Herpes Simplex Virus Infection, Acyclovir and IVIG Treatment All Independently Cause Gut Dysbiosis.

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Abstract.

Herpes simplex virus 1 (HSV) is a ubiquitous human virus resident in a majority of the global population as a latent infection. Acyclovir (ACV), is the standard of care drug used to treat primary and recurrent infections, supplemented in some patients with intravenous immunoglobulin (IVIG) treatment to suppress deleterious inflammatory responses. We found that HSV, ACV and IVIG can all independently disrupt the gut bacterial community in a sex biased manner when given to uninfected mice. Treatment of HSV infected mice with ACV or IVIG alone or together revealed complex interactions between these drugs and infection that caused pronounced sex biased dysbiosis. ACV reduced *Bacteroidetes* levels in male but not female mice, while levels of the Anti-inflammatory Clostridia (AIC) were reduced in female but not male mice, which is significant as these taxa are associated with protection against the development of GVHD in hematopoietic stem cell transplant (HSCT) patients. Gut barrier dysfunction is associated with GVHD in HSCT patients and ACV also decreased *Akkermansia muciniphila,* which is important for maintaining gut barrier functionality. Cumulatively, our data suggest that long-term prophylactic ACV treatment of HSCT patients may contribute to GVHD and potentially impact immune reconstitution. These data have important implications for other clinical settings, including HSV eye disease and genital infections, where ACV is given long-term.

Author Summary.

Primary and reactivated HSV and VZV infections are treated with Acyclovir (ACV), an antiviral drug that blocks viral DNA synthesis. In some patients IVIG is used as adjunctive therapy to block deleterious inflammation. Long term preventative treatment of patients who receive stem transplants for various blood cancers has been successful in preventing life threatening reactivated HSV and VZV infections, but GVHD remains a major factor limiting transplant

success. Studies reported here reveal that HSV infection, ACV and IVIG given alone can all disrupt the gut microbiota and that complex interactions between these drugs and infection results in even more pronounced sex biased changes in the gut bacteria community structure. Importantly, ACV treatment decreased the levels of specific bacterial taxa, including the anti-inflammatory *Clostriodia* and *Bacteroidetes* that have been shown to protect against development of GVHD in stem cell transplant patients. These data suggest that long term preventative treatment of patients with ACV may contribute to GVHD in transplant patients and have negative consequences in other HSV induced diseases treated long term with ACV. The health effects of long term ACV and IVIG treatments warrant further clinical studies.

Introduction.

Herpes Simplex Virus type 1 (HSV), a ubiquitous human virus is the major cause of HSV encephalitis (HSE), the most prevalent sporadic encephalitis resulting from either primary infection or reactivation of latent virus. However, despite improved diagnostic procedures and effective antiviral therapies, most HSE survivors have persistent neurological impairments, including memory and behavior disturbances, dysphasia and seizures, and only 50-65% of these survivors return to independent living [1, 2]. A delay in initiating Acyclovir (ACV) treatment past the second hospital day is associated with poor neurological outcomes [3, 4]. Recent clinical trials evaluating prolonged oral ACV/valaciclovir (VACV) treatment following standard 14-day intravenous ACV treatment reported improved neurocognitive outcomes in neonates but not adults for reasons that are obscure [5, 6]. Although, it is generally accepted that replication induced pathology underlies HSV related neurological dysfunction, supporting experimental or clinical evidence is lacking. Overwhelming evidence has linked inflammation to the development of various neurological disorders and neuropsychiatric diseases, including Alzheimer's disease

82 (AD), schizophrenia, autism spectrum disorder (ASD), multiple sclerosis (MS), Parkinson's
83 disease (PD), depression and anxiety [7-9].
84
85 Having unequivocally established that HSE arises from exaggerated CNS inflammatory
86 responses and that the immunomodulatory activities of intravenous immunoglobulins (IVIG) can
87 prevent HSE in a mouse model [10], we tested the hypothesis that persistent inflammation, which
88 is documented in humans and mice after HSE [11-14], causes neurobehavioral impairments in
89 survivors, that should be impeded by IVIG's anti-inflammatory activity [10]. Compared to treatment
90 of HSV infected mice with ACV or PBS alone, treatment with ACV+IVIG from day 4 pi reduced
91 CNS inflammation and anxiety, consistent with our hypothesis. Strikingly, development of learning
92 and memory (LM) deficits that were evident only in female PBS treated mice, were inhibited by
93 ACV treatment and counterintuitively, aggravated by ACV+IVIG treatment. Treatment of infected
94 male mice with ACV+IVIG also impaired LM compared to ACV or PBS alone, revealing that IVIG
95 antagonized the beneficial effects of ACV [15]. Intriguingly, the differential antagonistic effects of
96 ACV+IVIG on cognitive behavior in HSV infected mice, compared to ACV and PBS treatment
97 alone, were reflected in differential serum proteomic profiles [15]. These reported antagonistic
98 effects of ACV and IVIG on LM present a conundrum, since they are at odds with the known
99 mechanisms of action of these drugs.
100
101 Rapidly accumulating evidence is revealing the critical role of the microbiome in regulating
102 brain homeostasis and function such that perturbation of the gut bacteria community structure
103 and function is increasingly being implicated in a variety of neurodegenerative and
104 neuropsychiatric diseases. In an effort to gain insight into how HSV induces LM impairment and
105 the paradoxical effects of ACV and IVIG, we investigated a role for the gut microbiota. HSV
106 infection, ACV and IVIG were all associated with significant disruption of the gut bacterial
107 community structure that was sex biased. Furthermore, treating HSV infected mice with either

108 ACV or IVIG alone or both drugs together resulted in more pronounced sex-biased shifts in the
109 gut bacterial community structure compared to uninfected mice. These results have significant
110 clinical implications, particularly when patients receive prolonged ACV or IVIG treatment.
111
112 **Results**.
113
114 Equal numbers (n=8) of female and male C57BL/6 mice were bilaterally inoculated with
115 virulent HSV1 strain 17+ ($1x10^5$ PFU/eye) by corneal scarification as previously described [15].
116 At day 4 post infection (pi), ACV was administered at 1.25 mg / mouse by intraperitoneal injection
117 (ip) daily for 3 days, while IVIG was given as single dose of 25 mg/mouse by ip injection on day
118 4pi [15]. Fresh fecal pellets (n=1-2/ mouse) were collected on day 7 pi and stored at -80°C until
119 processed for Illumina 16S rRNA gene sequencing to determine the effects of infection and drug
120 treatment on the gut microbiome. Normal male and female mice differed in gut bacteria
121 composition and unexpectedly, HSV ocular infection caused further shifts in the gut bacteria
122 community and amplified this sex difference, as shown in a PCoA plot of Hellinger beta diversity
123 distance values for infected compared to uninfected male and female mice (**Figure 1A**; $P<0.05$,
124 Adonis Tests). In addition, HSV infection had a greater effect on gut bacterial communities in
125 males ($P=0.003$) compared to females ($P=0.011$) (**Figure 1A**). Significant differences were
126 observed at the phyla level, particularly for firmicutes (**Figure 1B**) with more marked differences
127 evident at the species level for *Clostridium aerotolerans* and other clostridial species, for example
128 *Clostridium XIVa* that ferment carbohydrates in the gut resulting in production of short chain fatty
129 acids (SFCs) that contribute to barrier integrity and also exhibit anti-inflammatory properties
130 (**Figure 1C**). A notable difference was also observed for *Akkermansia muciniphila* that has many
131 health promoting activities, including maintaining gut barrier health (**Figure 1C**).
132

133 Treating HSV infected mice with ACV from day 4 pi for three days resulted in even more
134 drastic shifts in the gut bacteria composition and exaggerated sex differences (**Figure 2A**), than
135 for infection alone. Considerable abundance changes were evident at the Phyla level for
136 *Bacteroidetes*, *Firmicutes* and *Verrucomicrobia* (**Figure 2B**) and at the species level (**Figure 2C**).
137 Notably, whereas HSV infection reduced the abundance of *Firmicutes* significantly in male but
138 not female mice (**Figure 1B**), ACV reversed this effect restoring the abundance to the level in
139 uninfected male mice, while also increasing the abundance in female mice (**Figure 2B** and **Figure**
140 **1B**). Notable abundance changes at the species level included drastic suppression of *Clostridium*
141 *aerotolerans* in infected male mice compared to increased abundance in females (**Figure 1C**),
142 while ACV treatment further increased this abundance only in females (**Figure 2C**). *Akkermansia*
143 *muciniphila* abundance was increased by infection in male mice but reduced in females (**Figure**
144 **1C**), while ACV treatment resulted in total suppression of this species in female mice compared
145 to a marked reduction in male mice (**Figure 2C**). There are many other similar changes in species
146 abundance that are differentially impacted by ACV treatment in a sex-biased manner, indicative
147 of complex interactions between infection, ACV effects on infected host cells, and bacteria, as
148 well as metabolites produced by bacterial metabolism of ACV.
149
150 Treatment of uninfected mice with IVIG alone also shifted the gut bacteria community
151 composition with a notable marked sex effect as determined by a beta diversity analysis (**Figure**
152 **3**). Males and females showed a major reduction in *A. muciniphila,* and a lesser reduction of
153 *Verrucomicrobia* in males, compared to females that showed increased abundance of this phylum
154 in response to IVIG treatment (**Figure 4**). The abundance of many other bacterial species was
155 differentially altered by IVIG treatment of males and females, for example, *Clostridium*
156 *aerotolerans*, *Bacteroides acidifaciens* and *Porphyromonadaceae* (**Figure 4B**). The response to
157 IVIG was distinct in HSV infected mice, and the complex interactions between infection, ACV and
158 IVIG were also evident at the phyla and species levels and were strongly sex biased as well

159 (**Figure 4A and 4B**). IVIG treatment decreased *A. muciniphila* abundance markedly in infected
160 males and females as did ACV, whereas in contrast, treatment with ACV+IVIG caused a notable
161 increase in its abundance, indicative of antagonistic effects of these two drugs in the context of
162 infection (**Figure 4B**) In a similar vein, *C. aerotolerans* abundance increased markedly in males,
163 but was unchanged in females treated with IVIG, while in contrast, it was strongly decreased in
164 males but slightly increased in females treated with ACV alone. In contrast, treatment with
165 ACV+IVIG suppressed an IVIG-induced increase in males and an ACV-induced increase in
166 females, revealing antagonism between ACV and IVIG in the context of HSV infection (**Figure**
167 **4B**).
168
169 Patients with hematologic and other malignancies have benefited immensely from
170 allogeneic hematopoietic stem cell transplantation (allo-HSCT or HSCT), which can be a potent
171 curative immunotherapy. However, life threatening complications such as graft-versus-host
172 disease (GVHD), relapse, and infections that include reactivated HSV and VZV limit its application
173 [16]. HSV and varicella zoster (VZV) reactivation has been successfully suppressed by
174 prophylactic ACV treatment, though ACV-resistant (ACVr) HSV is an emerging problem [17, 18].
175 Long term ACV prophylactic treatment is now routine for HSCT patients, because it was found to
176 correlate with reduced HSV and ACVr HSV disease in those treated for longer than 1 year [19].
177
178 Given this routine clinical practice, we evaluated the effects of ACV on fecal bacteria,
179 because gut microbes have been implicated in GVHD pathophysiology and because we posit that
180 ACV contributes to the development of GVHD by changing the gut microbiota. First, we identified
181 gut bacterial changes in humans with GVHD [20-30]. Next, we determined whether the ACV-
182 induced changes that we detected in this mouse study matched those GVHD-associated
183 changes. Whenever we identified taxa that were altered in both types of studies, the direction of
184 the change was the same, and it was consistent with our hypothesis that ACV contributes to the

185 development of human GVHD by changing the gut microbiota. In the following, we describe these
186 results, and we note that these ACV-induced changes were only observed in the HSV-infected
187 mice and not in the uninfected mice.
188
189 Reduced levels of several taxa belonging to the phylum *Bacteroidetes* have been shown
190 to be associated with GVHD, indicating that these gut bacteria may play a protective role. In a
191 pediatric study, GVHD patients had lower levels of the family *Bacteroidaceae* and the genus
192 *Parabacteroides* [30]. In a longitudinal study, pediatric patients that had lower levels of
193 *Bacteroidetes* prior to HSCT were more likely to develop GVHD [24]. In our study, all three if these
194 taxa were reduced by ACV treatment in male but not female mice (**Figure 5A**).
195
196 Reduced levels of Anti-Inflammatory Clostridia (AIC) have also been detected in human
197 GVHD patients [20, 23-25, 27-30], indicating that these gut bacteria may play a protective role.
198 This terminology was first introduced by Piper et al. [31] in the context of short bowel syndrome,
199 and then introduced to the GVHD literature by Simms-Waldrip et al. [30]. AIC taxa include
200 members of the families *Clostridiaceae*, *Erysipelotrichaceae*, *Eubacteriaceae*, *Lachnospiraceae*
201 and *Ruminococcaceae*. In a pediatric study, decreases in *Blautia* and *Clostridium bolteae* were
202 associated with the development of GVHD [30]. In an adult study, lower levels of *Blautia*, *Blautia*
203 *hansenii*, and *Blautia stercoris* were associated with the development of GVHD [28]. In a
204 longitudinal study, reduced levels of the *Blautia* before HSCT was shown to be a predictive marker
205 for the development of GVHD [27]. In our study, all of these taxa were reduced by ACV treatment
206 in female but not male mice (**Figure 5B**).
207
208 In a more detailed analysis of AIC bacteria, we observed that while HSV infection
209 increased the abundance of *Blautia hansenii* only in males, ACV treatment reduced its abundance
210 in females but had no effect on its abundance in males (**Supplemental Figure 1**). Remarkably,

211 a dramatic increase in *B. hansenii* in uninfected females was observed after IVIG treatment, and
212 this increase was abrogated by ACV (compare NoHSV_F, NoHSV_IVIG_F and
213 NoHSV_ACVplusIVIG_F) (**Supplemental Figure 1**), a result that supports sex-based differential
214 effects of these drugs. However, during HSV infection, both IVIG and ACV reduced *B. hansenii*
215 in females, whereas only IVIG reduced abundance in males. Interestingly, HSV infection
216 significantly increased the abundance of the AIC genera *Blautia, Allobaculum,* and *Clostridium*
217 XVIII but not *Turicibacter* in both males and females (**Supplemental Figure 2**). ACV treatment of
218 HSV infected female mice resulted in significant decreases in the abundances of 4 AIC genera:
219 *Blautia*, *Allobaculum*, *Clostridium* XVIII and *Turicibacter,* whereas in infected males, ACV
220 decreased the abundance of *Marvinbryantia* and *Oscillibacter* (**Supplemental Figure 2**). In
221 addition, ACV increased the abundance of *Turicibacter* in uninfected females but not males.
222
223 Finally, the two most abundant operational taxonomic units (OTUs), which exhibited a
224 change in their relative abundances due to ACV treatment, were assigned to the family
225 *Porphyromonadaceae* and the species *A. muciniphila* (**Figure 5C**). While we did not find these
226 taxa associated with GVHD in prior human studies, GVHD has been associated with intestinal
227 barrier dysfunction [32-36]. Supporting our hypothesis that ACV contributes to the development
228 of GVHD by changing the gut microbiota, members of the *Porphyromonadaceae* have been
229 shown to cause gut barrier dysfunction [37, 38], and our *Porphyromonadaceae* OTU was
230 increased in its abundance by ACV. In addition, *A. muciniphila* was decreased by ACV treatment
231 in our study, and it has been shown to strengthen gut barrier functioning [39-41].
232
233 **Discussion**.
234
235 Our intention in this brief report is to alert the scientific community and especially clinicians
236 to the fact that HSV infection, the antiviral drug ACV, and the immunomodulatory biological, IVIG,

237 can all independently result in significant perturbations of the gut bacterial communities. Our data
238 reveal complex interactions between HSV infection and ACV or/and IVIG treatment that result in
239 marked alterations to gut bacterial communities. Although the clinical consequences of these
240 changes have not yet been elucidated, they could have profound implications in several settings
241 including HSCT-associated GVHD.
242
243 Though the mechanisms by which ocular HSV infection causes gut dysbiosis are unclear,
244 neuroinflammatory mechanisms and effects on the enteric nervous system via connected
245 brainstem neuronal circuits can be envisaged [15, 42]. Indeed, recent paradigm-shifting reports
246 reveal that peripheral neurons, including nociceptive and sensory neurons, can directly sense and
247 respond to environmental alarms by releasing neuropeptides that can regulate immune responses
248 in target organs including the gut [43, 44]. Persistence of gut dysbiosis was not evaluated here,
249 but results from a behavioral study alluded to earlier suggest long-term effects of infection and
250 drug treatment on gut bacterial ecology should be investigated [15]. Sex biased effects on HSV
251 induced dysbiosis merit further study, as these may involve microglial responses to HSV infection
252 and the microglial compartment is known to be regulated by the microbiota in a sex biased manner
253 [45-47].
254
255 The mechanism by which ACV, the standard antiviral for HSV infections, changes the gut
256 microbiota likely involves its uptake into bacteria. ACV is preferentially phosphorylated by the viral
257 encoded thymidine kinase (Tk) resulting in cell retention and eventual incorporation into viral DNA
258 resulting in inhibition of viral replication via DNA chain termination. Because Tk is conserved in
259 numerous bacterial species, ACV can be taken up and incorporated into DNA, resulting in
260 bactericidal effects [48-51]. Indeed, early studies on DNA replication mechanisms relied on
261 labeling bacterial DNA with tritiated thymidine and many bacterial taxa can be imaged using
262 nucleoside analogues such as 1-(2_-deoxy-2_-fluoro-_-D-arabinofuranosyl)-5-[125I] iodouracil

263 ([125I]FIAU) that are substrates for HSV Tk [52-55]. Incorporation of [*methyl*-^{3}H]thymidine into
264 DNA has been unequivocally demonstrated for members of the *Clostridium* genus [56] and our
265 data show ACV reduced the abundance of the *Blautia* genus (order *Clostridiales;* [57]) *Blautia*
266 *hansenii*, *Blautia stercoris*, and *Clostridium bolteae* in females but not males. Additionally,
267 interrogating the NCBI reference genome sequence for *Blautia hansenii* confirmed the presence
268 of a thymidine kinase enzyme. Our data are therefore consistent with ACV causing dysbiosis by,
269 at least in part, inhibiting the growth of various bacteria taxa via the Tk mechanism, though other
270 mechanisms involving bacterial metabolism of ACV cannot be excluded. Clearly, the mechanisms
271 by which ACV affects gut bacterial ecology are complex, which is further supported by the sex-
272 biased effects.
273
274 We also explored the effects of IVIG treatment alone and in combination with ACV in HSV-
275 infected and uninfected mice, because IVIG has been used to treat HSV encephalitis (HSE) and
276 is also a frontline therapy for autoimmune encephalitis, which is triggered by HSE and other insults
277 [58-60]. Moreover, IVIG is being evaluated in a randomized control trial for children with all-cause
278 encephalitis to determine whether neurological outcomes are improved compared to standard
279 antiviral therapy alone, which is similar to our behavioral study that generated paradoxical results
280 [15, 61]. Reports that IVIG's antigenic repertoire includes reactivities to a variety of gut commensal
281 antigens and metabolites have increased recently [62-64], which is consistent with a report that
282 gut commensals can somehow trigger systemic IgG responses under homeostatic conditions that
283 protect against systemic infection [65, 66]. We speculate that by neutralizing bacterial/host
284 antigens/metabolites, IVIG is able to influence host immunity, the nervous system, and other
285 physiological processes, resulting in perturbation of gut bacteria ecology. We speculate that the
286 disparate and complex effects of ACV and IVIG alone and in combination on the gut bacteria
287 ecology likely account for their antagonistic effects on cognitive behavior in mice latently infected
288 with HSV that we alluded to earlier [15].

289

290 This study has several limitations. Being exploratory in nature, analyses of the gut bacteria
291 were done at a single time point immediately after infection or drug treatment, rather than as a
292 longitudinal study that would have provided information on the persistence of the dysbiotic state
293 as well as mechanistic insights as to how HSV, ACV and IVIG provoke dysbiosis. Ideally, the
294 effects of ACV should be tested in latently infected mice, since virtually all HSCT patients harbor
295 latent HSV. However, because HSV infection alone disrupts the gut bacterial community,
296 assessing the effects of ACV on the gut bacteria community structure in the latently infected mice
297 would likely be difficult. Because ACV was given ip to mice but usually orally to HSCT patients
298 [67], its effects on the gut bacteria community maybe underestimated in our study.

299

300 Notwithstanding these caveats, our finding that ACV treatment of HSV infected mice
301 decreased the relative abundances of several bacterial taxa is important because these bacteria
302 have been negatively correlated with the induction of and mortality from GVHD in HSCT patients
303 [24, 27, 28, 30]. These results are also consistent with our hypothesis that ACV contributes to the
304 development of GVHD by changing the gut microbiota. In the context of allo-HSCT, GVHD occurs
305 when donor immune cells recognize recipient tissues as foreign, leading to immune-mediated
306 damage to several organs and tissues including the gastrointestinal tract. This has led
307 researchers to posit that the reduction of anti-inflammatory bacteria such as AIC contribute to
308 GVHD pathology [30]. The results from our study extend this hypothesis to include ACV treatment
309 as a putative contributor to GVHD, because ACV reduced AIC bacteria in the gut. ACV treatment
310 also decreased the relative abundances of several members of the *Bacteroidetes*, some of which
311 have been shown to exhibit anti-inflammatory properties [68-71]. More relevantly, the capsular
312 polysaccharide A (PSA) from *Bacteroides fragilis* reduced HSV-associated mortality in mice by
313 dramatically reducing immune-mediated inflammation [72]. In addition, the two most abundant
314 OTUs identified in our study, whose relative abundances were positively (*Porphyromonadaceae*)

315 and negatively (*A. muciniphila*) correlated with ACV treatment, have been shown to weaken [37,
316 38] and strengthen [39-41] gut barrier function, respectively. These results provide an additional
317 link between ACV treatment and GVHD, because barrier dysfunction, which can cause systemic
318 inflammation, is a hallmark of GVHD [32-36]. Finally, long-term ACV prophylaxis initiated early
319 after HSCT might also impair immune reconstitution based on results from a study of antibiotic
320 depletion of gut bacteria in a murine model of syngeneic bone marrow transplantation [73]. These
321 tantalizing results warrant independent validation and further detailed studies using a murine
322 autologous BMT model to more rigorously evaluate the impact of long-term ACV prophylaxis on
323 GVHD and engraftment, because results from such studies might eventually lead to improved
324 outcomes for HSCT patients. Ideally, such future studies should be performed with mice harboring
325 wild microbiota, because several recent reports show that immune responses in mice with wild
326 microbiomes model human immune responses more closely than conventional mice with SPF
327 microbiota [74-76].
328
329 **Materials and Methods.**
330
331 **Ethics Statement**
332 All animal procedures were performed with prior approval of the City of Hope Institutional Animal
333 Care and Use Committee (IACUC) under protocol # 07043 and within the framework of the Guide
334 for the Care and Use of Laboratory Animals. C57BL6/J (B6) were bred in the vivarium at City of
335 Hope.
336
337 **Mouse Studies**
338
339 Master stocks of HSV1 strain 17 composed of only of cell-released virus were prepared in
340 and their titers determined on mycoplasma-free CV-1 cell monolayers. Single use aliquots of virus

341 in Hanks balanced salt solution supplemented with 2% fetal bovine serum were stored at -80°C.
342 Male and female mice, 6–8 weeks of age, were infected with HSV1 17^+, a virulent strain. Mice
343 were sedated with ketamine (60 mg/kg) and xylazine (5 mg/kg) prior to HSV inoculation by corneal
344 scarification. B6 mice were bilaterally inoculated with 1x 10^5 PFU per eye and monitored daily as
345 previously described [15, 77].
346
347 **Administration of Acyclovir and Intravenous Immunoglobulins.**
348
349 ACV obtained from (APP Pharmaceuticals, Schaumburg, IL) was given at 50 mg/kg of
350 body weight by intraperitoneal (ip) injection daily for 3 days starting on day 4 pi and PBS was
351 given according to the same schedule to control mice. IVIG (Carimune, NF) obtained from CSL
352 Behring (King of Prussia, PA, USA) was given ip as a single 0.5 ml dose (25 mg/mouse) on day
353 4 pi or it was given in combination with a 3 day course of ACV.
354
355 **Illumina Bacterial 16S rRNA gene sequencing**.
356
357 Illumina bacterial 16S rRNA gene libraries were constructed as follows. PCRs were
358 performed in an MJ Research PTC-200 thermal cycler (Bio-Rad Inc., Hercules, CA, USA) as 25
359 µl reactions containing: 50 mM Tris (pH 8.3), 500 µg/ml bovine serum albumin (BSA), 2.5 mM
360 $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP), 400 nM of the forward PCR primer,
361 200 nM of each reverse PCR primer, 1 µl of DNA template, and 0.25 units JumpStart Taq DNA
362 polymerase (Sigma-Aldrich, St. Louis, MO, USA). PCR primers 515F
363 (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to
364 targeted the 16S rRNA gene containing portions of the hypervariable regions V4 and V5, with the
365 reverse primers including a 12-bp barcode [78]. Thermal cycling parameters were 94°C for 5 min;
366 35 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s, and followed by 72°C for 5 min. PCR

367 products were purified using the MinElute 96 UF PCR Purification Kit (Qiagen, Valencia, CA,
368 USA).
369
370 **16S rRNA gene data processing**.
371
372 We used the UPARSE pipeline for de-multiplexing, length trimming, quality filtering and
373 operational taxonomic units (OTU) picking using default parameters or recommended guidelines
374 that were initially described in [79] and which have been updated at
375 https://www.drive5.com/usearch/manual/uparse_pipeline.html. Briefly, after demultiplexing,
376 sequences were trimmed to a uniform length of 249 bp, then filtered at the recommended 1.0
377 expected error threshold. Sequences were then dereplicated and clustered into zero-radius OTUs
378 using the UNOISE3 algorithm [80], which also detects and removes chimeric sequences; this
379 method is based on making OTUs at 100% identity. An OTU table was then generated using the
380 otutab command. OTUs having non-bacterial DNA were identified by performing a local BLAST
381 search [81] of their seed sequences against the nt database. OTUs were removed if any of their
382 highest-scoring BLAST hits contained taxonomic IDs within Rodentia, Viridiplantae, Fungi, or
383 PhiX. Taxonomic assignments to the OTUs were performed with SINTAX [82] using RDP
384 Classifier 16S training set number 16 [83] as the reference database.
385
386 **16S rRNA gene data analyses**.
387 Beta diversity was measured using QIIME 1.9.1 [84] to calculate a Hellinger beta diversity
388 distance matrix, which was depicted using principle coordinates analysis (PCoA), and statistically
389 assessed by performing Adonis tests. Statistical differences among the taxa were determined
390 using edgeR [85, 86]. Taxa relative abundance figures were made using Prism (GraphPad, La
391 Jolla, CA). Comparative analyses of the bacterial taxa between human GVHD studies and our
392 mouse study excluded sequence-selective qPCR, because the selectivity of such assays is

393 questionable given the conserved nature of the 16S rRNA gene, and because the results of such
394 studies are not typically validated by sequence analyses. The bacterial sequences have been
395 deposited in the National Center for Biotechnology Information (NCBI)'s Sequence Read Archive
396 (SRA) under the BioProject Accession Number PRJNA549765.
397
398

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400

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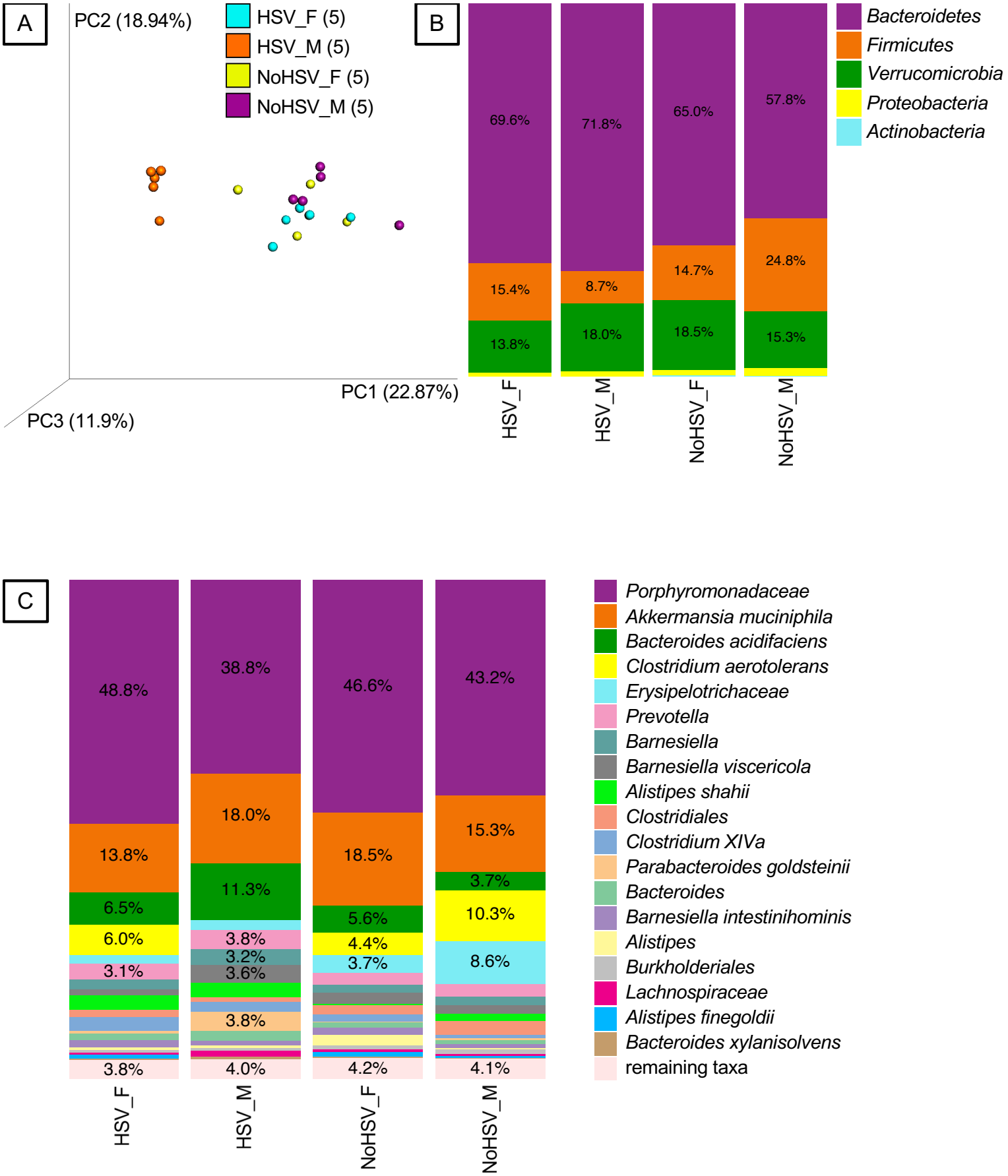

Figure 1. Fecal Bacteria from HSV-Infected and Uninfected Mice. A. Principal-coordinates analysis (PCoA) of Hellinger beta diversity distance values generated from 16S rRNA gene sequences. All four groups were different ($P<0.05$, Adonis Tests). The number of mice (n) in each genotype-microbiota group are shown in parentheses. B. Bacteria phyla associated with HSV-infected and uninfected mice. C. Bacterial species (or higher taxa) associated with HSV-infected and uninfected mice. Females = _F and Males = _M.

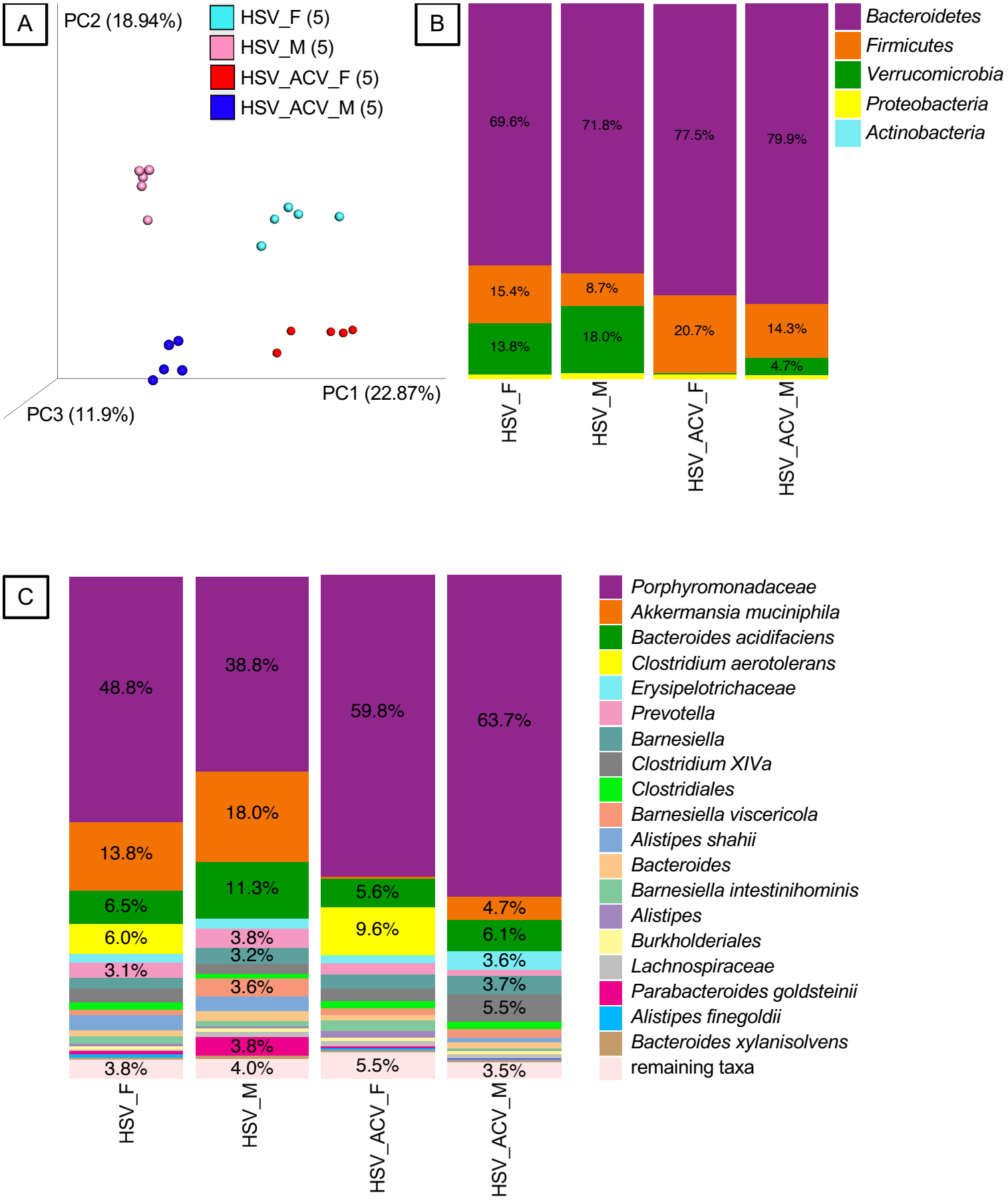

Figure 2. Fecal Bacteria from HSV-Infected Mice Treated and Not Treated with ACV. A. Principal-coordinates analysis (PCoA) of Hellinger beta diversity distance values generated from 16S rRNA gene sequences. All four groups were different (P<0.05, Adonis Tests). The number of mice (n) in each genotype-microbiota group are shown in parentheses. B and C. Bacteria phyla and species (or higher taxa), respectively, associated with HSV-infected mice treated and not treated with ACV. Females = _F and Males = _M.

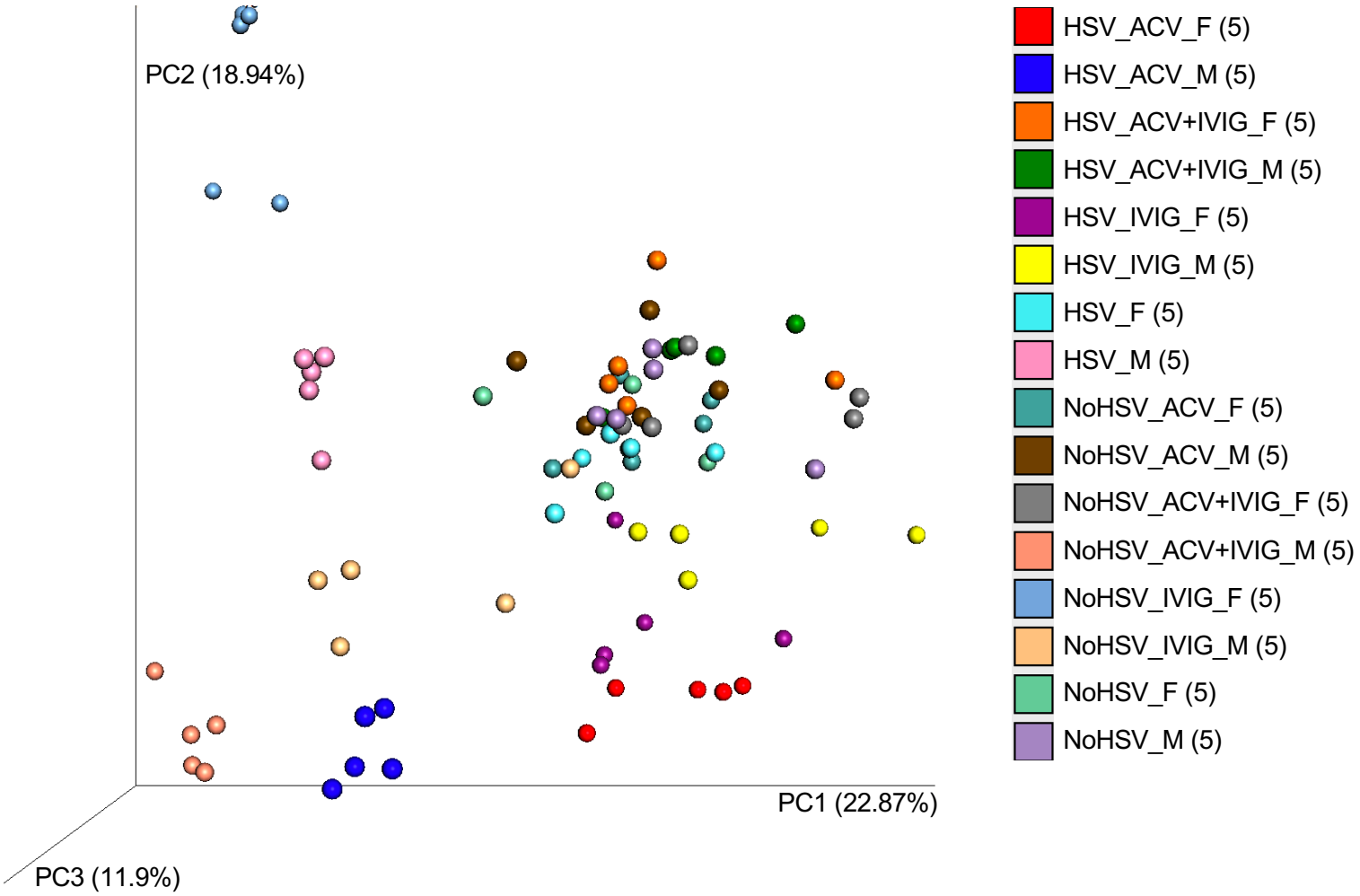

Figure 3. Beta Diversity Analysis of Fecal Bacteria from HSV-Infected and Uninfected Mice Treated and Not Treated with ACV and/or IVIG. Principal-coordinates analysis (PCoA) of Hellinger beta diversity distance values generated from 16S rRNA gene sequences. The number of mice (n) in each genotype-microbiota group are shown in parentheses. Females = _F and Males = _M.

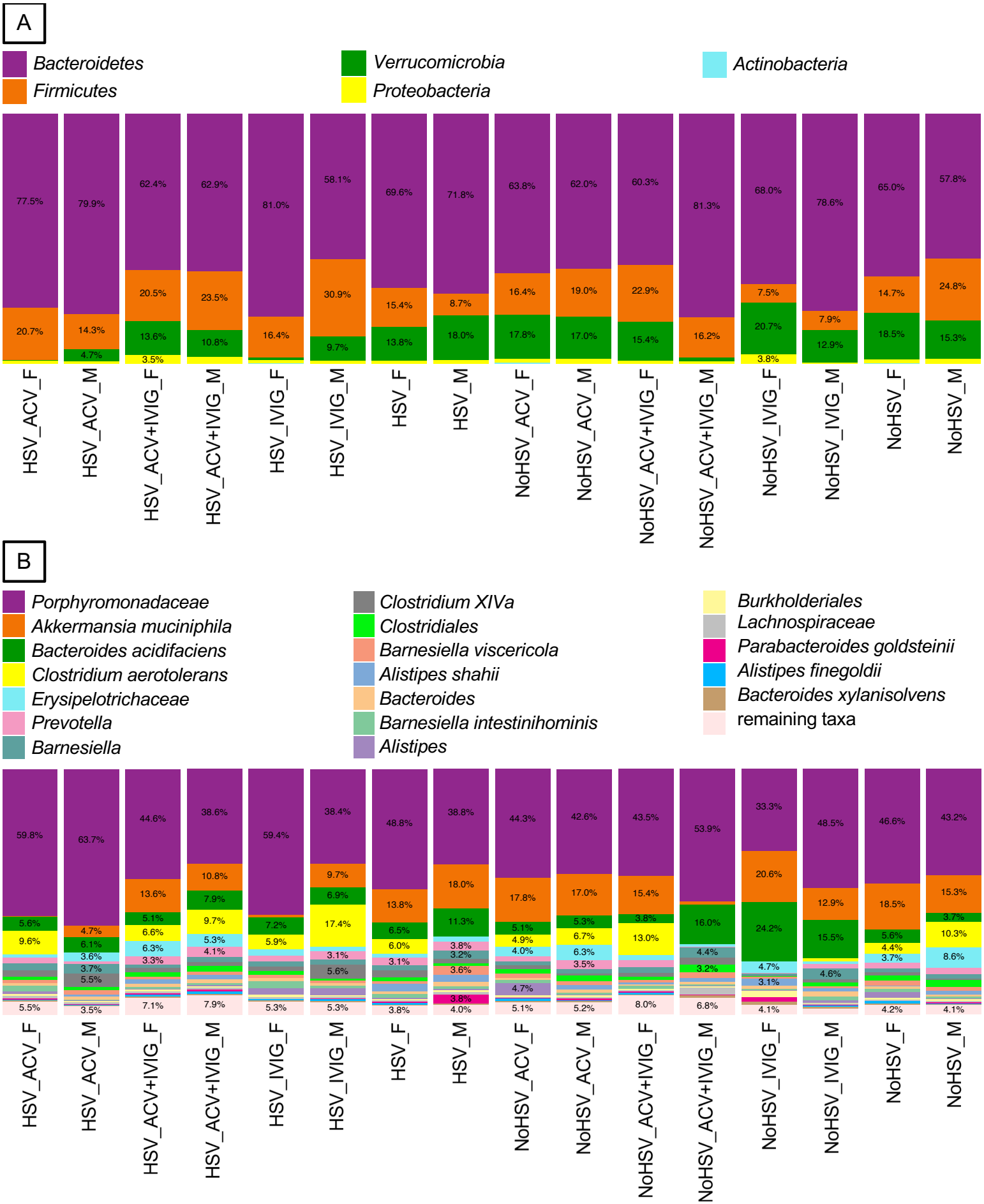

Figure 4. Fecal Bacterial from HSV-Infected and Uninfected Mice Treated and Not Treated with ACV and/or IVIG. A and B. Bacteria phyla and species (or higher taxa), respectively, associated with HSV-infected and uninfected mice treated and not treated with ACV, IVIG, or ACV+IVIG. Females = _F and Males = _M.

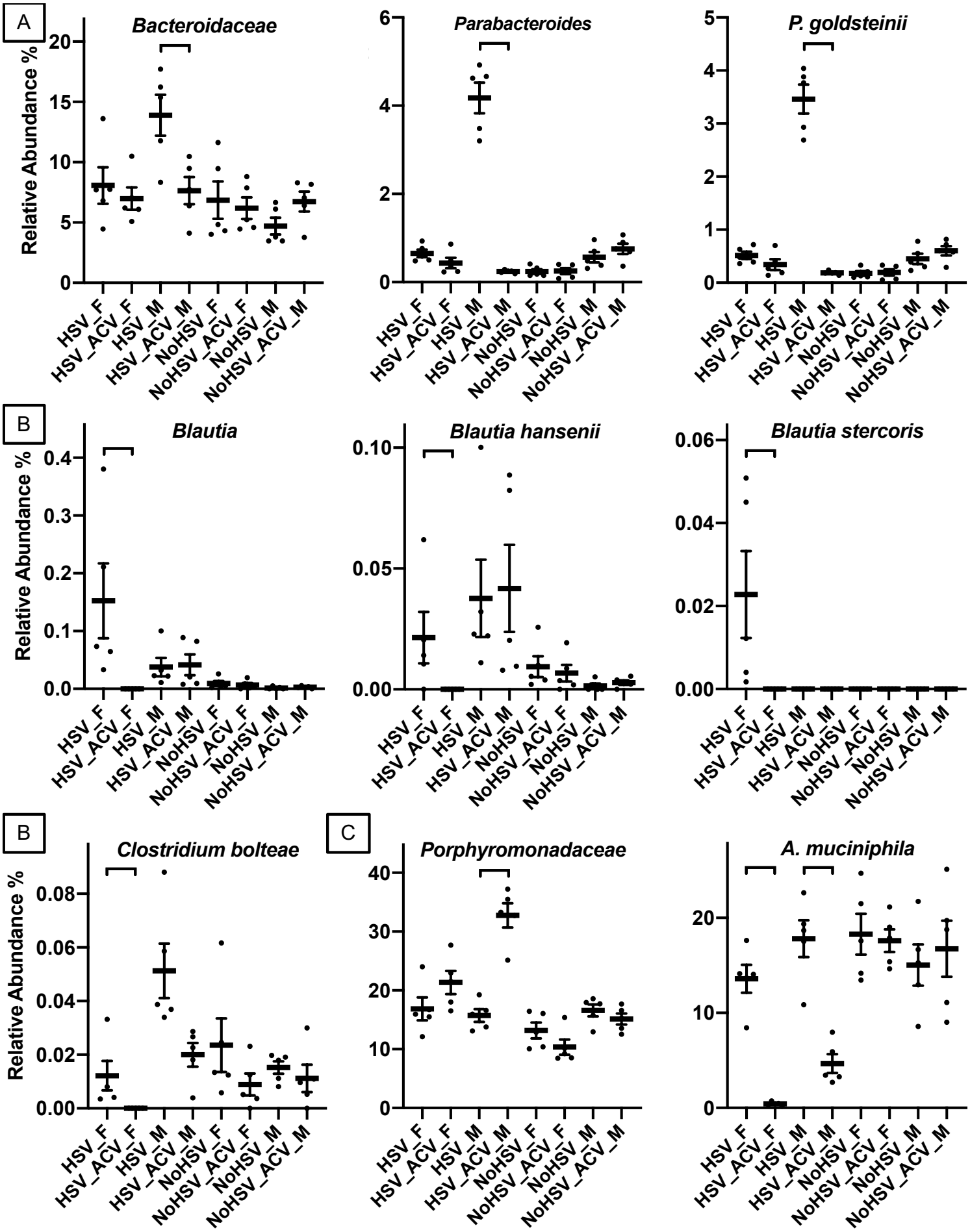

Figure 5. Fecal Bacterial from HSV-Infected and Uninfected Mice Treated and Not Treated with ACV. A and B. Fecal bacterial taxa that were were changed in both human GVHD studies and by ACV in this study. A and B. Members of the *Bacteroidetes* and AIC, respectively. C. The two most abundant bacterial OTUs. The only pairwise differences shown are between ACV treated and untreated mice for each sex (FDR-adjusted P values < 0.05). Bars = standard error. Females = _F and Males = _M.