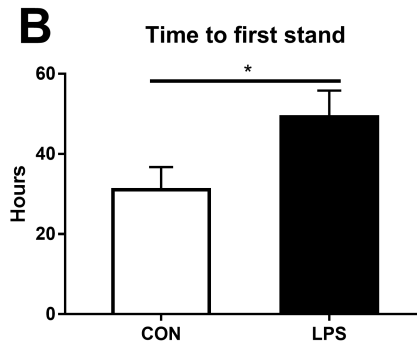
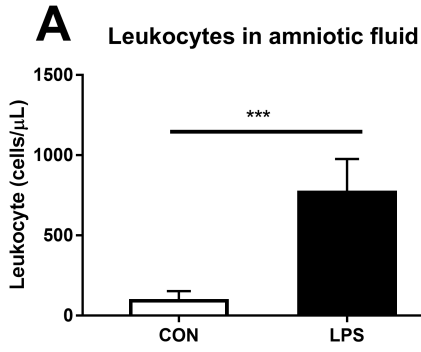
578 **Fig. 5.** Gut inflammatory cytokines and mediators and goblet cell density in IA LPS-exposed preterm pigs
579 following five days of postnatal feeding with various bioactive diets. (A-D) Distal small intestinal IL-1 β , IL-8,
580 IL-10 and I-FABP, respectively. (E-F) Goblet cell density in distal small intestine and colon. All treatment
581 groups compared with FOR group (control). n = 10-12 in each group. Values are mean \pm SEM. * and *** P <
582 0.05 and 0.001. IA: intra-amniotic.

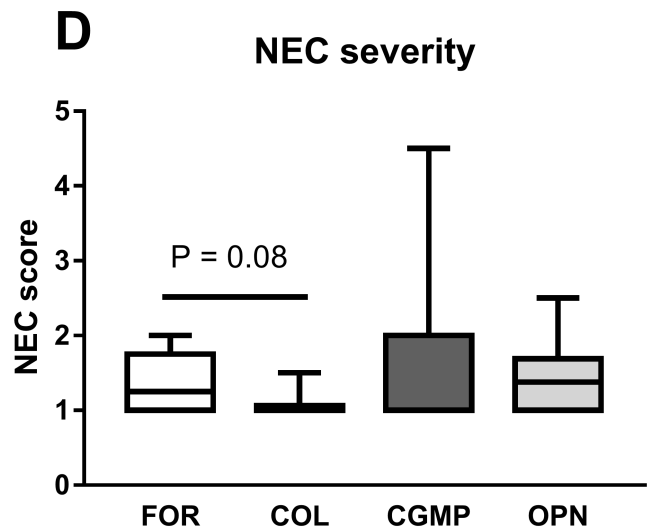
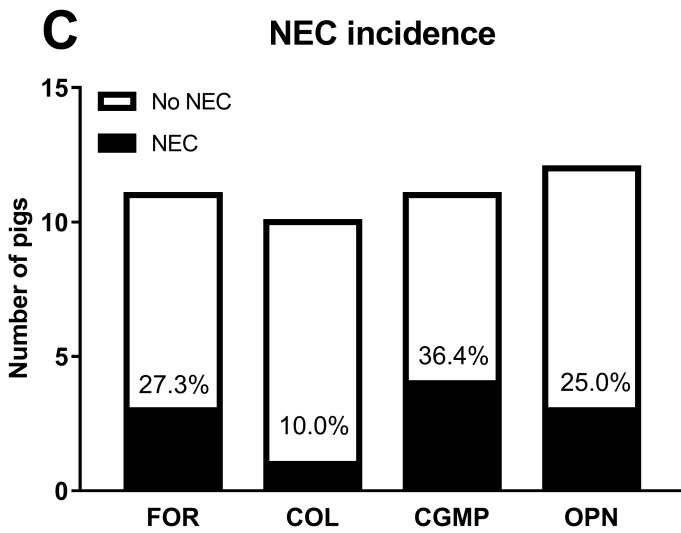
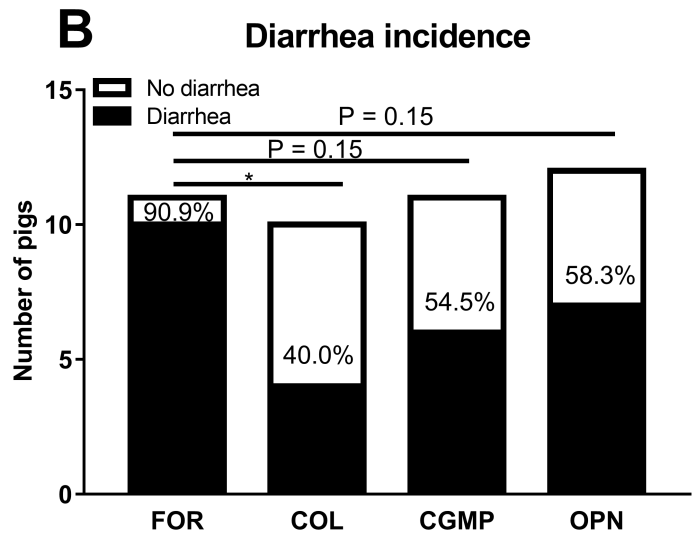
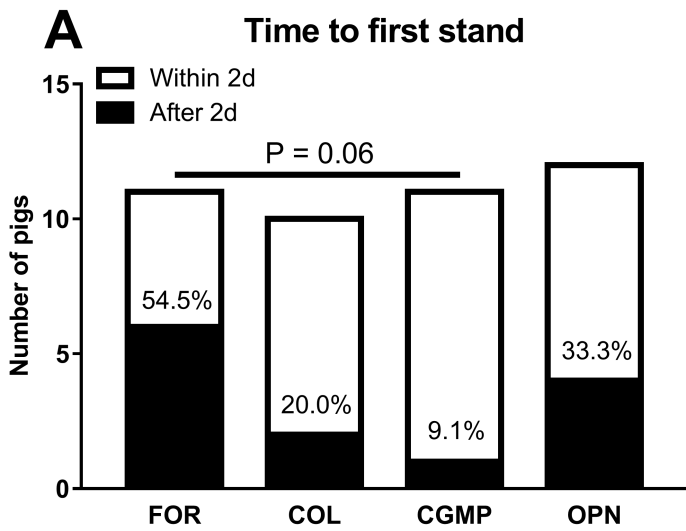
583 **Fig. 6.** Systemic immune parameters in IA LPS-exposed preterm pigs following five days of postnatal feeding
584 with various bioactive diets. (A-C) Serum/plasma levels of glucose, IL-1 β and iron at euthanasia on d 5,
585 respectively. (D-E) Percentage of helper T cells and fluorescent intensity of CD4 on d 3. (F) Neutrophil
586 phagocytic capacity on d 5. All treatment groups were compared with FOR group (control). n = 8-12 in each
587 group for (A-C) and n = 5-10 in each group for (D-F). Values are mean \pm SEM. *, p<0.05. IA: intra-amniotic;
588 MFI: median fluorescent intensity

589 **Fig. 7.** Colonic microbiota composition in IA LPS-exposed preterm pigs following five days of postnatal
590 feeding with various bioactive diets. (A) Number of operational taxonomic units (OTUs). (B) Relative
591 abundance of specific genera, with species with an relative abundance below 1% being grouped to "others".
592 (C-D) Principle coordinates analysis (PCoA) plots based on unweighted and weighted Unifrac distance
593 matrix. n = 6-10 in each group.

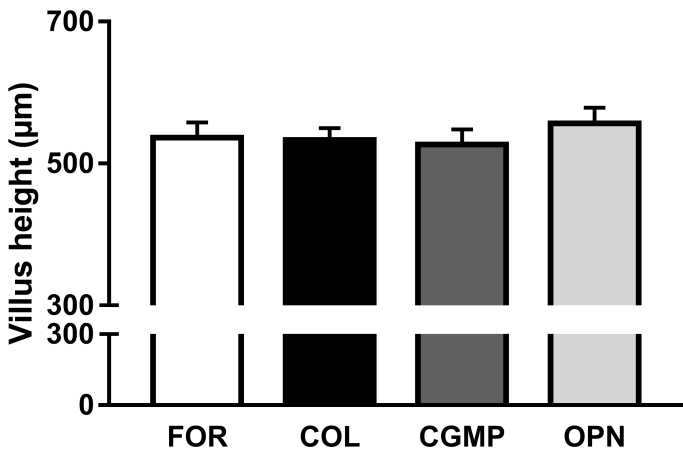
594 **Fig. 8.** *In vitro* effects of OPN and CGMP on intestinal epithelial cells (IECs). (A) *In vitro* IEC proliferation
595 stimulated by different concentrations of CGMP and OPN (0, 0.01, 0.1 and 1 g/L) for 24 h. (B) IL-8 secretion
596 in IECs following cell stimulation with CGMP or OPN with or without LPS presence. n = 3-4 in each
597 treatment group. Values (mean \pm SEM) not sharing the same letters are significantly different (P < 0.05). *
598 and **, P < 0.05 and 0.01, respectively. IEC: intestinal epithelial cell.



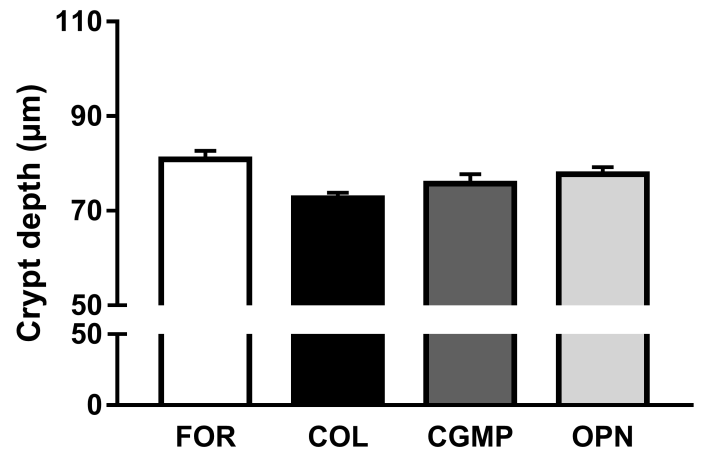




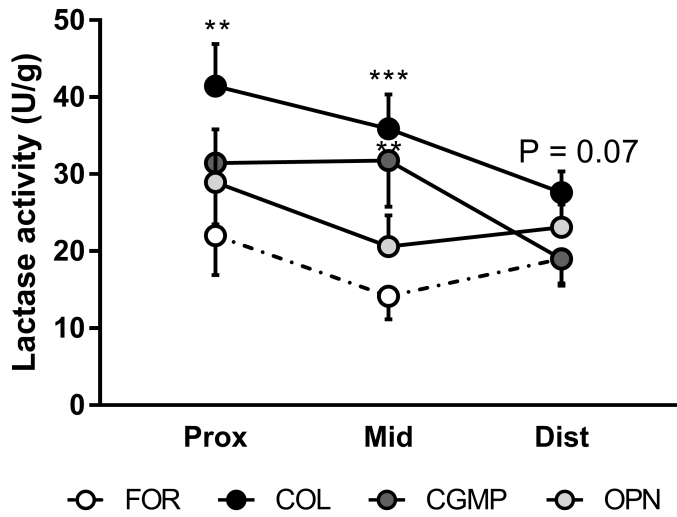
A Small intestinal villus height



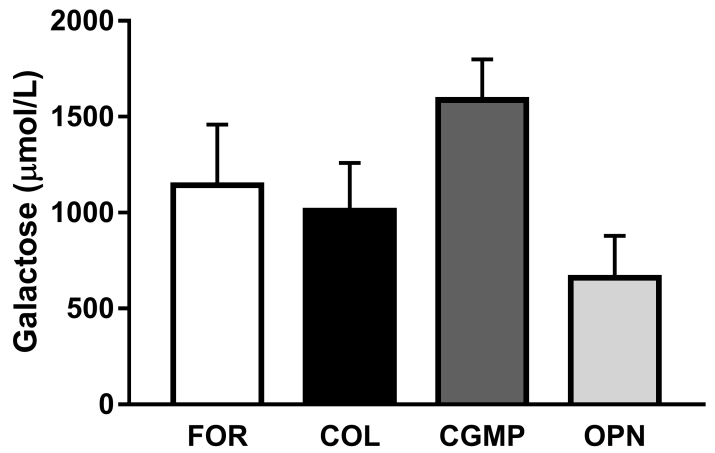
B Small intestinal crypt depth

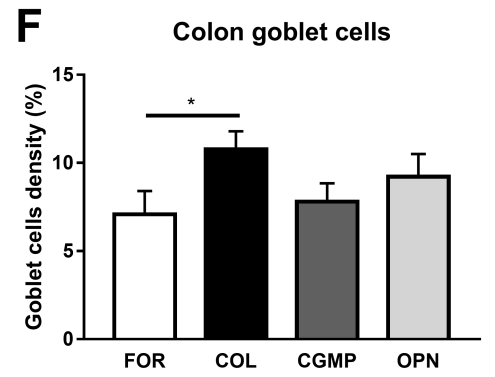
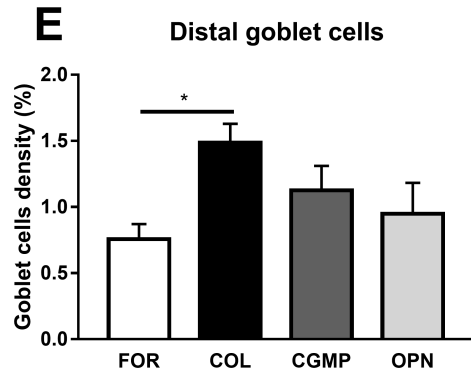
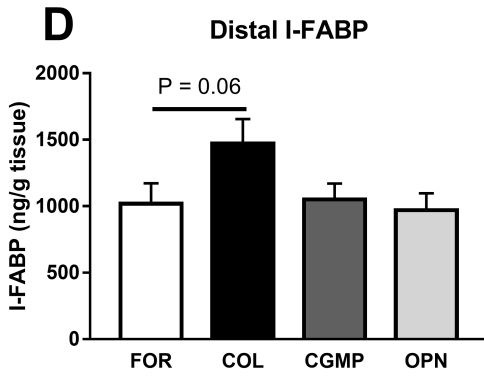
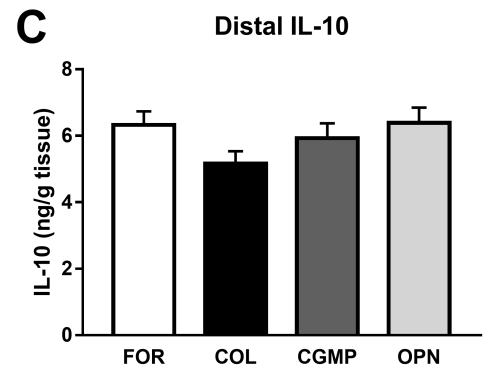
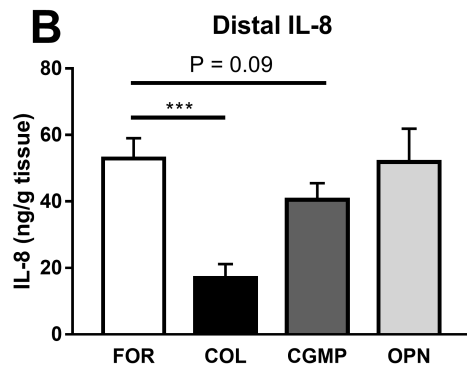
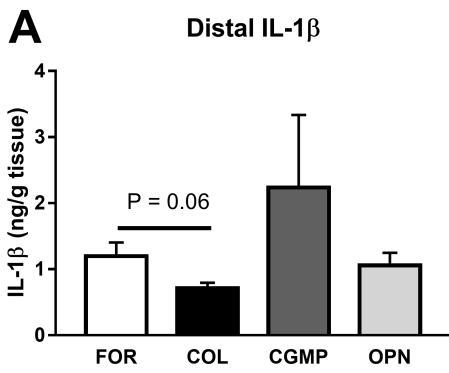


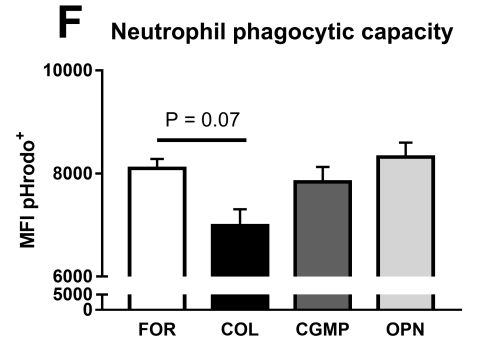
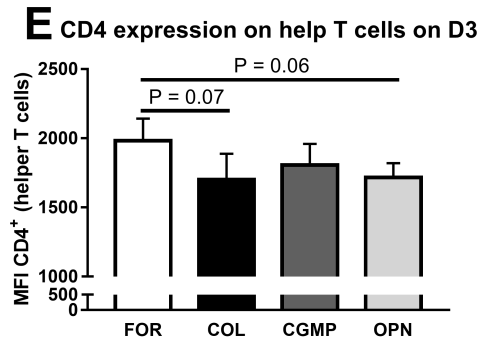
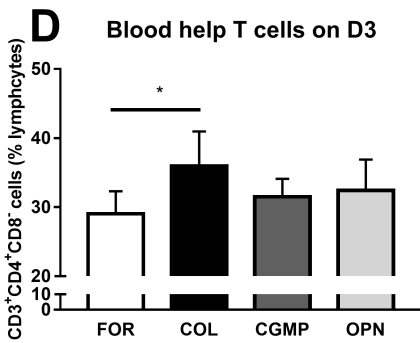
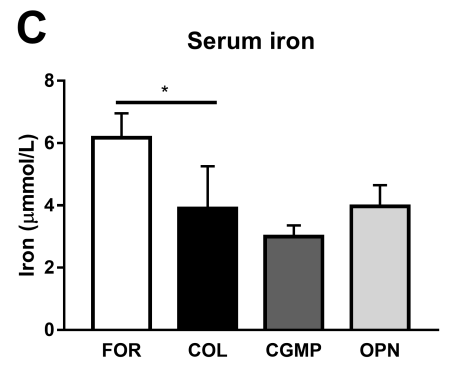
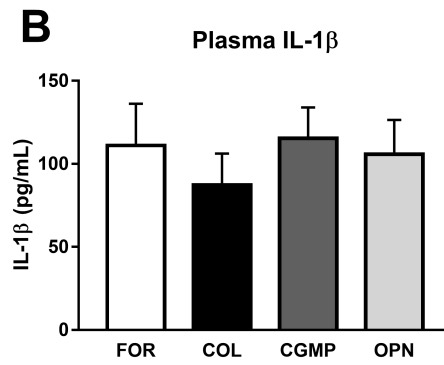
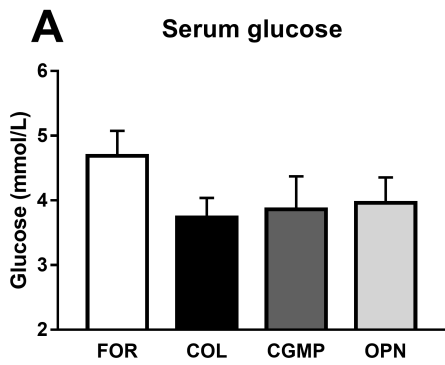
C Lactase activity

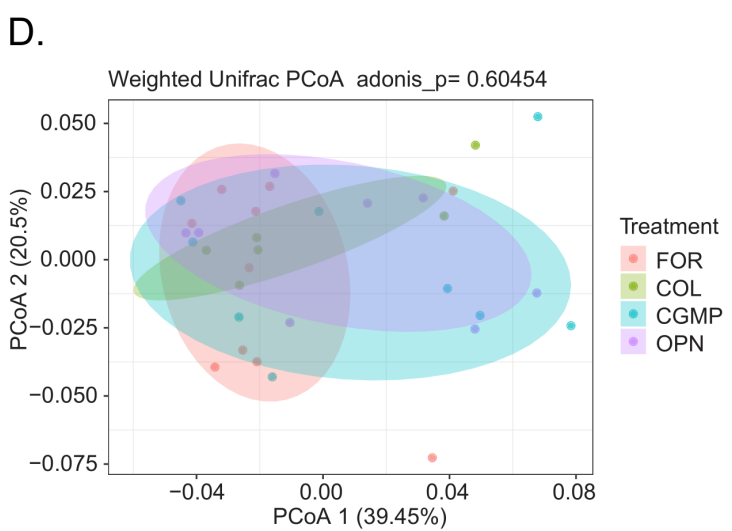
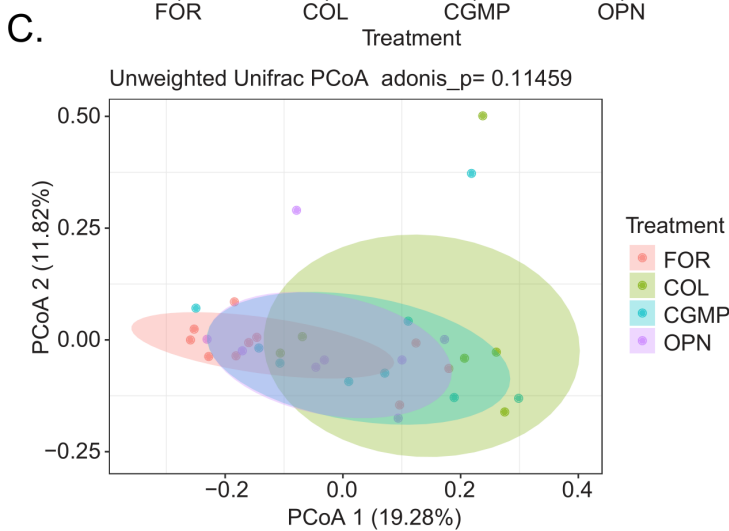
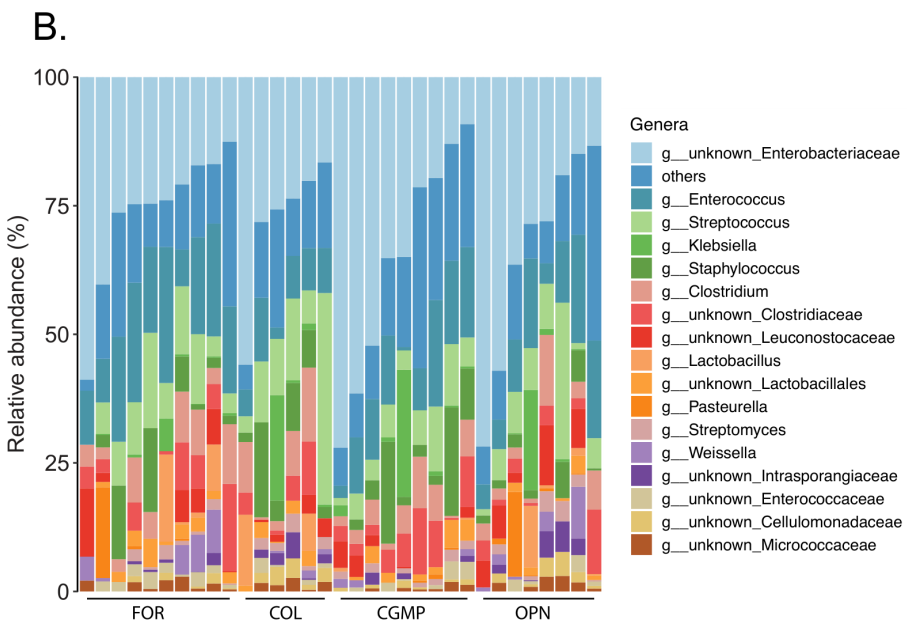
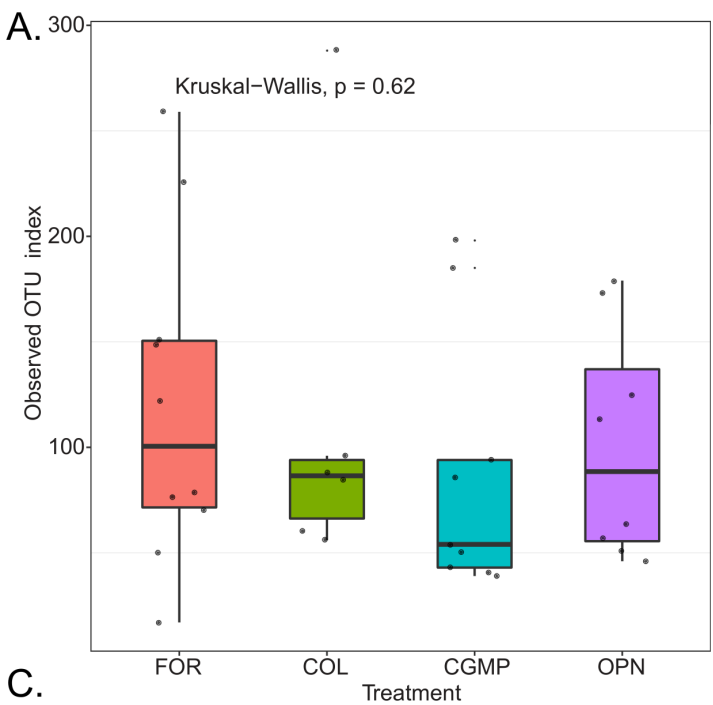


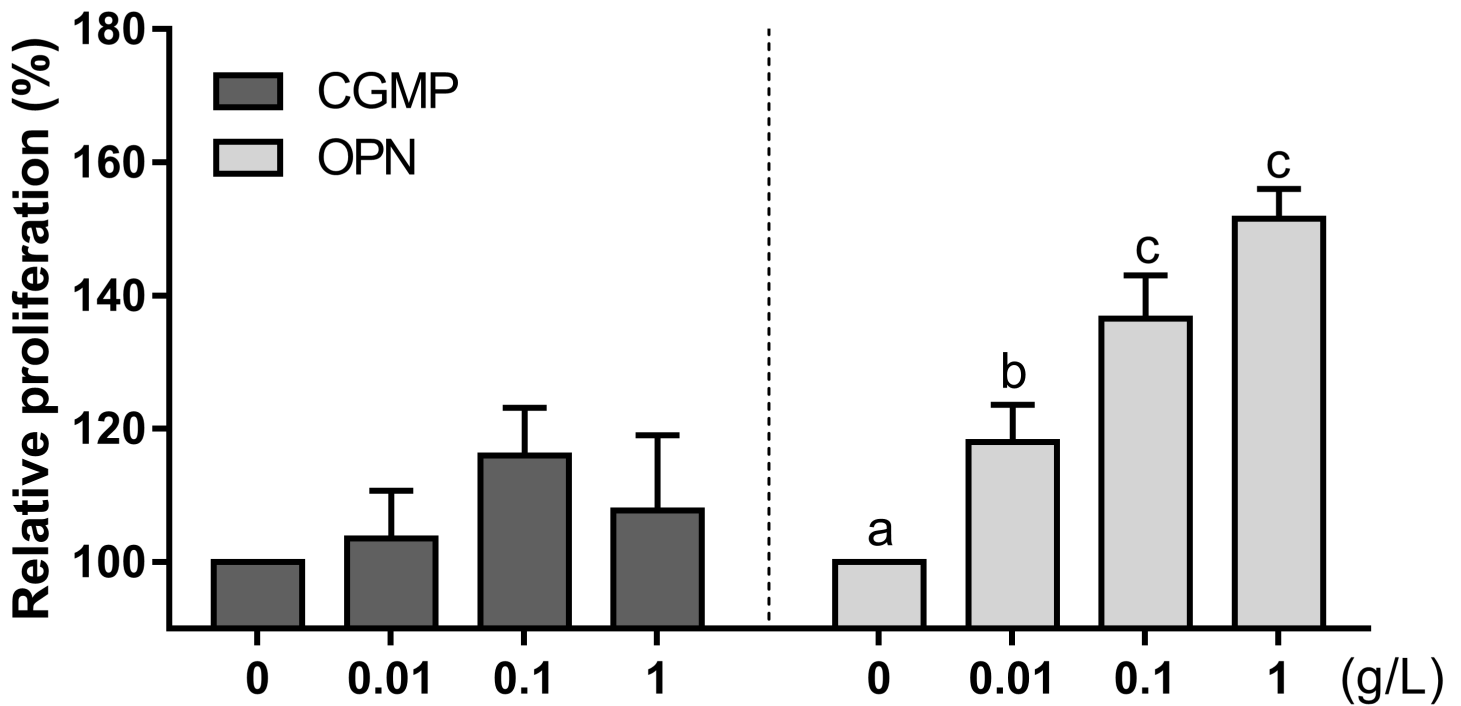
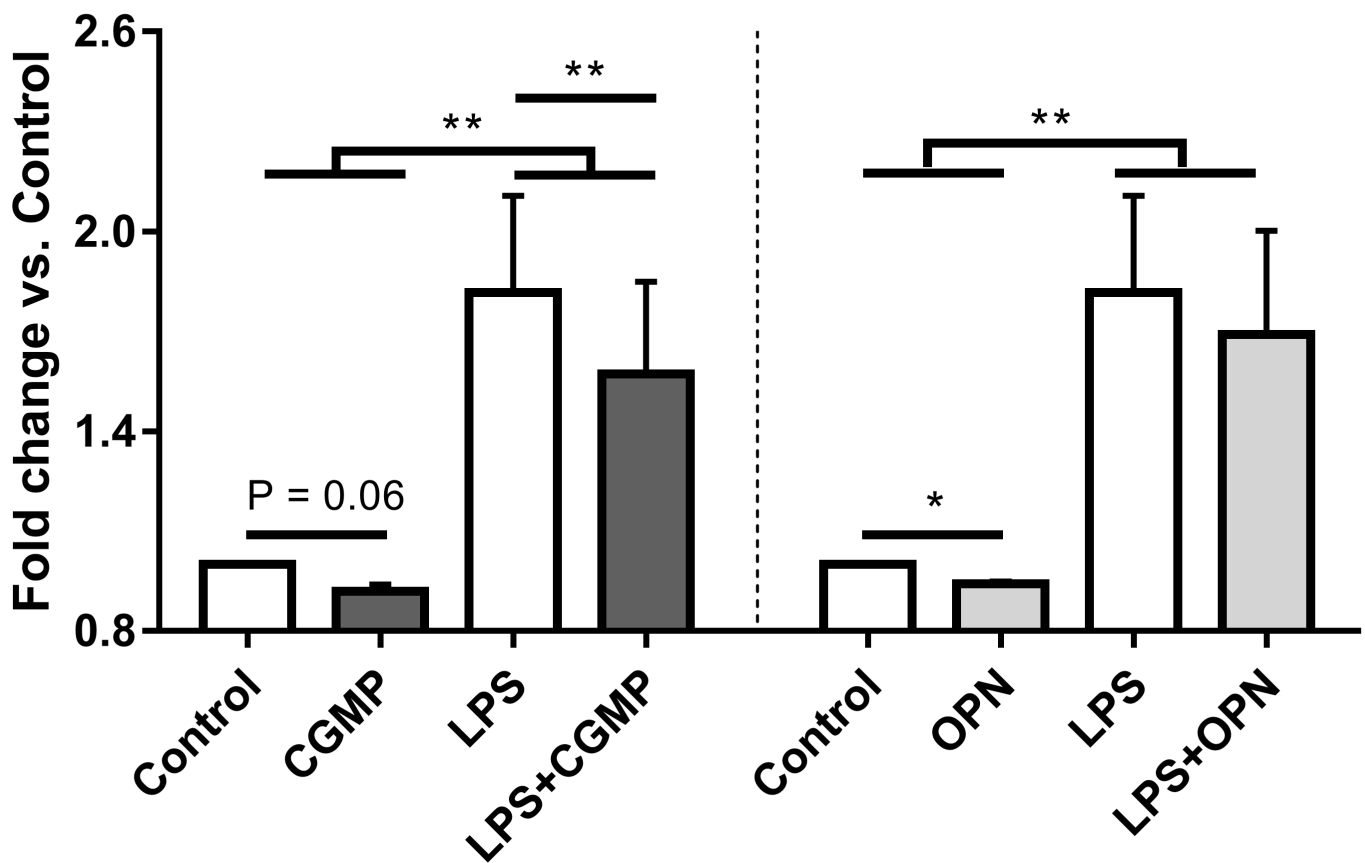
D Plasma galactose





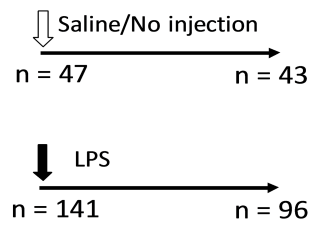




A***In vitro* IEC proliferation****B****IL-8 secretion**

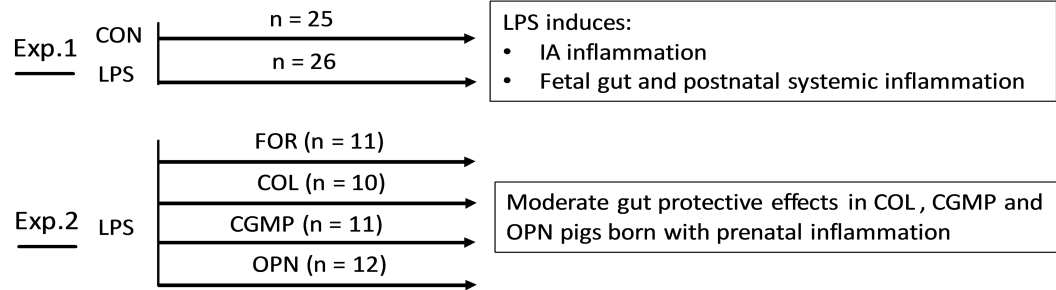
(Postnatal day) d -3

d 1



d 1

d 5



(Gestational age) 103d

106d

106d

110d