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1 # Parasite-probiotic interactions in the gut: *Bacillus* sp. and
2 # *Enterococcus faecium* regulate type-2 inflammatory responses and
3 # modify the gut microbiota of pigs during helminth infection

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25 **Abstract**

26 Dietary probiotics may enhance gut health by directly competing with pathogenic agents and
27 through immunostimulatory effects. These properties are recognized in the context of bacterial
28 and viral pathogens, but less is known about interactions with eukaryotic pathogens such as
29 parasitic worms (helminths). In this study we investigated whether two probiotic mixtures
30 (comprised of *Bacillus amyloliquefaciens, B. subtilis*, and *Enterococcus faecium* [BBE], or
31 *Lactobacillus rhamnosus* LGG and *Bifidobacterium animalis* subspecies *Lactis* Bb12 [LB])
32 could modulate helminth infection kinetics as well as the gut microbiome and intestinal immune
33 responses in pigs infected with the nodular worm *Oesophagostomum dentatum*. We observed
34 that neither probiotic mixture influenced helminth infection levels. BBE, and to a lesser extent
35 LB, changed the alpha- and beta-diversity indices of the colon and faecal microbiota, notably
36 including an enrichment of faecal *Bifidobacterium* spp. by BBE. However, these effects were
37 muted by concurrent *O. dentatum* infection. BBE (but not LB) significantly attenuated the *O.*
38 *dentatum*-induced upregulation of genes involved in type-2 inflammation and restored normal
39 lymphocyte ratios in the ileo-caecal lymph nodes that were altered by infection. Moreover,
40 inflammatory cytokine release from blood mononuclear cells and intestinal lymphocytes was
41 diminished by BBE. Collectively, our data suggest that selected probiotic mixtures can play a
42 role in maintaining immune homeostasis during type 2-biased inflammation. In addition,
43 potentially beneficial changes in the microbiome induced by dietary probiotics may be
44 counteracted by helminths, highlighting the complex inter-relationships that potentially exist
45 between probiotic bacteria and intestinal parasites.

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48 **Introduction**

49 The mammalian gut environment is maintained in a complex homeostasis encompassing
50 interactions between dietary compounds, the commensal gut microbiota (GM) and the mucosal
51 immune system. Dysregulation of this balanced ecosystem can lead to increased susceptibility to
52 pathogen infection and chronic inflammation, and is a major source of disease and morbidity in
53 humans and decreased productivity in livestock. To this end, dietary supplementation with
54 probiotic bacteria has gained increasing attention as a safe method to maintain intestinal
55 homeostasis, subsequently improving gut health. Beneficial effects of probiotics are strain-
56 specific and dose-dependent, and can be achieved by modulating intestinal motility and barrier
57 function, outcompeting enteropathogens, or by modifying the composition of host GM,
58 subsequently affecting host mucosal immune responses [1, 2].

59

60 Pigs are a key species in the food production industry and also serve as an important model for
61 human biomedical research due to similarities in gastrointestinal physiology and microbiota
62 composition [3]. Supplementation of pig diets with probiotics has revealed beneficial effects such
63 as improved growth, carcass quality, and enhanced host protective responses against different
64 pathogens, with pronounced efficiency at reducing bacterial load of enterotoxigenic *Escherichia*
65 *coli* (F4) in weaned piglets [4-8]. Additional studies against eukaryotic pathogens have also
66 reported beneficial effects of probiotics. For example, *in vitro* and murine models of *Giardia*
67 infection have shown that *Lactobacillus* spp. and *Enterococcus faecium* can eliminate infection
68 and reinforce host immune responses [9-11].

69

70 Parasitic worms (helminths) are among the most widespread gut pathogens, infecting more than
71 a billion humans worldwide and being commonly found in nearly all farmed livestock [12, 13].
72 Infection can result in marked immunopathology and a reduction in mucosal barrier function and

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73 poses a significant risk to health and productivity [14, 15]. Moreover, mucosal-dwelling helminths
74 induce strongly polarized T helper (Th) type-2 immunity and thus serve as a useful model for
75 Th2-mediated intestinal immune responses, such as those elicited by food allergens [16]. Studies on
76 the trilateral interactions between parasites, the GM and the immune system may therefore shed
77 light on the role of gut bacteria in regulating host-parasite and immune interactions at mucosal
78 barrier surfaces. Several studies have reported that feeding prebiotic dietary fibres (e.g. inulin) or
79 administration of microbial metabolites (short-chain fatty acids (SCFA) or lactic acid) can
80 strongly influence infection dynamics and immune responses induced by the large intestinal-
81 dwelling parasites *Trichuris suis* (porcine whipworm) and *Oesophagostomum dentatum* (porcine
82 nodular worm) [17-19]. These effects are thought to be mediated by GM changes in the caecum and
83 colon [19, 20], as inulin is known to increase the abundance of microbes such as *Lactobacillaceae*
84 and *Bifidobacterium* during helminth infection [19].
85
86 Reports on the effects of dietary probiotic supplementation during helminth infection are limited,
87 and whether probiotics can modulate helminth infection and associated inflammatory and
88 immunopathological changes in the large intestine, as appears to be the case with inulin and
89 other prebiotics, remains unknown. Supplementation with *Bifidobacterium animalis* subspecies
90 *Lactis* Bb12 was shown to modulate mucosal immune responses and enhance jejunal barrier
91 function in pigs infected with *Ascaris suum* [21], whilst *Lactobacillus rhamnosus* LGG intake
92 supressed the development of type-2 related immune responses in the tracheal-bronchial lymph
93 nodes of *A. suum*-infected pigs [22]. Thus, probiotic bacteria may exert immunomodulatory effects
94 in the context of type-2 immune function. In light of this, porcine models of helminth infection
95 may represent a valuable model for studying the interactions between probiotic bacteria and gut
96 pathogens, and assessing if probiotics have potential as health-promoting dietary additives that
97 can prevent or alleviate the effects of enteric helminth infection.
98

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99 Here, we investigated the effects of two different probiotic mixtures on *O. dentatum*
100 establishment and infection dynamics in pigs. In addition, we explored the interactions between
101 these probiotics and infection on GM composition throughout the intestinal tract, as well as
102 peripheral and local mucosal immune responses. We show that a dynamic relationship exists
103 between probiotic supplementation, the GM and the immune system during helminth infection,
104 which may have significant implications for our understanding of the regulation of type-2
105 inflammation in mucosal tissues, and for the application of probiotics for prevention or control of
106 intestinal diseases.
107

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108 **Results**

109

110 **Effects of probiotics on the intestinal environment and *O. dentatum* infection levels**

111 Pigs (n=48) were divided into three groups (**Supplementary Figure 1**). 16 pigs received only
112 the basal control diet (based on ground barley and soybean meal) throughout the study, 16 pigs
113 received the basal diet supplemented with a mixture of *Bacillus amyloliquefaciens B. subtilis*,
114 and *Enterococcus faecium* (hereafter BBE), and 16 pigs received the basal diet supplemented
115 with a mixture of *Lactobacillus rhamnosus* LGG and *Bifidobacterium animalis* subsp. *Lactis*
116 BB-12 (hereafter LB). The BBE mixture was selected based on its development specifically to
117 improve gut health in pigs, whilst LB was chosen as it contained two well-studied probiotic
118 strains that have previous been shown to induce immunomodulatory activity in pigs [21, 23]. Within
119 each dietary group, following a 14 day acclimatization period, half the pigs (n=8) were either
120 trickle-infected throughout the study with *O. dentatum* larvae (n=24), or remained uninfected
121 (n=24).

122 To explore the effects of probiotics on the response to helminth infection, we quantified the
123 effect of probiotic supplementation on intestinal physicochemical parameters and parasite
124 establishment and development. We first assessed the concentrations of SCFA and D-lactic acid
125 in the proximal colon (**Figure 1A**), with a separate analysis conducted for the two different
126 probiotic-supplemented groups, relative to those with no supplementation. Acetic and propionic
127 acid concentrations were unaffected by either infection or probiotic supplementation. *O.*
128 *dentatum* infection significantly increased n-Butyric acid levels ($p < 0.05$) in pigs fed either the
129 control diet alone or in those supplemented with BBE. However, there was no effect of *O.*
130 *dentatum* when analysing LB-supplemented pigs, indicating that the effect of infection varied
131 according to specific probiotic intake (**Figure 1A**). Total SCFA levels were not different
132 between any of the groups. In contrast, D-lactic acid levels were significantly increased by LB

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133 supplementation, and tended also to be increased by BBE supplementation (p = 0.08),
134 independently of infection (**Figure 1A**). Neither probiotic supplementation nor infection
135 influenced the pH in the jejunum or ileum (data not shown), or the caecum or proximal colon
136 (Figure 1B). However, infection resulted in a lower pH in the distal colon ($p < 0.05$; **Figure 1B**).
137
138 Supplementation with either of the probiotic mixtures did not significantly influence infection
139 levels or parasite infection kinetics, with average worm numbers (adult and larval *O. dentatum*)
140 of 15,843 ± 2,128 and 17,425 ± 2,185 (mean ± SEM) for pigs fed BBE and LB probiotics
141 respectively, compared to 18,455 ± 2,598 for the control-fed group (**Figure 2**). Moreover,
142 probiotic supplementation had no effect on worm length (data not shown) nor egg production;
143 with similar eggs per gram faeces (EPG) scores observed for all diet treatment groups. In
144 addition, no significant differences in body weight gain were observed between the dietary
145 treatment/infection groups, with all pigs gaining weight consistently over the course of the
146 experimental period (data not shown).
147
148 ***O. dentatum* infection changes the response of the faecal microbiota to probiotic**
149 **supplementation**
150
151 To examine if the two probiotic mixtures and/or *O. dentatum* affected the composition of the
152 prokaryotic GM, we conducted longitudinal sampling and analyses of faeces over the course of
153 the study. Across the time period, α-diversity remained stable in pigs with no probiotic
154 supplementation, regardless of whether they were infected with *O. dentatum* or not, with no
155 significant differences in Faith phylogenetic diversity (PD) (**Figure 3A).** In contrast, in both
156 uninfected and *O. dentatum*-infected pigs, BBE or LB supplementation tended to increase the
157 Faith PD over time (indicative of a more diverse microbiota at the end of the study than at the
158 start), (p = 0.065 for LB in infected pigs; p = 0.05 in other cases) (**Figure 3A**).

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159

160 There was also a significant shift in β-diversity in the faecal GM as a result of probiotic
161 supplementation, but this was dependent on infection status. Non-parametric microbial
162 interdependence testing (NMIT) indicated that infected pigs fed BBE differed in β-diversity from
163 infected pigs without probiotic supplementation ($p < 0.05$; **Figure 3B**). However, this was not
164 the case for uninfected pigs ($p = 0.26$; Figure 3B). A contrasting effect was observed for LB,
165 where uninfected pigs fed LB diverged from uninfected pigs without probiotic supplementation
166 ($p < 0.05$), yet infected pigs fed LB did not differ from infected pigs without LB (**Figure 3C**). In
167 the absence of probiotic supplementation, infection did not influence β-diversity. Analyses on
168 pooled data revealed a similar story, with both LB and BBE-fed pigs (independent of infection
169 status) significantly diverging from control-fed pigs ($p < 0.05$), whereas infection status
170 (independent of probiotic supplementation) had no effect (**Supplementary Figure 2**). Taken
171 together, these data suggest that over the course of the seven week experiment, both BBE and
172 LB probiotics induced modest but significant changes in the composition of the faecal
173 microbiota, yet these probiotic-induced changes were further influenced by concurrent *O.*
174 *dentatum* infection.

175

176 To explore which bacterial taxa were responsible for the divergence between probiotic-fed pigs
177 and their respective controls without probiotics, Feature Volatility analysis was performed.
178 Within uninfected pigs, six taxa were enriched in pigs receiving BBE compared to those that did
179 not, most notably the *Bifidobacterium* genus, whilst a single family (*Succinivibrionaceae*)
180 belonging to the Proteobacteria phylum decreased in abundance (**Figure 3D**). However, in
181 infected pigs fed BBE, relative abundance of *Bifidobacterium* spp. was lower compared to
182 infected pigs without BBE, indicating that the infection abrogated the probiotic-stimulated
183 increase in *Bifidobacteria*. *Turicibacter* sp., a genus we have previously observed to be enriched
184 in the colon of pigs infected with *Ascaris suum* [24], was elevated in infected pigs fed BBE

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185 compared to uninfected controls. Similarly, the effects of LB varied depending on infection
186 status (**Figure 3D**). In uninfected pigs, only two taxa differed between LB-fed pigs and control-
187 fed pigs without LB. In contrast, relative to the control group (uninfected pigs without
188 probiotics), infected pigs fed LB had higher relative abundance of several members of the
189 Firmicutes phylum including two *Lactobacillus* species, as well as *Mitsuokella multacida,* a
190 putative butyrate producer and beneficial microbe [25]. Collectively, these data suggest that BBE
191 tended to enrich beneficial bacteria such as *Bifidobacterium* in faeces over the course of the
192 experiment in uninfected pigs, but these effects were reversed in *O. dentatum*-infected pigs.
193 Conversely, LB tended to enrich beneficial bacteria such as *Lactobacillus* more strongly in the
194 faeces of *O. dentatum*-infected pigs than uninfected pigs. Thus, *O. dentatum* alone did not
195 change the composition of the faecal microbiota over the course of the study, but instead
196 modulated the effect of BBE and LB in two distinct ways, indicating a complex interaction
197 between probiotics and the parasitic infection.
198
199 **Probiotics and *O. dentatum* infection interact to change the intestinal microbiota in a site-**
200 **specific manner**
201
202 We next investigated how infection and/or probiotics influenced the microbiota composition
203 throughout the intestinal tract. Similarly to the longitudinal faecal samples, α-diversity (Faiths
204 PD) was increased by both BBE and LB in comparison to control pigs, mainly in the distal
205 colon, with a comparable effect in both infected and uninfected pigs (p = 0.093 for infected pigs
206 fed LB; $p < 0.05$ for other comparisons; **Figure 4**). Notably, *O. dentatum* infection was also
207 associated with increased α-diversity in the distal colon **(Figure 4; Supplementary Table 1**).
208 Effects of infection and treatment were not as pronounced in the other gut segments (**Figure 4;**
209 **Supplementary Table 1**).
210

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211 Analysis of unweighted Unifrac distance metrics showed that, in the absence of probiotic
212 supplementation, the only intestinal site where *O. dentatum* infection significantly changed β-
213 diversity, relative to uninfected pigs, was the proximal colon (the predilection site of the worms)
214 ($p < 0.05$ by PERMANOVA; **Figure 4B**). β-diversity in the gut was also considerably altered by
215 probiotic supplementation. Changes were primarily observed via unweighted Unifrac analysis,
216 indicating that most differences were driven by low-abundance species. In uninfected pigs, BBE
217 supplementation altered β-diversity compared to pigs without probiotic supplementation in the
218 ileum, caecum and both proximal and distal colon ($p = 0.096$ for caecum, $p < 0.05$ for all other
219 segments by PERMANOVA; **Figure 4C; Supplementary Table 2**). However, this effect was
220 less evident when the BBE-supplemented pigs were infected with *O. dentatum*. In these animals,
221 supplementation with BBE resulted in no significant difference in β-diversity in the ileum or
222 caecum relative to control pigs (uninfected and without probiotics). Furthermore, lesser (albeit
223 still significantly different) changes were observed in the colon between control pigs and
224 infected pigs receiving BBE (**Figure 4C; Supplementary Table 2**). Thus, infection appeared to
225 attenuate the BBE-induced changes in GM composition.

226

227 LB also tended to alter β-diversity in the jejunum and caecum, with similar changes in both
228 uninfected and infected pigs ($p < 0.1$ by PERMANOVA; **Figure 4C; Supplementary Table 3**).
229 LB had a stronger effect in the colon (both proximal and distal). Here, significant divergence was
230 observed between control and LB-fed pigs, regardless of infection status ($p < 0.05$ by
231 PERMANOVA). However, within LB-fed pigs, infected pigs were significantly diverged from
232 uninfected pigs with infected pigs clustering closer to the control animals ($p < 0.05$ by
233 PERMANOVA; **Figure 4C; Supplementary Table 3**), again indicating that infection tended to
234 limit the modulatory effects of the probiotics on the GM.

235

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236 We attempted to identify specific taxa responsible for the differences between treatment groups,
237 however ANCOM analysis yielded no significant differences in any gut segment ($p > 0.05$).
238 Thus, the changes in the GM community within the gut segments appeared to derive from the
239 cumulative effect of subtle alterations across multiple taxa, rather than substantial alterations in
240 the abundance of precise bacterial species.

241

242 **Both probiotics and *O. dentatum* infection influence peripheral and local immune function**

243

244 We next assessed how probiotic supplementation modulated the development of the systemic
245 and mucosal response to *O. dentatum* infection. Serum IgA and IgG_1 antibody levels were
246 measured weekly until day 28 p.i. All pigs were sero-negative for *O. dentatum* prior to study
247 start at day 0. Infection with *O. dentatum* resulted in increased *O. dentatum*-specific antibody
248 titres compared to uninfected pigs (**Figure 5A**). Both IgA and IgG_1 antibody titre levels
249 increased from day 7 through until day 28 p.i. There was a significant interaction between time
250 and LB probiotics at day 21 p.i., whereby LB-fed infected pigs had higher IgA levels compared
251 to the other infected groups ($p < 0.005$), however this difference was not apparent at other time
252 points. BBE probiotics did not influence IgA titres, and there was no effect of probiotic
253 supplementation on IgG_1 titres.

254

255 Analysis of CLN lymphocyte populations revealed a significant interaction between BBE
256 probiotic supplementation and *O. dentatum* infection. In control-fed pigs, infection increased the
257 percentage of T cells ($p < 0.01$), and reduced the percentage of B-cells ($p < 0.05$) resulting in an
258 altered T-cell/B-cell ratio (**Figure 5B**). However, this effect was not apparent in infected pigs
259 fed the BBE probiotics, with the T-cell/B-cell ratio equivalent to uninfected pigs, indicating that
260 *O. dentatum*-induced alterations in lymphocyte populations were attenuated in these animals
261 (**Figure 5B**). In contrast, LB probiotic supplementation did not have this modulatory effect, with

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262 no significant interaction and only a main effect of infection in analysis of both T-cell and B-cell
263 populations (**Figure 5B**). Analysis of other cell populations, namely $CD3^{+}CD4^{+}$ helper and
264 $CD3^{+}CD8^{+}$ cytotoxic T cells, or monocytes, showed no significant effects of either diet or
265 infection (data not shown).
266
267 To assess functional cellular immune responses in peripheral and lymphoid tissues, PBMCs and
268 CLN cells were stimulated with LPS or PHA, respectively, and cytokine secretion quantified.
269 Infection did not consistently change the cytokine secretion pattern (**Figure 6**). In contrast, BBE
270 supplementation substantially modulated cytokine profiles, although the effect was dependent on
271 infection status. There was an interaction ($p < 0.05$) between probiotics and infection on
272 mitogen-induced TNFα secretion from CLN cells, with BBE supplementation significantly
273 reducing TNFα production in uninfected pigs, but not in infected animals. In contrast, IL-10
274 production tended to be enhanced by BBE in both infected and uninfected pigs ($p = 0.06$ for
275 main effect of probiotic supplementation; **Figure 6A**). In PBMCs, BBE significantly suppressed
276 LPS-induced IL-1β in both uninfected and infected pigs ($p < 0.05$; **Figure 6B**), with a similar
277 tendency for IL-10 secretion ($p = 0.06$; **Figure 6B**). TNFα followed the same pattern but the
278 differences were not significant (**Figure 6B**). There was an interaction ($p < 0.05$) between
279 probiotics and infection for IL-6 production, with secretion reduced in uninfected pigs fed with
280 BBE, but tended to be enhanced in infected pigs (**Figure 6B**). The effects of LB probiotics were
281 less apparent. LB supplementation resulted in lower ($p < 0.05$) TNFα secretion from CLN cells,
282 independently of infection status, but there were no effects on the other cytokines measured in
283 either CLN or PBMC (**Supplementary Figure 3**). Collectively, these data suggest that BBE
284 probiotics have an anti-inflammatory effect in the absence of parasite infection. However this
285 effect was modulated in infected pigs. Whereas IL-1β was strongly suppressed in PBMC from
286 both uninfected and infected animals receiving BBE, the effect on other cytokines such as IL-6
287 appeared to be influenced by the parasitic infection, with the suppressive effect less evident in

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288 infected pigs. These data suggest that concurrent helminth infection may restrict the anti-
289 inflammatory properties of BBE probiotics.
290

291 **Probiotics attenuate *O. dentatum*-induced inflammatory gene expression in the proximal**
292 **colon**
293

294 To explore in more detail if the dietary probiotics modulated local host immune responses, we
295 investigated changes in gene expression in the proximal colon during *O. dentatum* infection. A
296 panel of genes was selected to represent Th1-, Th2- and regulatory immune responses, as well as
297 mucosal barrier and innate immunity-related genes. Principal component analysis (PCA) of the
298 relative expression of all genes analysed in the proximal colon illustrated a marked effect of *O.*
299 *dentatum* infection (**Figure 7A**), and a lesser influence of probiotic supplementation (**Figure**
300 **7B**). In the absence of probiotic supplementation, there was a prototypical type-2 polarised
301 immune gene expression profile in the proximal colon of pigs infected with *O. dentatum*, relative
302 to uninfected animals. Infection with *O. dentatum* significantly increased expression of *IL4*,
303 *IL13*, *ARG1*, *CCL17* and *CCL26,* with a concurrent trend for down-regulation of the expression
304 of Th1-related genes such as *IL8* (**Figure 7C**; **Supplementary Table 4**). In addition, increased
305 expression of mucosal barrier-related genes, such as *RETNLB*, *FFAR2*, and *DCLK1,* and innate
306 immune genes such as *IL6*, *C3* and *PTGS2* (encoding cyclooxygenase-2) were also observed in
307 infected, control-fed pigs (**Figure 7C**; **Supplementary Table 4**).
308

309 We noted a moderately enhanced Th1 polarization as a result of probiotic supplementation. Both
310 probiotic treatments increased the expression of *IL8*, *IL12B* and *INOS* in both uninfected and *O.*
311 *dentatum*-infected animals. LB supplementation also significantly increased *IFNG* expression
312 (**Figure 7C**; **Supplementary Table 4**), as well as *CXCL10* expression but only in males ($p <$
313 0.05 for interaction between sex and LB supplementation).

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314

315 Strikingly, in *O. dentatum*-infected pigs, BBE supplementation markedly attenuated the
316 helminth-induced increases in gene expression relative to control-fed animals In BBE-fed pigs,
317 Th2 genes were still up-regulated as a result of helminth infection, but to a lesser degree
318 compared to *O. dentatum* infected pigs fed only the control diet (**Figure 7C**). For genes where
319 there was a significant interaction ($p < 0.05$) between BBE supplementation and infection, in
320 every case this resulted in significant down-regulation of expression in infected, BBE-fed pigs
321 compared to infected, control-fed pigs (**Supplementary Table 4**). This included key Th2 and
322 epithelial/ mucosal barrier related genes, including those coding for the short-chain fatty acid
323 receptor *FFAR2,* the epithelial cell kinase and tuft cell marker *DCLK1* the interleukin-4 receptor
324 *IL4*, and the eosinophil chemoattractant *CCL26* (**Figure 7D**). Moreover, the helminth-induced
325 expression of other immune related genes such as *TNF, CTLA4* and *PLA2G4A* was significantly
326 attenuated by BBE supplementation (**Figure 7D**). This was evident in PCA analysis which
327 showed that *O. dentatum*-infected pigs administered BBE clustered closer to uninfected control
328 pigs than *O. dentatum*-infected pigs without probiotic supplementation, suggesting that the
329 response to infection was muted in these animals , and that BBE acted to restrain the localized
330 inflammatory response to the parasite (**Figure 7E**). A similar pattern was evident in infected pigs
331 with LB supplementation, but the effect was less pronounced, with the immune gene expression
332 profiles with the immune gene profile more closely resembling that of *O. dentatum*-infected pigs
333 fed the control diet (**Figure 7E**). However, we did note a trend ($p < 0.1$) for interactions between
334 infection and LB supplementation for the expression, of *ARG1, TLR3, IL1B,* and *CTLA4*, with
335 the infection-induced expression of these genes being attenuated to some extent by LB (**Figure**
336 **7C; Supplementary Table 4)**. Thus, probiotic supplementation (most primarily with BBE)
337 acted to attenuate parasite-induced, type-2 biased inflammatory responses in the colon.

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345 **Discussion**
346
347 The beneficial effect of probiotics on health and control of bacterial infections is well-
348 documented, however the potential interactions of probiotics with helminth infection and the
349 mechanisms by which they can influence mucosal immune responses is not well understood. We
350 found that probiotics (BBE in particular) were capable of supressing *ex vivo* inflammatory
351 cytokine production and attenuating the host mucosal immune responses elicited in response to
352 infection. Neither probiotic mixture modulated the establishment or infection kinetics of *O.*
353 *dentatum*. However, both mixtures appeared to beneficially modulate the intestinal microbiota
354 composition, as evidenced by increased bacterial diversity in both faecal and large intestinal
355 samples. Interestingly, we noted that these effects were to some extent modulated by *O.*
356 *dentatum* infection, suggesting a novel interaction of parasite infection on probiotic activity.
357 Furthermore, we observed attenuation of the prototypical type-2 inflammation induced by *O.*
358 *dentatum* by BBE probiotics.
359
360 *O. dentatum* infection is highly prevalent in pigs worldwide. Whereas dietary prebiotics, such as
361 inulin, have been shown to be highly effective in reducing parasite burdens, our results here
362 show that supplementation of these specific probiotic strains did not have an anti-parasitic effect.
363 The mode-of-action of prebiotics against *O. dentatum* is hypothesized to result from a selective
364 enrichment of lactic acid producing bacteria, and production of GM-derived metabolites such as
365 SCFA, which lower the colon pH and create an inhospitable environment for helminths [26].

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366 Despite an increase in D-lactic acid induced by LB, we did not observe changes in gut pH (or
367 total SCFA levels) as a result of either probiotic mixture. Thus, the administration of certain
368 probiotic bacteria was insufficient to have an anthelmintic effect, although associated effects on
369 the immune system or GM may still markedly impact gut health.
370
371 Helminth infection is typically associated with a rise in antibody secretion and the initiation of a
372 characteristic Th2 immune response. Similarly to Andreasen *et al*. (2015) [27] we observed a type-
373 2 immune response in control-fed pigs infected with *O. dentatum*, with increased antibody
374 secretion, peripheral T cell activation, and type-2 immune gene expression profiles in the
375 proximal colon confirming an active host immune response was elicited. Interestingly, infected
376 pigs fed BBE probiotics exhibited a reduction in epithelial immune genes, such as *TSLP*, *IL4R*
377 and *FFAR2*, compared to the *O. dentatum*-infected pigs fed only the control diet. In addition,
378 BBE treatment alone tended to reduce expression of key Th2 immune genes, such as *IL4*, *IL5*
379 and *CCL26*, and appeared to diminish the parasite-induced increase in the expression of these
380 genes in infected pigs fed BBE. Together, this suggests that the typical polarised helminth-
381 mediated Th2 immune response is attenuated by the supplementation of *Bacillus* spp. plus *E.*
382 *faecium*-based probiotics. This attenuation of prototypical helminth-induced immune response
383 has been observed previously in *A. suum*-infected pigs fed *L. rhamnosus* LGG [22]. Jang *et al.*
384 (2017) [22] reported reduced IgG_2 antibody titres and reduced expression of *IL13*, eosinophil
385 peroxidase *EPX*, and *CCL26* in *A. suum*-infected pigs supplemented with LGG. The observed
386 suppression of Th2 and epithelial gene expression profiles in this study may have been the result
387 of the probiotics exerting a regulatory effect to maintain intestinal immune homeostasis.
388
389 We observed that probiotic supplementation appeared to significantly alter the intestinal
390 microbiota, with both mixtures (BBE and LB) improving the microbial diversity and richness
391 over the course (day 28 post-infected compared to 7 days pre-infection) of the study and at

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392 different segments of the intestinal tract. PERMANOVA analysis confirmed that probiotic
393 supplementation did have a modulatory effect on the microbiota, although the changes could not
394 be ascribed to specific taxa. The modest impact of probiotics on the composition of the GM
395 appears to be in keeping with several studies that reported minor compositional alterations as a
396 result of supplementation with a range of probiotic strains [6, 28]. Interestingly, both probiotic
397 mixtures induced subtle alterations to SCFA and lactic acid levels present in intestinal digesta,
398 suggesting that even with limited changes in the GM, potentially beneficial outcomes to
399 intestinal health can still be achieved, as was evident by the modulation of intestinal immune
400 gene expression profiles.
401
402 To our knowledge this is the first time the porcine GM has been characterised during *O.*
403 *dentatum* infection. Consistent with previous observations in pigs infected with *T. suis* [19, 20], *O.*
404 *dentatum* infection altered β-diversity in the caecum and colon. However, unlike *T. suis,* this
405 modulation did not appear to be associated with defined bacterial taxa, and significant changes
406 were not observed in faeces or the small intestine. This suggests that *O. dentatum* infection had a
407 localised impact on the GM without inducing changes throughout the intestinal tract. The most
408 striking observation was the apparent ability of *O. dentatum* to suppress the changes in the GM
409 brought about by probiotics that were observed in uninfected pigs. Thus, concurrent parasitic
410 infections, which are common in livestock and humans in developing countries, may be a
411 previously unappreciated factor influencing the health benefits of dietary probiotics.
412
413 The mechanisms by which probiotics alter the response to helminth infection requires further
414 investigation. Various modes-of-action have been proposed for the health benefits of probiotic
415 bacteria. Probiotics may adhere to intestinal epithelial cells and thereby prevent the attachment
416 of potentially pathogenic bacteria such as *E. coli*, as well as inducing mucus production and the
417 stimulation of antimicrobial peptides [29]. Furthermore, probiotics may regulate inflammatory

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418 responses by binding to PRRs on immune cells and promoting secretion of IL-10 or TGF-β,
419 which can supress inflammatory cytokine production [30]. Moreover, probiotics such as LGG have
420 previously been shown to promote Th1 responses in pigs, and the Th1-stimulating properties of
421 probiotics has been suggested to underlie the ability of probiotics to supress symptoms of
422 allergies in humans and animal models [30, 31]. Indeed, our gene expression data in the colon
423 indicated a modest Th1-polarizing effect of both probiotic mixtures in the absence of infection,
424 suggesting that host pattern recognition receptors recognize the bacteria and respond with
425 production of type-1 cytokines and innate immune mediators that are typically produced in
426 response to TLR or NOD receptor binding. Probiotics have also been shown to induce regulatory
427 responses that can alleviate inflammation during pathogen challenge in pigs [32], and thus the
428 attenuation of the helminth-induced type-2 response may then derive from the ability of the
429 probiotic bacteria to restore homeostasis in the face of acute pathogen-driven inflammation.
430 Interestingly, we observed that BBE appeared to be more efficient than LB in modulating host
431 immune responses, which may reflect the inclusion of porcine-derived strains in the BBE
432 mixture.
433

434 In conclusion, we show here that probiotics, in particular the strains *Bacillus amyloliquefaciens,*
435 *B. subtilis*, and *Enterococcus faecium*, do not appear to directly affect worm establishment and
436 development but do regulate inflammatory responses and attenuate host mucosal immune
437 function during *O. dentatum* infection, which may serve to regulate host intestinal function and
438 maintain immune homeostasis. This probiotic-mediated regulation of host immune responses is
439 also indicative of the ability of probiotics to potentially dampen Th2-mediated pathology as a
440 result of, for example, food allergies [33-35]. Moreover, the ability of these probiotic strains to
441 attenuate pathogen-induced inflammatory responses may have relevance for dietary interventions
442 that seek to maintain intestinal homeostasis during infectious challenge.
443

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452 ## Materials and methods

453

454 ### Experimental design

455 A total of 48 Yorkshire-Landrace pigs (females and castrated males, 8-10 weeks old, initial body
456 weight approximately 20 kg) were sourced from a specific pathogen-free farm. After
457 stratification based on sex and weight, pigs were randomly allocated to one of six groups. Each
458 treatment group contained eight pigs housed in a separate pen. Two groups (each n=8) received
459 the basal diet only (based on ground barley and soybean containing 16.2% crude protein). Two
460 groups (each n=8) received the same diet supplemented with BBE containing the strains *Bacillus*
461 *amyloliquefaciens* 516 (porcine origin), *B. subtilis* 541 (human origin), and *Enterococcus*
462 *faecium* 669 (human origin). The final two groups (each n=8) received the basal diet
463 supplemented with LB, containing the strains *Lactobacillus rhamnosus* LGG® (human origin;
464 DSM33156) and *Bifidobacterium animalis* subsp. *Lactis* BB-12® (food origin; DSM15954). All
465 probiotic strains were supplied by Chr.Hansen A/S, Denmark. Pigs were fed twice a day with the
466 probiotic-supplements mixed with the standard feed immediately before feeding. For both
467 probiotic mixtures, pigs received 2×10^{10} CFU per day.

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469 After two weeks of diet adaptation, a total of 24 pigs (8 pigs from each diet treatment group)
470 were each inoculated with 25 *O. dentatum* third stage larvae (L3)/ kg body weight, by oral
471 gavage. These pigs subsequently continued to receive the same *O. dentatum* L3 dose three days a
472 week until study end (a total of four weeks). Infection doses were provided during the morning
473 feeding, and were uniformly distributed on top of the feed. The dosed feed was provided in
474 troughs that allowed all pigs' adequate space to feed equally and simultaneously. The dosing
475 regime was chosen to mimic a natural moderate exposure level and the average approximate
476 theoretical total dose during the study was 22,000 *O. dentatum* L3/ pig. The remaining 24 pigs
477 were uninfected for the duration of the study.
478
479 All pigs had been vaccinated against *L. intercellularis* with one dose of a live, attenuated vaccine
480 (Enterisol® Ileitis, Boehringer Ingelheim) on farm four weeks prior to arriving on the
481 experimental premises (which was six weeks prior to infection with *O. dentatum*). All pigs were
482 confirmed negative for *O. dentatum* infection upon arrival by McMaster faecal egg count and
483 serology. For the duration of the study, all pigs were housed on concrete floored pens with wood
484 chips and water provided *ad libitum*. Welfare checks were performed daily, with body weight
485 monitored and reported weekly. At day 28 post-infection (p.i.), 48 pigs were sacrificed over the
486 course of three days by stunning with captive bolt followed by exsanguination.
487
488 This study was approved by the Danish Animal Experimentation Inspectorate (License number
489 2015-15-0201-00760), and performed at the Experimental Animal Unit, University of
490 Copenhagen according to FELASA guidelines and recommendations.
491
492 **Digesta sampling and *O. dentatum* isolation**
493 Weekly blood and faecal samples were taken between arrival (day -14) and until the end of the
494 study (day 28 p.i.). Blood samples were taken in order to collect serum for ELISA (see below),

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495 and isolate peripheral blood mononuclear cells (PBMCs; day 28 p.i. only). Faecal samples were
496 scored following a 5-point scale (1 – hard; 2 – normal; 3 – soft; 4 – watery; 5 – diarrhoea) in
497 order to monitor changes in faecal consistency as a result of probiotic supplementation. After
498 scoring, samples were cooled to ~4°C immediately upon collection for subsequent enumeration
499 of *O. dentatum* egg counts per gram of faeces (EPG) using a McMaster faecal egg count method
500 (as described in Roepstorff & Nansen, 1998) [36].

501

502 At necropsy, fresh intestinal digesta samples were collected from specific intestinal sections:
503 jejunum (mid-point of the small intestine), ileum (10 cm proximal from ileocaecal junction),
504 caecum, proximal colon (20 cm distal from ileocaecal junction) and distal colon (central part of
505 the spiral) colon) for microbiota and pH measurement, with additional samples taken from the
506 proximal colon for SCFA analysis, as previously described [19]. Following this, *O. dentatum* larvae
507 and adults were recovered according to the agar-gel migration technique described previously by
508 Slotved *et al*. (1996) [37]. Briefly, luminal contents of caecum and colon were collected and diluted
509 to a total volume of 10 litres using 0.9% saline (37°C). A 5% sub-sample was then embedded in
510 2% agar on cloths that were then suspended in saline and incubated for 24 hours at 37°C to
511 isolate immature and adult *O. dentatum* from each pig. Worms were isolated on a 38 µm mesh
512 and stored in 70% ethanol for later enumeration. For each pig, ten adult female and male worms
513 were selected for length measurement, using Leica Application Suite version 4.7 (Leica
514 Microsystems, Germany), as a measure of *O. dentatum* fitness.

515

516 **Cell isolation, flow cytometry and assessment of cytokine production**

517 Ileo-caecal lymph nodes (CLNs) were dissected and passed through a 70 µM cell strainer to
518 obtain single cell suspensions. After a series of washing, the cells were prepared for flow
519 cytometric phenotypic analysis of T cells, B cells and monocyte populations as described in
520 Myhill *et al.* (2018) [15]. Flow cytometry was performed using a BD Accuri C6 flow cytometer

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521 (BD Biosciences), and data were analysed using Accuri CFlow Plus software (Accuri®
522 Cytometers Inc., MI, USA). PBMCs were isolated from heparinised whole blood using
523 Histopaque-1077 (Sigma-Aldrich) and centrifugation. To assess cytokine production, isolated
524 CLN cells were cultured for 48 hours in complete media (RPMI 1640 supplemented with 2 mM
525 L-glutamine, 10% calf serum, 100µg/mL streptomycin and 100 U/mL penicillin) together with
526 10 µg/mL phytohemagglutinin (Sigma-Aldrich). Measurement of secreted TNFα and IL-10 was
527 assessed
528 using commercial ELISA kits (R&D systems). Isolated PBMCs in complete media were
529 stimulated with LPS (1 µg/mL), cultured for 24 hours, and concentrations of IL-6, TNFα, IL-10
530 and IL-1β assessed by ELISA. Values below the detection limit were assigned an arbitrary value
531 of half the lowest value of the standard curve.
532

533 ***O. dentatum* culture**

534 *O. dentatum* larvae were isolated from infected control-fed pigs, and washed extensively in 37°C
535 saline. The exsheathed larvae were cultured in complete media containing antibiotics and
536 fungicide for 3 days at 37°C to obtain excretory/secretory (E/S) products. Every day the culture
537 media was removed and stored at -80°C, and replaced with fresh media. Pooled culture media
538 containing E/S was concentrated by centrifugation using Amicon ultra centrifugal filter units
539 (MWCO 10 kDa, Sigma-Aldrich, Denmark), and filtered prior to testing of protein content by
540 bicinchoninic (BCA) assay (Thermo Fisher Scientific).
541

542 ***O. dentatum* ELISA**

543 Anti-*O. dentatum* IgA and IgG_1 levels in serum were quantified by ELISA as described in
544 Myhill *et al*. (2018) [19]. Briefly, plates (Nunc Maxisorb) were coated with 5 µg/mL *O. dentatum*
545 larval E/S overnight at 4°C. Serum antibodies were then detected using goat anti-pig IgA-

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546 horseradish peroxidase (HRP; BioRad, Germany), or mouse anti-pig IgG_1 (clone K139-3C8;
547 BioRad) followed by goat anti-mouse IgG-HRP conjugate (BioRad). Incubations were for 1 hour
548 at 37°C, and between all steps, plates were washed four times with PBS plus 0.02% Tween 20.
549 After development with tetramethylbenzidine (TMB) substrate, the reaction was stopped with
550 0.2M H_2SO_4, and the plates read at 450 nM with a Multiskan FC plate reader (Waltham,
551 Massachusetts, USA).
552
553 **Quantitative real-time PCR**
554 Total RNA was extracted from proximal colon tissue using a miRNAeasy® Mini kit (Qiagen,
555 CA, USA) according to manufacturer's guidelines, and as described in Myhill *et al.* (2018) [19].
556 Synthesis of cDNA and pre-amplification was conducted as described in Williams *et al*. (2017)
557 [24]. A panel of 77 genes of interest, including key Th1/Th2/Treg/innate immune response-related
558 genes and epithelial/mucosal barrier function-related genes, were examined on a BioMark HD
559 Reader (Fluidigm). First, a thermal mix and hot start protocol was performed to mix primers,
560 samples and reagents (50°C for 2 min, 70°C for 30 min, 25°C for 10 min, 50°C for 2 minutes,
561 95°C for 10 min), followed by qPCR using the following cycling conditions of: 35 cycles at
562 95°C for 15 seconds and 60°C for 1 min. After data pre-processing, 68 genes of interest passed
563 quality control criteria and were statistically analysed. Normalization using several validated
564 reference housekeeping genes and data pre-processing, was carried out as described in
565 Skovgaard *et al.* (2009) [38]. Primer sequences are presented in **Supplementary Table 5.**
566
567 **16S rRNA sequencing of microbiota**
568 DNA was extracted from faeces or intestinal content in a randomized order using the Bead-Beat
569 Micro AX Gravity Kit (A&A Biotechnology, Poland) according to manufacturer's instructions.
570 Prior to extraction, samples were lysed in LSU buffer supplemented with Lysozyme (4000 U)
571 and Mutanolysin (50 U), and incubated at 50°C for 20 min. The concentration and purity of

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572 extracted DNA were determined using a NanoDrop ND-1000 spectrophotometer and normalized
573 to 10 ng/µl. High throughput sequencing based 16S rRNA gene amplicon (V3-region)
574 sequencing was carried out on an Illumina NextSeq platform as previously described [39].
575
576 The raw dataset containing pair-ended reads with corresponding quality scores were merged and
577 trimmed using fastq_mergepairs and fastq_filter scripts implemented in the USEARCH pipeline as
578 described previously [39]. Purging the dataset from chimeric reads and constructing zero radius
579 Operational Taxonomic Units (zOTU) was conducted using UNOISE. The Greengenes (13.8) 16S
580 rRNA gene collection was used as a reference database. Quantitative Insight Into Microbial
581 Ecology (QIIME) open source software package (v2019.7.0) was used for subsequent analysis steps
582 [40]. Alpha diversity measures: observed species (number of zOTUs) and Shannon diversity indices
583 were computed for rarefied OTU tables (10,000 reads/sample) using the alpha rarefaction
584 workflow. Differences in alpha diversity were determined using a t-test-based approach employing
585 the non-parametric (Monte Carlo) method (999 permutations) implemented in the compare alpha
586 diversity workflow. Principal Coordinates Analysis (PCoA) plots were generated with the
587 Jackknifed Beta Diversity workflow based on 10 distance metrics calculated using 10 sub-sampled
588 OTU tables. The number of sequences taken for each jackknifed subset was set to 85% of the
589 sequence number within the most indigent sample (~10,000). Community differences (beta-
590 diversity) were revealed by weighted and unweighted Unifrac distance metrics visualised as
591 Principle Coordinate Analysis (PCoA) plots. Permutational Multivariate Analysis of Variance
592 (PERMANOVA) and Non-parametric microbial interdependence test (NMIT) were used to evaluate
593 group differences based on weighted and unweighted UniFrac distance matrices. Taxa-level
594 differences were assessed using longitudinal feature-volatility analysis and analysis of composition
595 of microbes (ANCOM).
596

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597 **Statistical analysis**

598 Data were analysed using general linear model (GLM) using IBM SPSS Statistics 28. For each
599 separate probiotic mixture (BBE or LB), the effects of probiotic supplementation and parasite
600 infection, and their interaction, were compared to control-fed animals using a separate factorial
601 analysis. The model included infection status, probiotic supplementation and sex as fixed
602 factors, together with their first-order interactions. Sex was removed from the model when not
603 significant. For analysis of ELISA data, time was included as an additional fixed factor to
604 account for repeated measurements. Assumptions of normality were checked through inspection
605 of histogram plots and Shapiro-Wilk and Kolmogorov-Smirnov tests of GLM residuals, and data
606 that did not conform to normality was transformed with either square-root or $\log_{10}$
607 transformations prior to analysis. Significance was taken at $p < 0.05$, and a trend at $p < 0.1$.

608

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613 with qPCR. In addition, we would like to thank Bea Nielsen (Chr. Hansen A/S) for providing all
614 probiotic strains/ mixtures used within this study, and for technical advice.

615

616 **Conflicts of interest**

617 The authors have no conflicts of interest to declare.

618

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623 **Data Availability Statement**

624 Raw sequence data is available at Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/)
625 under the accession number PRJNA746763. All other data is available within the manuscript or
626 supplementary material.

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630

631 **Figure Legends**

632

633 **Figure 1. Effect of probiotics and *Oesophagostomum dentatum* infection on the intestinal**
634 **environment**

635 **(A)** Microbial metabolite (short-chain fatty acids and D-lactic acid) concentrations from
636 proximal colon digesta after 28 days of *O. dentatum* infection, in pigs fed a control diet or a diet
637 supplemented with either a mixture of *Enterococcus faecium* and *Bacillus* sp. (BBE), or LGG
638 and Bb12 (LB). Metabolite concentrations are expressed in mmol/kg wet sample. **(B)** pH of
639 digesta sampled throughout the intestinal tract.

640 Statistical analysis was conducted separately for each probiotic treatment, using a GLM analysis
641 comparing the effect of probiotic supplementation and infection (and their interaction) to the
642 control-diet groups (no probiotics). Data presented as means ± SEM (*$p \leq 0.05$, ***$p \leq 0.005$,
643 by GLM). n=8 pigs per treatment group.

644

645 **Figure 2. *Oesophagostomum dentatum* burden is not affected by probiotic treatment.**

646 *O. dentatum* adult and larval worm burdens, at day 28 post-infection in pigs fed a control diet or
647 a diet supplemented with either a mixture of *Enterococcus faecium* and *Bacillus* sp. (BBE), or
648 LGG and Bb12 (LB). Data presented as means ± SEM. n=8 pigs per treatment group.

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649

650 **Figure 3. Probiotics modulate the faecal gut microbiota over time**

651 **A)** Alpha-diversity (Faith PD) in faeces samples over time from -7 to day 28 post-infection (p.i.).
652 Pigs were either uninfected or infected with *O. dentatum* (Od) and fed a control diet or a diet
653 supplemented with either a mixture of *Enterococcus faecium* and *Bacillus* sp. (BBE), or LGG
654 and Bb12 (LB). (**B-C**) NMIT PCoA showing effect of infection and diet in pigs fed BBE (**B**) or
655 LB (**C**) from day -7 to day 28 p.i. **D)** Taxa where abundance was significantly altered in faeces
656 across the course of the experiment as a result of infection or diet, as identified by Feature
657 Volatility Analysis. n=8 pigs per treatment group.

658

659 **Figure 4. Probiotics and parasite infection modulate the gut microbiota in different**
660 **gastrointestinal compartments.**

661 **A)** Alpha-diversity (Faith PD) in different gut segments at day 28 post-infection. Pigs were either
662 uninfected or infected with *O. dentatum* (Od), and fed a control diet or a diet supplemented with
663 a either mixture of *Enterococcus faecium* and *Bacillus* sp. (BBE), or LGG and Bb12 (LB). *P*-
664 values are shown in Supplementary Table 1. n=8 pigs per treatment group.

665 **B)** Unweighted PCoAs for pairwise comparisons of uninfected and *Oesophagostomum dentatum*
666 (Od)-infected pigs fed only the control diet (no probiotics) in the caecum and proximal and distal
667 colon.

668 **C)** Unweighted PCoAs for pairwise comparisons of uninfected and *O. dentatum* (Od)-infected
669 pigs fed only the control diet (no probiotics), or a diet supplemented with either a mixture of
670 *Enterococcus faecium* and *Bacillus* sp. (BBE), or LGG and Bb12 (LB).

671

672 **Figure 5. Systemic and peripheral immune responses elicited towards *Oesophagostomum***
673 ***dentatum* infection.**

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674 **A)** *O. dentatum* specific IgA and IgG_1 serum antibody production over the 28 days of infection in
675 pigs fed a control diet or a diet supplemented with either a mixture of *Enterococcus faecium* and
676 *Bacillus* sp. (BBE), or LGG and Bb12 (LB).
677 **(B)** Flow cytometric analysis of ileo-caecal lymph node cells obtained at day 28 post-infection.
678 % CD3+ T cells and % CD79α B-cells. Statistical analysis was conducted separately for each
679 probiotic treatment, using a GLM analysis comparing the effect of probiotic supplementation and
680 infection (and their interaction) to the control-diet groups (no probiotics). Data presented as
681 means ± SEM (*$p \leq 0.05$, ***$p \leq 0.005$, by GLM). n=8 pigs per treatment group.
682

683 **Figure 6. *Ex vivo* cytokine secretion is modulated by probiotics and *Oesophagostomum***
684 ***dentatum* infection**
685 **A)** Phytohaemagglutinin-induced secretion of TNFα and IL-10 in ileal-caecal lymph node
686 cultures. Pigs were either uninfected or infected with *O. dentatum* for 28 days, with or without
687 supplementation of a mixture of *Enterococcus faecium* and *Bacillus* sp. (BBE). **B)** LPS-induced
688 secretion of IL-1β, IL-6, TNF α and IL-10 in peripheral blood mononuclear cells from pigs
689 infected with *O. dentatum* for 28 days or uninfected pigs, with or without supplementation of
690 BBE. * $p < 0.05$ by GLM analysis. n=8 pigs per treatment group.
691

692 **Figure 7. Probiotics and *Oesophagostomum dentatum* infection alters immune gene**
693 **expression profiles.**
694 **A-B)** Principal component analysis of immune gene expression in the proximal colon at day 28
695 post-infection as a result of *O. dentatum* infection **(A)** or diet supplementation with probiotic
696 mixtures *Enterococcus faecium* and *Bacillus* sp. (BBE). or LGG and Bb12 (LB) **(B)**. **C)**
697 Expression of genes involved in different biological function as a result of *O. dentatum* infection
698 (Od), BBE or LB supplementation, or *O. dentatum* infection combined with BBE or LB
699 supplementation. The control group received no infection or probiotic treatment. Data presented

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700 as Z-scores of relative gene expression data. **D)** Fold changes in expression of genes from
701 proximal colon tissue significantly altered ($p < 0.05$) by the interaction of *Oesophagostomum*
702 *dentatum* infection and dietary supplementation with a mixture of *Enterococcus faecium* and
703 *Bacillus* sp. (BBE), in comparison to control-fed, *O. dentatum*-infected controls. n=8 pigs per
704 treatment group.

705 **E)** Principal component analysis showing immune gene expression in the proximal colon at day
706 28 post-infection in control pigs (no infection or probiotics), *O. dentatum* infection without
707 probiotics, and *O. dentatum* with BBE supplementation.

708

709

710 **References**

711

712 1. FAO, WHO. Probiotics in food - Health and nutritional properties and guidelines for evaluation. FAO Food
713 and Nutrition Paper 85. Rome: Food and Agriculture Organization of the United Nations and World Health
714 Organization, 2006:http://www.fao.org/3/a-a0512e.pdf.

715 2. Travers MA, Florent I, Kohl L, Grellier P. Probiotics for the control of parasites: an overview. Journal of
716 parasitology research 2011; 2011:610769.

717 3. Xiao L, Estellé J, Kiilerich P, Ramayo-Caldas Y, Xia Z, Feng Q, et al. A reference gene catalogue of the pig
718 gut microbiome. Nature microbiology 2016; 1:16161.

719 4. Böhmer BM, Kramer W, Roth-Maier DA. Dietary probiotic supplementation and resulting effects on
720 performance, health status, and microbial characteristics of primiparous sows. J Anim Physiol Anim Nutr (Berl) 2006;
721 90:309-15.

722 5. Suo C, Yin Y, Wang X, Lou X, Song D, Wang X, et al. Effects of lactobacillus plantarum ZJ316 on pig
723 growth and pork quality. BMC Vet Res 2012; 8:89.

724 6. Sato Y, Kuroki Y, Oka K, Takahashi M, Rao S, Sukegawa S, et al. Effects of Dietary Supplementation With
725 Enterococcus faecium and Clostridium butyricum, Either Alone or in Combination, on Growth and Fecal Microbiota
726 Composition of Post-weaning Pigs at a Commercial Farm. Frontiers in Veterinary Science 2019; 6.

727 7. Wang K, Chen G, Cao G, Xu Y, Wang Y, Yang C. Effects of Clostridium butyricum and Enterococcus
728 faecalis on growth performance, intestinal structure, and inflammation in lipopolysaccharide-challenged weaned piglets.
729 J Anim Sci 2019; 97:4140-51.

730 8. Konstantinov SR, Smidt H, Akkermans ADL, Casini L, Trevisi P, Mazzoni M, et al. Feeding of Lactobacillus
731 sobrius reduces Escherichia coli F4 levels in the gut and promotes growth of infected piglets. FEMS Microbiol Ecol
732 2008; 66:599-607.

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733 9. Pérez PF, Minnaard J, Rouvet M, Knabenhans C, Brassart D, De Antoni GL, et al. Inhibition of Giardia
734 intestinalis by extracellular factors from Lactobacilli: an in vitro study. Appl Environ Microbiol 2001; 67:5037-42.

735 10. Shukla G, Bhatia R, Sharma A. Prebiotic inulin supplementation modulates the immune response and restores
736 gut morphology in Giardia duodenalis-infected malnourished mice. Parasitol Res 2016; 115:4189-98.

737 11. Benyacoub J, Pérez PF, Rochat F, Saudan KY, Reuteler G, Antille N, et al. Enterococcus faecium SF68
738 Enhances the Immune Response to Giardia intestinalis in Mice. The Journal of Nutrition 2005; 135:1171-6.

739 12. Freeman MC, Akogun O, Belizario V, Jr., Brooker SJ, Gyorkos TW, Imtiaz R, et al. Challenges and
740 opportunities for control and elimination of soil-transmitted helminth infection beyond 2020. PLOS Neglected Tropical
741 Diseases 2019; 13:e0007201.

742 13. Morgan ER, Aziz N-AA, Blanchard A, Charlier J, Charvet C, Claerebout E, et al. 100 Questions in Livestock
743 Helminthology Research. Trends in Parasitology 2019; 35:52-71.

744 14. McKay DM, Shute A, Lopes F. Helminths and intestinal barrier function. Tissue barriers 2017; 5:e1283385.

745 15. Jensen TK, Christensen CM. Dose related mucosal hyperplasia induced by Oesophagostomum dentatum
746 infection in pigs. Can J Vet Res 1997; 61:315-8.

747 16. Thomas DJ, Husmann RJ, Villamar M, Winship TR, Buck RH, Zuckermann FA. Lactobacillus rhamnosus
748 HN001 Attenuates Allergy Development in a Pig Model. PLOS ONE 2011; 6:e16577.

749 17. Petkevicius S, Bach Knudsen KE, Murrell KD, Wachmann H. The effect of inulin and sugar beet fibre on
750 oesophagostomum dentatum infection in pigs. Parasitology 2003; 127:61-8.

751 18. Petkevicius S, Murrell KD, Bach Knudsen KE, Jørgensen H, Roepstorff A, Laue A, et al. Effects of short-
752 chain fatty acids and lactic acids on survival of Oesophagostomum dentatum in pigs. Vet Parasitol 2004; 122:293-301.

753 19. Myhill LJ, Stolzenbach S, Hansen TVA, Skovgaard K, Stensvold CR, Andersen LO, et al. Mucosal Barrier and
754 Th2 Immune Responses Are Enhanced by Dietary Inulin in Pigs Infected With Trichuris suis. Front Immunol 2018;
755 9:2557.

756 20. Stolzenbach S, Myhill LJ, Andersen LO, Krych L, Mejer H, Williams AR, et al. Dietary Inulin and Trichuris
757 suis Infection Promote Beneficial Bacteria Throughout the Porcine Gut. Front Microbiol 2020; 11:312.

758 21. Solano-Aguilar G, Shea-Donohue T, Madden KB, Quinoñes A, Beshah E, Lakshman S, et al. Bifidobacterium
759 animalis subspecies lactis modulates the local immune response and glucose uptake in the small intestine of juvenile
760 pigs infected with the parasitic nematode Ascaris suum. Gut microbes 2018; 9:422-36.

761 22. Jang S, Lakshman S, Beshah E, Xie Y, Molokin A, Vinyard BT, et al. Flavanol-Rich Cocoa Powder Interacts
762 with Lactobacillus rhamnossus LGG to Alter the Antibody Response to Infection with the Parasitic Nematode Ascaris
763 suum. Nutrients 2017; 9.

764 23. Mao X, Gu C, Hu H, Tang J, Chen D, Yu B, et al. Dietary Lactobacillus rhamnosus GG Supplementation
765 Improves the Mucosal Barrier Function in the Intestine of Weaned Piglets Challenged by Porcine Rotavirus. PLOS
766 ONE 2016; 11:e0146312.

767 24. Williams AR, Krych L, Fauzan Ahmad H, Nejsum P, Skovgaard K, Nielsen DS, et al. A polyphenol-enriched
768 diet and Ascaris suum infection modulate mucosal immune responses and gut microbiota composition in pigs. PLoS
769 One 2017; 12:e0186546.

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770 25. Kalyana Chakravarthy S, Jayasudha R, Ranjith K, Dutta A, Pinna NK, Mande SS, et al. Alterations in the gut
771 bacterial microbiome in fungal Keratitis patients. PLoS One 2018; 13:e0199640.

772 26. Petkevičius S, Murrell KD, Bach Knudsen KE, Jørgensen H, Roepstorff A, Laue A, et al. Effects of short-
773 chain fatty acids and lactic acids on survival of *Oesophagostomum dentatum* in pigs. Veterinary Parasitology 2004;
774 122:293-301.

775 27. Andreasen A, Petersen HH, Kringel H, Iburg TM, Skovgaard K, Dawson H, et al. Immune and inflammatory
776 responses in pigs infected with Trichuris suis and Oesophagostomum dentatum. Vet Parasitol 2015; 207:249-58.

777 28. Li P, Niu Q, Wei Q, Zhang Y, Ma X, Kim SW, et al. Microbial shifts in the porcine distal gut in response to
778 diets supplemented with Enterococcus Faecalis as alternatives to antibiotics. Sci Rep 2017; 7:41395.

779 29. Ballou MA, Davis EM, Kasl BA. Nutraceuticals: An Alternative Strategy for the Use of Antimicrobials.
780 Veterinary Clinics of North America: Food Animal Practice 2019; 35:507-34.

781 30. Plaza-Diaz J, Ruiz-Ojeda FJ, Gil-Campos M, Gil A. Mechanisms of Action of Probiotics. Adv Nutr 2019;
782 10:S49-S66.

783 31. Klei TR. Symposium: New approaches in the study of animal parasites. Veterinary Parasitology 2004;
784 125:147-61.

785 32. Zhou D, Zhu Y-H, Zhang W, Wang M-L, Fan W-Y, Song D, et al. Oral administration of a select mixture of
786 *Bacillus* probiotics generates Tr1 cells in weaned F4ab/acR– pigs challenged with an F4+ ETEC/VTEC/EPEC strain.
787 Veterinary Research 2015; 46:95.

788 33. Shin HS, Eom JE, Shin DU, Yeon SH, Lim SI, Lee SY. Preventive Effects of a Probiotic Mixture in an
789 Ovalbumin-Induced Food Allergy Model. J Microbiol Biotechnol 2018; 28:65-76.

790 34. Huang C-H, Shen C-C, Liang Y-C, Jan T-R. The probiotic activity of Lactobacillus murinus against food
791 allergy. J Funct Foods 2016; 25:231-41.

792 35. Schiavi E, Barletta B, Butteroni C, Corinti S, Boirivant M, Di Felice G. Oral therapeutic administration of a
793 probiotic mixture suppresses established Th2 responses and systemic anaphylaxis in a murine model of food allergy.
794 Allergy 2011; 66:499-508.

795 36. Roepstorff A, Nansen P. Chapter 3: Faecal examinations for parasites. FAO Animal Health Manual 3:
796 Epidemiology, Diagnosis and Control of Helminth Parasites in Swine, 1998:35-41.

797 37. Slotved HC, Barnes EH, Bjørn H, Christensen CM, Eriksen L, Roepstorff A, et al. Recovery of
798 Oesophagostomum dentatum from pigs by isolation of parasites migrating from large intestinal contents embedded in
799 agar-gel. Vet Parasitol 1996; 63:237-45.

800 38. Skovgaard K, Mortensen S, Boye M, Poulsen KT, Campbell FM, Eckersall PD, et al. Rapid and widely
801 disseminated acute phase protein response after experimental bacterial infection of pigs. Vet Res 2009; 40:23.

802 39. Krych L, Kot W, Bendtsen KMB, Hansen AK, Vogensen FK, Nielsen DS. Have you tried spermine? A rapid
803 and cost-effective method to eliminate dextran sodium sulfate inhibition of PCR and RT-PCR. J Microbiol Methods
804 2018; 144:1-7.

805 40. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis
806 of high-throughput community sequencing data. Nat Methods 2010; 7:335-6.

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807

A

Acetic Acid
BBE
Control
LB
O. dentatum

Propionic Acid
BBE
Control
LB
O. dentatum

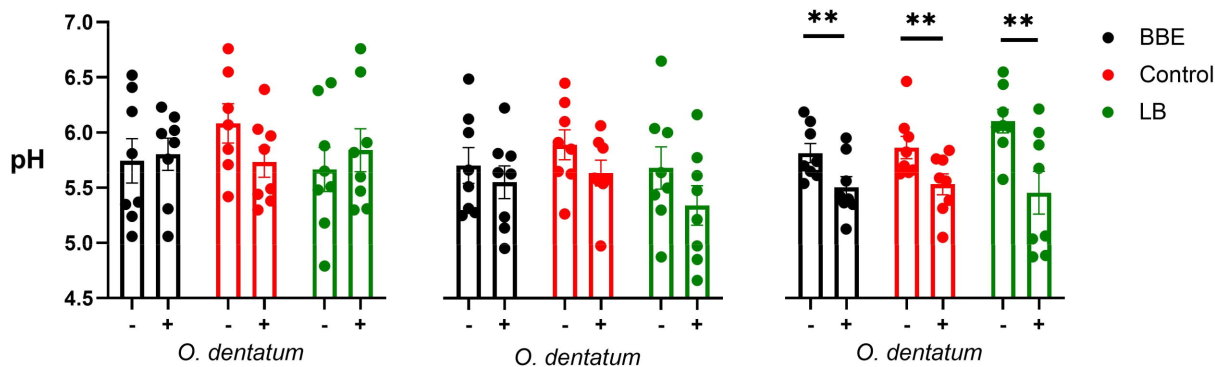
n-Butyric Acid
BBE
Control
LB
O. dentatum

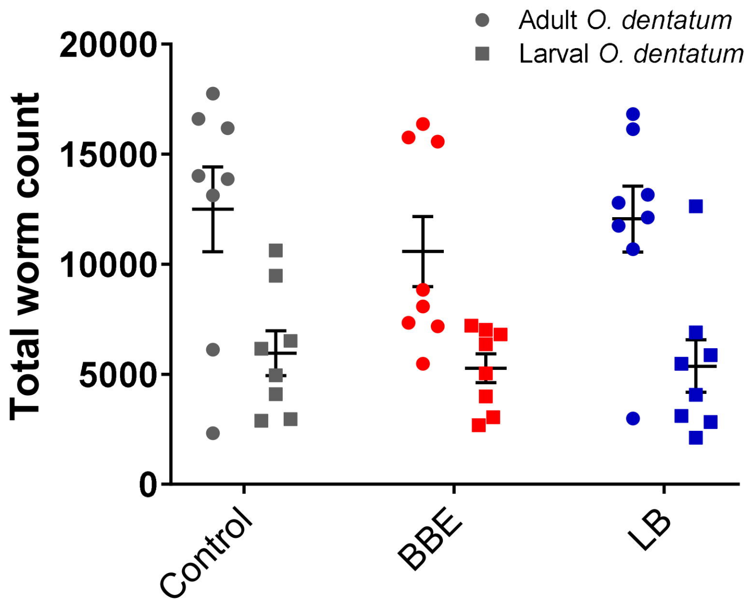
n-Valeric acid
BBE
Control
LB
O. dentatum

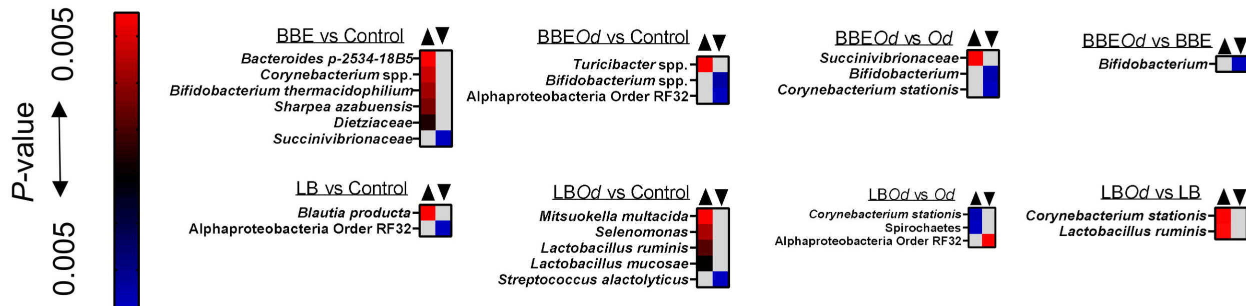
Total SCFA
BBE
Control
LB
O. dentatum

D-lactic acid
BBE
Control
LB
O. dentatum

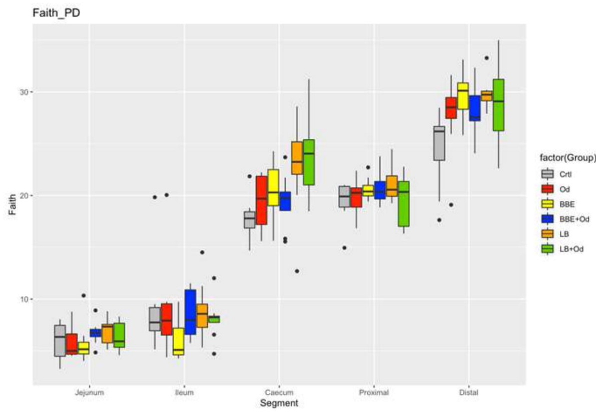
B




Adult O. dentatum
Larval O. dentatum
Total worm count
20000
15000
10000
5000
0
Control
BBE
LB

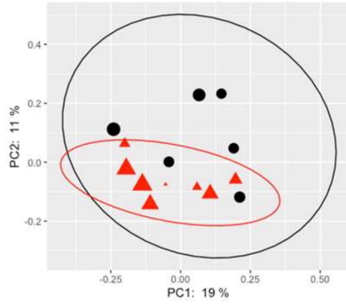

A
FaithPD
Difference
Group
factor(Group)
Crtl
Od
BBE
BBE+Od
LB
LB+Od
B
NMIT
PC1: 5 %
Group
BBE
BBE+Od
Crtl
Od
C
NMIT
PC1: 5 %
PC2: 5 %
Group
Crtl
LB
LB+Od
Od
D
P-value
0.005
0.005
BBE vs Control
Bacteroides p-2534-18B5
Corynebacterium spp.
Bifidobacterium thermacidophilium
Sharpea azabuensis
Dietziaceae
Succinivibrionaceae
BBEOd vs Control
Turicibacter spp.
Bifidobacterium spp.
Alphaproteobacteria Order RF32
BBEOd vs Od
Succinivibrionaceae
Bifidobacterium
Corynebacterium stationis
BBEOd vs BBE
Bifidobacterium
LB vs Control
Blautia producta
Alphaproteobacteria Order RF32
LBOd vs Control
Mitsuokella multacida
Selenomonas
Lactobacillus ruminis
Lactobacillus mucosae
Streptococcus alactolyticus
LBOd vs Od
Corynebacterium stationis
Spirochaetes
Alphaproteobacteria Order RF32
LBOd vs LB
Corynebacterium stationis
Lactobacillus ruminis

A

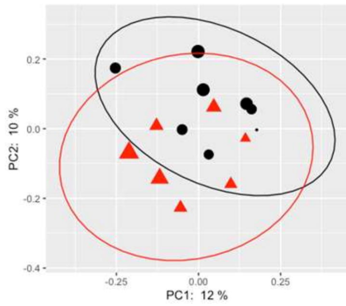


B

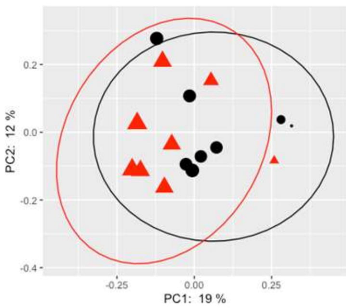
CAECUM



PROXIMAL



DISTAL



C

BBE
LB
JEJUNUM
PC2: 9 %
PC1: 15 %
PC2: 9 %
PC1: 14 %
ILEUM
PC2: 9 %
PC1: 16 %
PC2: 10 %
PC1: 16 %
CAECUM
PC2: 9 %
PC1: 16 %
PC2: 9 %
PC1: 19 %
PROXIMAL
PC2: 7 %
PC1: 10 %
PC2: 7 %
PC1: 14 %
DISTAL
PC2: 10 %
PC1: 14 %
PC2: 10 %
PC1: 17 %
BBE
BBE+Od
Crtl
LB
LB+Od

A

IgA

IgG$_1$

OD (450nm)

0.4
0.3
0.2
0.1
0.0
-0.1

Day 0
Day 7
Day 14
Day 21
Day 28

Control
BBE
LB
O. dentatum
O. dentatum + BBE
O. dentatum + LB

B

% CD3$^+$ T-cells

80
70
60
50
40
30

**
**

BBE
Control
LB

% CD79α B-cells

60
40
20
0

*
*

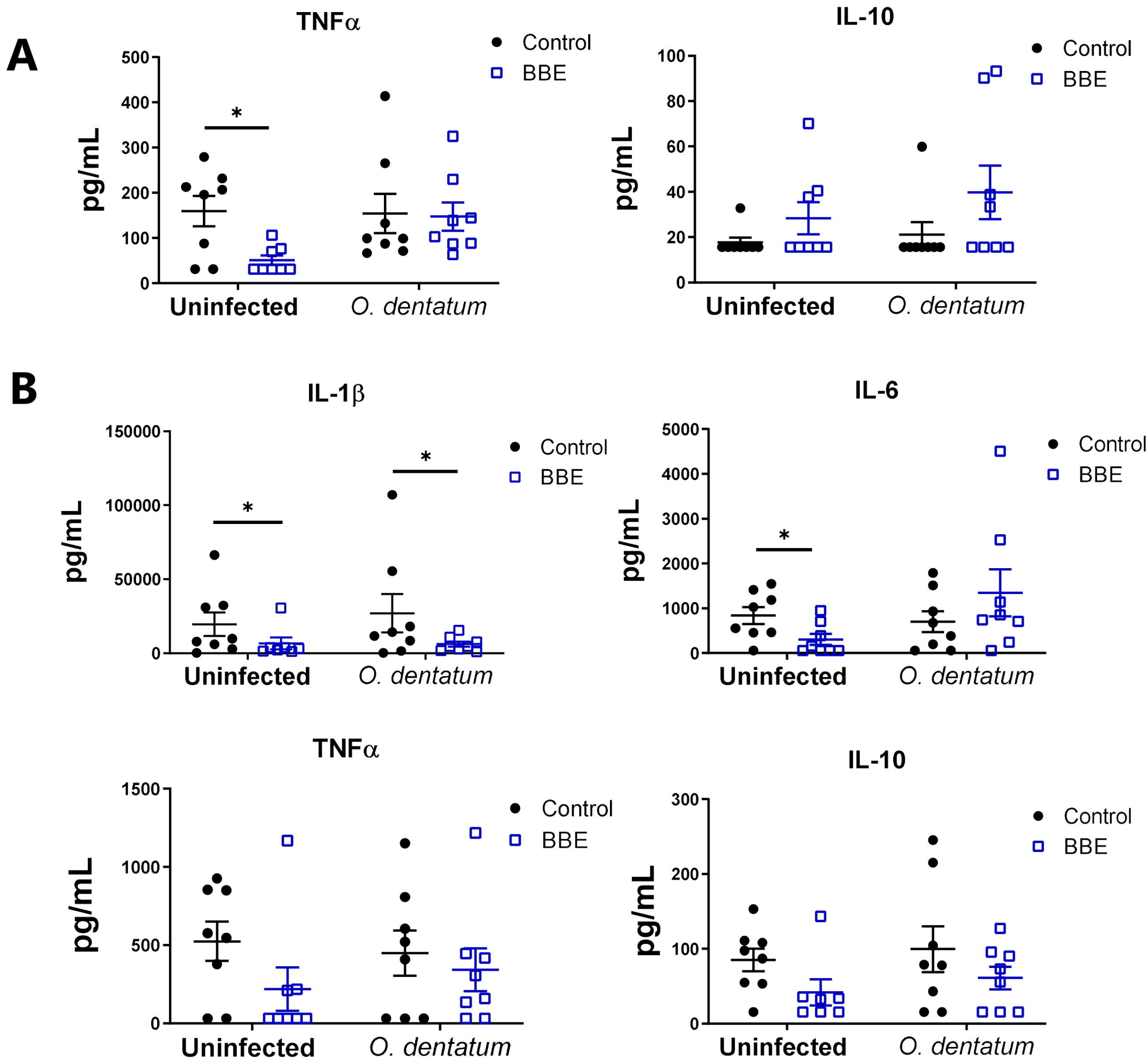
BBE
Control
LB

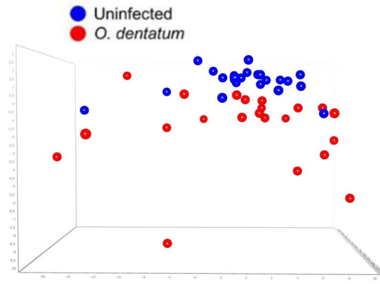
- + - + - +

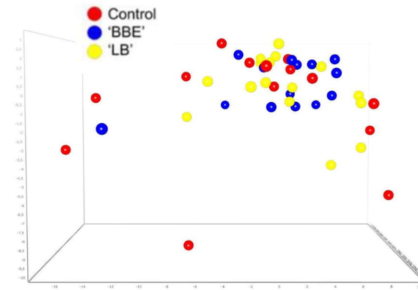
O. dentatum

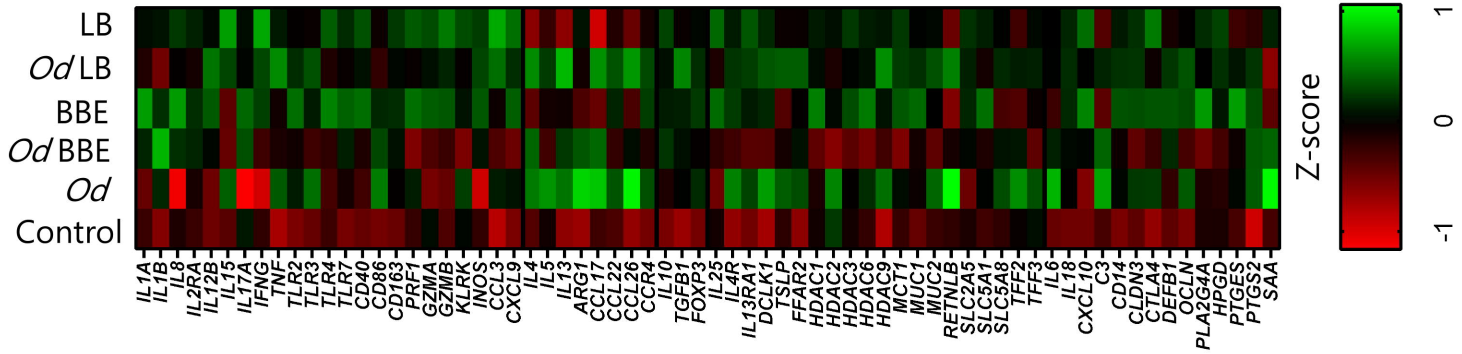
- + - + - +

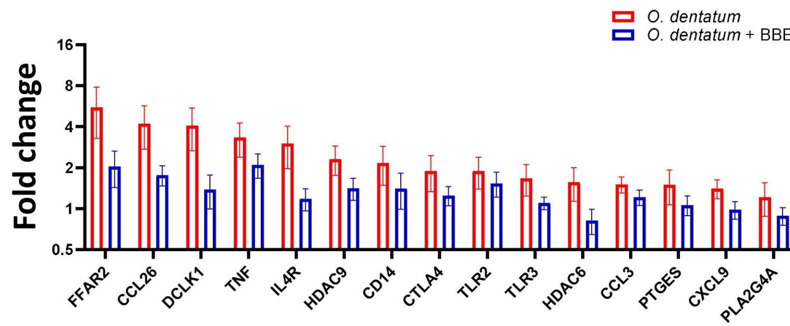
O. dentatum

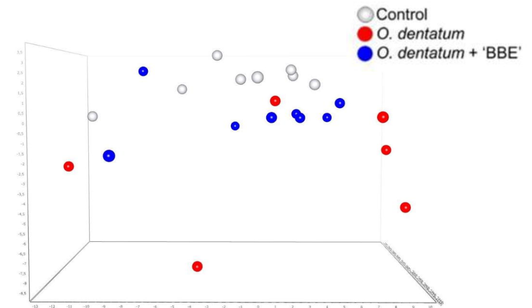

A
TNFα
Control
BBE
pg/mL
500
400
300
200
100
0
*
Uninfected
O. dentatum
IL-10
100
80
60
40
20
B
IL-1β
150000
100000
50000
IL-6
5000
4000
3000
2000
1000
1500
1000
500
300
200


A
Uninfected
O. dentatum


B
Control
'BBE'
'LB'


C
Th1
Th2
Treg
Epithelial Function
Innate Defence
LB
Od LB
BBE
Od BBE
Od
Control
IL1A
IL1B
IL8
IL2RA
IL12B
IL15
IL17A
IFNG
TNF
TLR2
TLR3
TLR4
TLR7
CD40
CD86
CD163
PRF1
GZMA
GZMB
KLRK
INOS
CCL3
CXCL9
IL4
IL5
IL13
ARG1
CCL17
CCL22
CCL26
CCR4
IL10
TGFB1
FOXP3
IL25
IL4R
IL13RA1
DCLK1
TSLP
FFAR2
HDAC1
HDAC2
HDAC3
HDAC6
HDAC9
MCT1
MUC1
MUC2
RETNLB
SLC2A5
SLC5A1
SLC5A8
TFF2
TFF3
IL6
IL18
CXCL10
C3
CD14
CLDN3
CTLA4
DEFB1
OCLN
PLA2G4A
HPGD
PTGES
PTGS2
SAA
Z-score
1
0
-1


D
O. dentatum
O. dentatum + BBE
Fold change
16
8
4
2
1
0.5
FFAR2
CCL26
DCLK1
TNF
IL4R
HDAC9
CD14
CTLA4
TLR2
TLR3
HDAC6
CCL3
PTGES
CXCL9
PLA2G4A


E
Control
O. dentatum
O. dentatum + 'BBE'