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Effects of water decontamination methods and bedding material on the gut microbiota
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30 # Abstract

31 Rodent models are invaluable to understanding health and disease in many areas of biomedical
32 research. Unfortunately, many models suffer from lack of phenotype reproducibility. Our
33 laboratory has shown that differences in gut microbiota (GM) can modulate phenotypes of
34 models of colon cancer and inflammatory bowel disease. We and others have also shown that
35 a number of factors associated with rodent research, including vendor, cage system, and
36 bedding can alter GM. The objective of this study was to expand these studies to examine the
37 effect of additional bedding materials and methods of water decontamination on GM diversity
38 and composition. To this end, Crl:CD1 (ICR) mice were housed on corn cob or compressed
39 paper chip bedding and provided water that was decontaminated by four commonly used
40 procedures: reverse osmosis, autoclaving, sulfuric acid treatment, or hydrochloric acid
41 treatment. Feces was collected at day 0, and at day 28 (endpoint), fecal and cecal samples
42 were collected. DNA was extracted from samples, amplified by PCR using conserved bacterial
43 primer sets and subjected to next generation sequencing. Sequence data were analyzed using
44 Qiime and groups were compared using principal coordinate analysis (PCoA) and permutational
45 multivariate analysis of variance (PERMANOVA). Two factor PERMANOVA of cecal GM data
46 revealed significant changes when comparing bedding and water decontamination methods,
47 while no significant effects were noted in the fecal GM data. Subsequent PERMANOVA and
48 PCoA of cecal data revealed that several combinations of bedding and water decontamination
49 methods resulted in differing GM, highlighting the complexity by which environmental factors
50 interact to modulate GM.

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52 # Introduction

53 In recent years there has been a substantial increase in studies focusing on the microorganisms
54 present in the gastrointestinal tract (GIT). The gut microbiota (GM) is known to play crucial roles
55 in digestion, immune status development, and pathogen resistance, and differences in GM have
56 been associated with differences in health and disease susceptibility (1-3). Certain
57 characteristics in the GM have been associated with diseases of the GIT (4), as well as
58 diseases in other body systems such as the central nervous system (5, 6). Rodent models have
59 emerged as a highly valuable tool to determine the role of the GM in both health and disease.
60 Several studies have demonstrated that the highly dynamic GM is influenced by a variety of
61 environmental factors, and can in turn impact rodent model phenotypes (7). Recently, the use of
62 mouse models has been questioned due to the lack of reproducibility (8). These limitations have
63 spurred efforts from several institutions such as the National Institutes of Health (NIH) to
64 improve reproducibility of animal research (9). Our laboratory has focused on the microbial
65 composition of the GIT as an important contributing factor in phenotypic variability of rodent
66 disease models (10, 11). We previously found that the GM differs depending on the source and
67 genetic background of the mouse (12). Even mice of genetically similar backgrounds from the
68 same producer can have differing GM depending on the institution in which they are housed,
69 suggesting that the environment is a major factor in the determination of the GM (13). We have
70 also demonstrated significant changes in the GM in response to housing conditions (14), further
71 corroborating the importance of environmental factors in shaping the GM. Given that the GM
72 significantly impacts model phenotypes, these data substantiate the need to consider how
73 different husbandry factors may influence the GM.

74 Husbandry factors such as light cycle, temperature, bedding, and handling can be seen
75 as subtle factors that can affect the outcome of rodent experiments (7, 15). Factors such as

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76 temperature and light cycle can be controlled by proper building maintenance. Bedding, a factor
77 that is often overlooked, can greatly differ between facilities.

78 Another factor that can be overlooked is the water that is offered to rodents. Several
79 water decontamination methods are commonplace in contemporary rodent facilities. Methods
80 include filtering the water to physically remove contaminants (e.g., reverse osmosis), or
81 procedures to kill bacteria (e.g., UV light or acidification). Differing water decontamination
82 methods have been shown to impact model phenotypes. For example, the low pH of acidified
83 water was associated with phenotype changes in a mouse diabetic model (16, 17). Moreover,
84 water chlorination, when compared to tap water alone was shown to change the phenotype of a
85 mouse model of colorectal cancer (18). It is unclear whether different water decontamination
86 methods influence the GM, and in turn, become a potential experimental variable that
87 contributes to inadequate reproducibility of model phenotypes.

88 Bedding is another component of husbandry that varies between animal facilities. Corn
89 cob bedding is one of the most commonly used bedding materials because of its high
90 absorbency (19) and low cost; however other paper-based bedding materials are becoming
91 popular. Differing bedding materials have also been linked to model phenotype changes and
92 changes in the GM (20, 21), but controlled studies have yet to be performed. To address how
93 water decontamination and bedding shape the GM, we exposed mice to water decontaminated
94 by four different methods: autoclaving with reverse osmosis (RO), autoclaving with hydrochloric
95 acid (HCl), autoclaving with sulfuric acid (H_2SO_4), and autoclaving alone (Autoclaved). We also
96 exposed the mice to two different bedding materials, corn cob and paperchip, and evaluated the
97 interaction between water and bedding as drivers of GM composition change. Composition of
98 the GM was determined by targeted amplicon sequencing using DNA extracted from feces and
99 cecal content. Samples were collected upon arrival and four weeks after being exposed to either
100 of the water and bedding combinations. Robust statistical methods then were used to determine
101 main effects of, and interaction between water and bedding.

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102 Understanding how husbandry factors, such as water decontamination or choice of
103 bedding, can influence the GM is a critical first step toward improving reproducibility in animal
104 models.
105

106 Materials and Methods

107 Ethics statement

108 This study was performed in accordance with the recommendations put forth in the Guide for
109 the Care and Use of Laboratory Animals and were approved by the University of Missouri
110 Institutional Animal Care and Use Committee (MU ACUC protocol #8720).
111

112 Survey

113 A water decontamination survey (S1 Table) was sent out on December 15, 2015 using the
114 Compmed listserv (CompMed, AALAS, Memphis, TN), a listserv that is used for discussion of
115 subjects of comparative medicine, laboratory animals, and topics related to biomedical
116 research.
117 On March 21, 2016 a question survey about bedding was sent using the same email list. The
118 survey included the following; "I am conducting a survey on the different types of bedding used
119 in rodent facilities. Please send me a response with the type of bedding used in your facility.
120 Specifics on the bedding will be appreciated (size, form, etc.)." Results from both surveys were
121 recorded until the last response was received on March 29, 2016.
122

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123 ## Mice

124 Six to eight week-old female outbred Crl:CD1 (ICR) mice (*n* = 96) were purchased from Charles
125 River Laboratories (Wilmington, MA) in a single order, and housed in the same room and
126 maintained under barrier conditions in microisolator cages on individually ventilated cage-racks
127 (Thoren, Hazleton, PA), filled with either compressed paper (Paperchip® Brand Laboratory
128 Animal Bedding, Shepherd Specialty Papers, Watertown, TN) or corn cob bedding (Bed-o'Cobs
129 1/8″, The Andersons Inc., Maumee, OH), with *ad libitum* access to autoclaved rodent chow
130 (LabDiet 5008 Purina, St. Louis, MO) and water, under a 14:10 light/dark cycle. The cages
131 contained a nestlet for enrichment and 4 mice per cage. The water offered was municipal water
132 which was decontaminated using the methods explained below. Using a random number
133 generator, mice were randomly assigned to one of the water and bedding combinations. Mice
134 were determined to be free of overt and opportunist bacterial pathogens including *Citrobacter*
135 *rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Helicobacter* spp., *Mycoplasma*
136 spp., *Pasteurella pneumotropica*, *Pseudomonas aeruginosa,* *Salmonella* spp., *Streptococcus*
137 *pneumoniae*; MHV, MVM, MPV, MNV, TMEV, EDIM, LCMV, MAV1, MAV2, Polyomavirus, PVM,
138 REO3, Ectromelia virus, and Sendai virus; intestinal protozoa including *Spironucleus muris*,
139 *Giardia muris*, *Entamoeba muris*, trichomonads, and other large intestinal flagellates and
140 amoeba; intestinal parasites including pinworms and tapeworms; and external parasites
141 including all species of lice and mites, via quarterly sentinel testing performed by IDEXX
142 BioResearch (Columbia, MO). At the end of study, mice were humanely euthanized via inhaled
143 carbon dioxide, in accordance with the AVMA Guidelines for the Euthanasia of Animals: 2013
144 Edition, followed by cervical dislocation as a secondary means.
145

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146 ## Water decontamination methods

147 The four water decontamination methods selected were tap water (autoclaved), reverse
148 osmosis, and acidification with either hydrochloric acid or sulfuric acid. Filled water bottles from
149 all four groups were autoclaved prior to use. Reverse osmosis filtration used a Milli-Q® Direct
150 system (Merck KGaA, Darmstadt, Germany). Acidification was performed using an automated
151 bottle filler which titrated the water with sulfuric acid (model 9WEF, Tecniplast, Buguggiate,
152 Italy) or hydrochloric acid (model Basil 1100, Steris Corporation, Mentor, OH) to a target pH of
153 2.5 (range 2.3 to 2.7). Water pH was verified using a handheld pH meter (pHTestr® 10, Oakton
154 Instruments, Vernon Hills, IL).
155

156 ## Sample collection

157 Freshly evacuated fecal pellets were obtained from each mouse on the day of arrival. These
158 samples were collected by transferring each mouse to a separate clean microisolator cage
159 containing no bedding, and allowing the mouse to defecate normally. Fecal pellets were then
160 collected with a sterile wooden toothpick. At day 28 post-arrival mice were humanely euthanized
161 and cecal and fecal samples were collected using aseptic technique. Briefly, each region of the
162 gut was exteriorized to allow collection of samples from roughly the same site of each animal.
163 Cecal samples comprised the entire cecal contents; and fecal samples represented the most
164 distal fecal bolus present in the colon, excluding boli within the rectum proper. Instruments used
165 for collection were flamed and allowed to cool between all samples. Following collection, all
166 samples were placed in 2 mL round-bottom tubes containing a 0.5 cm diameter stainless steel
167 bead. All samples were stored in a -80°C freezer until extraction was performed.
168

169 For DNA extraction, 800 µL of lysis buffer (22) was added to each tube containing the
170 sample and then mechanically disrupted using a TissueLyser II (Qiagen, Venlo, Netherlands).

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171 After mechanical disruption, tubes were incubated at 70°C for 20 minutes with periodic
172 vortexing. Samples were then centrifuged at 5000×g for five minutes at room temperature, and
173 the supernatant transferred to a clean 1.5 mL Eppendorf tube. Two hundred µL of 10 mM
174 ammonium acetate was added to lysates, mixed thoroughly, incubated on ice for five minutes,
175 and then centrifuged as above. Supernatant was then mixed thoroughly with one volume of
176 chilled isopropanol and incubated on ice for 30 minutes. Samples were then centrifuged at
177 16000×g for 15 minutes at 4°C. The supernatant was aspirated and discarded, and the DNA
178 pellet washed several times with 70% ethanol and resuspended in 150 µL of Tris-EDTA. 15 µL
179 of proteinase-K and 200 µL of Buffer AL (DNeasy Blood and Tissue kit, Qiagen) were added
180 and samples were incubated at 70°C for 10 minutes. 200 µL of 100% ethanol was added and
181 the contents of each tube were transferred to a spin column from the DNeasy kit. DNA was then
182 purified according to the manufacturer's instructions and eluted in 200 µL of EB buffer (Qiagen).
183 Purity of DNA was assessed via spectrophotometry (Nanodrop, Thermo Fisher Scientific,
184 Waltham, MA); yield was determined via fluorometry (Qubit, Life Technologies, Carlsbad, CA)
185 using quant-iT BR dsDNA reagent kit (Invitrogen, Carlsbad, CA).
186

187 16S rRNA library preparation and sequencing

188 The extracted DNA was amplified and sequenced at the University of Missouri DNA Core
189 facility, as previously described (23). Briefly, an amplicon library of the V4 region of the 16S
190 rRNA gene was generated using normalized DNA as a template. Using single-indexed universal
191 primers (U515F/806R) flanked by Illumina standard adapter sequences with PCR parameters of
192 $98°C^{(3\ m)} + [98°C^{(15\ s)} + 50°C^{(30\ s)} + 72°C^{(30\ s)}] \times 25$ cycles + $72°C^{(7\ m)}$. Amplicons were then pooled
193 for sequencing using Illumina MiSeq and V2 chemistry with 2x250 bp paired-end reads.
194

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195 ## Informatics

196 All assembly, filtering, binning, and annotation of contiguous sequences was performed at the
197 University of Missouri Informatics Research Core Facility, as previously described (23), with one
198 exception: Operational taxonomic units (OTUs) were annotated using BLAST (24) against the
199 SILVA database (25) of 16S rRNA sequences and taxonomy rather than the Greengenes
200 database used in our previous studies.
201

202 ## Statistical Analysis

203 Samples receiving less than 10,000 sequence reads were omitted from analysis. Differences in
204 beta-diversity of all groups were tested via a two-way and one-way PERMANOVA of ranked
205 Bray-Curtis (shared abundances of OTUs) similarity index using the open access Past 3.14
206 software package (26) . Principal coordinate analysis (PCoA) was also performed using the
207 Past software package and the relative abundance data was fourth-root transformed to
208 normalize the data. OTU richness and diversity indices were tested for normality using the
209 Shapiro-Wilk method; differences were then tested via two-way ANOVA for normal data or
210 Kruskal-Wallis ANOVA on ranks for non-normal data using SigmaPlot 12.3 (Systat Software
211 Inc., San Jose, CA). Significant OTUs, hierarchical clustering, and random forest analysis were
212 performed using cube root-transformed sequence data using open access MetaboAnalyst 3.0
213 (http://www.metaboanalyst.ca) (27). We considered *p* values less than 0.05 significant.
214

215 ## Data availability

216 All reported data have been deposited in the National Center for Biotechnology Information
217 (NCBI) Sequence Read Archive (SRA) under BioProject accession number PRJNA453789.

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218 # Results

219 ## Water decontamination methods and bedding material survey results

220 To determine what methods are being used in contemporary housing, two surveys were
221 conducted. A survey was conducted to identify water decontamination methods that are
222 available and being used across different rodent facilities (Fig 1A). A total of 39 responses from
223 19 institutions were received and 13 different water decontamination methods were identified.
224 The most common method used was reverse osmosis (RO) followed by acidification with
225 hydrochloric acid. Autoclaving tap water was the third most common method and is a more
226 popular practice in smaller rodent facilities. Acidifying the water as a whole is a very common
227 practice, with a total of 10 facilities acidifying their water and three different acidifiers identified;
228 hydrochloric, sulfuric, and phosphoric acid. Due to barrier restrictions within the vivarium in
229 which these studies were conducted, water received by all groups was autoclaved, while certain
230 mice received water that was also purified via RO or acidified via HCl or H_2SO_4.

231 **Fig 1. Water decontamination technique and bedding material surveys.** Results of surveys
232 on different water decontamination techniques performed on December 2015 (A), and survey on
233 different bedding materials performed on March 2016 (B). Surveys were performed through
234 Compmed listserv. Darkened bars represent water and bedding used in this study.

235 A second survey was conducted to identify what bedding materials are being used in
236 different rodent facilities (Fig 1B). A total of 11 institutions responded, with a total of 21
237 responses. Corn cob bedding was the most common bedding material used (8/21), followed by
238 aspen chip (5/21). Three different paper-based beddings were identified; alpha dri (3/21),
239 carefresh (1/21), and paperchip (1/21). Based on availability at our institution, corn cob and
240 paperchip were used, and eight groups of mice (2 beddings × 4 water treatments) were
241 established in a fully crossed study design.

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242 ## Main effects of water decontamination methods and bedding material

243 When subjectively evaluating the composition of the fecal samples, it is difficult to observe
244 distinct differences in relative abundance of OTUs (S1 Fig A). In contrast, subjective evaluation
245 of the composition of cecal samples revealed a water-treatment dependent pattern in the most
246 abundant OTUs such as UC Family Bacteroidales S24-7 1 and Lachnospiraceae NK4A136
247 group sp. 2 (S1 Fig B). For evaluation of the overall main effects of water decontamination
248 methods and bedding material on the GM composition, a two-way PERMANOVA of ranked
249 Bray-Curtis similarity index was performed (Table 1). Surprisingly, there were no significant
250 differences in the microbiota composition of the fecal samples (FM, fecal microbiota). However,
251 when cecal samples (CM, cecal microbiota) were examined, significant main effects were
252 detected in both water decontamination methods (*p*=0.001) and bedding material (*p*=0.023). To
253 evaluate all of the different water and bedding combinations, a one-way PERMANOVA with
254 pairwise comparison of cecal communities was performed (Table 2). Out of 28 comparisons, 16
255 (57%) were significantly different demonstrating the complexity by which water decontamination
256 and bedding interactions can influence microbiota.

257 **Table 1. Main effects of bedding and water treatment on the fecal and cecal microbiota.**

Endpoint Data Two-way Permutational Multivariate Analysis of Variance (PERMANOVA)				
Source	**Fecal**		**Cecal**	
	F	*p*	F	*p*
Bedding	1.74	0.117	2.52	0.023
Water Decontamination Method	0.712	0.626	4.16	<0.001
Interaction	-0.500	0.095	0.24	0.106

258 Two-way PERMANOVA of ranked Bray-Curtis similarity indices results from endpoint (Day 28)
259 fecal and cecal samples. Considered *p*<0.05 significant.
260

261 **Table 2. Pairwise PERMANOVA of Bray-Curtis similarity indices between cecal samples.**

	CH	**CR**	**CS**	**CA**	**PH**	**PR**	**PS**	**PA**
CH	F / *p*	**3.40**	**2.90**	**1.13**	**1.393**	**5.93**	**2.92**	**0.65**

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CR	0.011		**3.02**	**4.47**	**1.27**	**2.67**	**4.02**	**1.85**
CS	0.020	0.015		**4.52**	**1.33**	**2.18**	**0.66**	**1.65**
CA	0.294	0.005	0.005		**2.38**	**7.55**	**4.82**	**0.71**
PH	0.188	0.236	0.213	0.047		**2.14**	**1.56**	**0.78**
PR	<0.001	0.017	0.037	<0.001	0.045		**2.55**	**2.96**
PS	0.010	<0.001	0.737	<0.001	0.128	0.014		**1.73**
PA	0.583	0.126	0.156	0.523	0.471	0.031	0.133	

Results of one-way PERMANOVA pairwise comparisons from cecal samples. F values (bolded) shown on upper right and *p* values are shown on lower left. The first letter represents bedding and the second letter represents water decontamination technique; C- Corn cob, P- Paperchip, A- Autoclaved, H- Hydrochloric acid, R- Reverse osmosis, S- Sulfuric acid. Considered $p<0.05$ significant.

Differences in CM composition between groups were also visualized by PCoA. All comparisons except for one revealed separation of groups with some overlap in PCo1 vs PCo2. Fig 2 shows selected comparisons and further emphasizes the complexity of interaction between the two factors examined.

Fig 2. Principal Coordinate Analysis (PCoA) of pairwise comparisons of selected groups. PCoAs of comparisons between groups housed in corn cob bedding offered either reverse osmosis or autoclaved treated water (A), paperchip or corn cob bedding offered reverse osmosis or autoclaved treated water respectively (B), and corn cob or paperchip bedding offered reverse osmosis or H2SO4 treated water respectively (C). Significant differences ($p<0.05$) in cecal microbiota composition between these group comparisons were detected via pairwise PERMANOVA (Table 2).

Differences in richness and alpha diversity

To measure richness of each group the number of distinct OTUs was counted for each sample. In both FM and CM there was a significant main effect of bedding on richness (S2 and S3 Tables). Overall there was a significant decrease in number of OTUs (i.e., richness) in mice housed on paperchip bedding when compared to corn cob (Fig 3 A and B). In the FM, some

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286 individual significant pairwise comparisons were demonstrated in groups offered the same water
287 source but different beddings; HCl, RO, and H_2SO_4 (Fig 3A). In the CM, there was also a
288 significant main effect of water (S3 Table), and several pairwise comparisons were significantly
289 different (Fig 3B). Within each bedding type, samples from mice receiving autoclaved water with
290 no additional treatment had the lowest richness, suggesting that all additional treatment
291 methods are associated with increased richness.
292

293 **Fig 3. Richness and diversity of fecal and cecal microbiota at endpoint.** Tukey's box plot of
294 endpoint fecal and cecal Richness (number of distinct OTUs) (A and B), Shannon Diversity
295 Index (C and D), and Simpson diversity index (E and F). Like letters indicate significantly
296 ($p<0.05$) different comparisons via two-way ANOVA followed by Tukey's post hoc test. Bottom
297 axis represents combination of bedding and water decontamination method: autoclaving with
298 reverse osmosis (RO), autoclaving with hydrochloric acid (HCl), autoclaving with sulfuric acid
299 (H2SO4), and autoclaving alone (Autoclaved).
300

301 Alpha diversity of the samples was calculated using the Shannon and Simpson diversity
302 indices, which combine richness and evenness of the OTUs. No significant differences were
303 observed in the FM in either of the diversity indices (S2 Table). In the CM however, there was a
304 significant effect of water decontamination method (S3 Table) on α-diversity, as measured via
305 the Shannon index. In pairwise comparisons, several significant differences were demonstrated
306 between groups housed with autoclaved or H_2SO_4-treated water and housed in paper or corn
307 cob bedding (Fig 3D). That said, no significant differences were observed in Simpson diversity
308 index of CM (Fig 3F). Collectively, these data indicate that both bedding and water treatment
309 methods primary influence the richness, but not the distribution of the CM, and that the bedding
310 dependent effects on richness are maintained in the FM.
311

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312 ## Variation in OTU abundances and group clustering

313 A hierarchical cluster analyses was performed to demonstrate how individuals within
314 experimental groups clustered according to the relative abundance of the 25 most variable
315 OTUs as determined by ANOVA (S2 Fig). All cecal samples were represented in the analysis
316 and were classified by treatment group. As in a PCoA, samples with similar composition cluster
317 more closely to each other. Based on these most variable taxa, samples from several treatment
318 groups clustered together loosely. Specifically, samples from the majority of mice receiving
319 autoclaved water were grouped on one distal arm of the dendogram, while samples from mice
320 receiving RO-treated water formed a separate distinct branch.

321 We also performed a Random Forest (RF) analysis as a means to predict OTUs that
322 were preferentially influenced by the different husbandry conditions (Fig 4). The analysis
323 selected 15 OTUs as important classifiers for the different husbandry conditions. Additionally, a
324 two-way ANOVA was performed to examine which factor influenced the relative abundance
325 most and compare to the RF results. Fourteen of the 15 OTUs selected by the RF were
326 significantly different in the two-way ANOVA (S4 Table). These OTUs (Family XIII UCG-001 sp.,
327 *Lachnoclostridium* sp. 1, UC Family *Clostridiales* vadinBB60 group 1, *Akkermansia sp.,*
328 *Ruminococcaceae* UCG-009 sp., *Ruminiclostridium* 5 sp. 4, UC Family *Clostridiales* vadinBB60
329 group 3, UC Family *Peptococcaceae* 1, *Ruminiclostridium* 5 sp. 2, *Shuttleworthia* sp.,
330 *Lactobacillus* sp., *Enterorhabdus* sp., UC Order *Mollicutes* RF9 1, and *Anaerostipes* sp.) were
331 detected in both analyses and thus represent candidate taxa most influenced by the
332 water/bedding combination. All OTUs determined to be different via ANOVA demonstrated a
333 main effect of water-treatment (S4 Table). The water main effect was visualized in several
334 OTUs with the RF analysis (Fig 4). For example, Family XIII UCG-001, *Akkermansia* sp., UC
335 Family *Peptococcaceae* 1, and *Anaerostipes* were all most abundant in both groups receiving
336 autoclaved water. *Enterorhabdus* was more abundant in both H_2SO_4 water groups, and less

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337 abundant in the RO groups. *Shuttleworthia* was more abundant in both H_2SO_4 water groups,
338 while least abundant in the autoclaved groups.
339

340 **Fig 4. Random forest analysis selection of operational taxonomic units (OTUs).**
341 Random forest analysis of the selected most important OTUs to classify groups. Scale on left
342 represents abundance of OTUs in each group. Asterisks represent OTUs that were significant in
343 two-way ANOVA (S3 Table).
344

345 Collectively, the data presented above show that bedding material and water
346 decontamination methods can influence the GM of laboratory mice and that interactions exist
347 between the two factors. As in other studies, these data also suggest that the CM is a more
348 sensitive indicator of environmental effects on the gut microbiota, as compared to the FM.
349 Specifically, while significant differences in richness were detected between treatment groups in
350 both FM and CM, significant compositional differences were detected in only the CM. These
351 findings reinforce the need to consider husbandry factors when comparing phenotypic data
352 generated at different institutions or at different times, and to collect and analyze other gut
353 regions when assessing the influence of environmental factors on the GM in general.

354 Discussion

355 There is growing evidence that variability in husbandry practices among rodent facilities can
356 influence rodent GM. Moreover, differences in GM have been shown to modulate model
357 phenotypes raising the possibility that these two factors are connected: differing husbandry yield
358 changes in GM that subsequently impact model phenotypes. Given the current concerns about
359 reproducibility of biomedical research models (9), studies further assessing this premise are

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360 warranted. To the authors' knowledge, this study was the first survey of changes in the GM
361 composition due to water decontamination methods and bedding material in research mice.
362 Results of this study, showing that water decontamination methods and bedding material can
363 change GM composition, provide additional potential sources of GM modulation that may in turn
364 explain why rodent phenotypes differ when experiments are performed in different housing
365 conditions.
366
367 As demonstrated in Fig 1, rodent facilities have a large variety of methods used for water
368 decontamination. When water acidification was introduced, several studies were performed to
369 assess animal health and reproduction with no adverse effects demonstrated (28, 29). One of
370 the first physiological changes associated with acidified water was decreased weight gain and
371 water consumption (30). Another study identified changes in immune responses when animals
372 were offered water acidified to a pH of 2.0 (31). More recently, studies have shown both
373 changes in GM and in disease model phenotypes as a result of water acidification (16, 17, 32).
374 In humans, while water acidification is not a commonly used practice for preventing pathogen
375 transmission, carbonated drinks can have a pH as low as 2.0, with no known immediate direct
376 effects on health (33).
377
378 One intriguing aspect of the present results was that even though no significant
379 differences were detected in the overall composition of the FM, there were significant
380 differences in richness. The changes in FM may be subtle between groups, but when the FM
381 from arrival was compared to that of endpoint there was a significant difference in composition
382 (S3 Fig). The significant changes in the CM composition can be an indication that cecal content
383 is a better sample for environmental influences on the GM. The demonstrated significant main
384 effects of both bedding and water indicate that our husbandry practices do influence the GM.

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385 The pairwise comparison (Table 2) also illustrated how different combinations of bedding and
386 water can influence the GM.
387
388 Bedding material varies greatly between facilities, with corn cob bedding being a more
389 popular choice, but paper based beddings are becoming increasingly common within the
390 laboratory animal community. There are many possible reasons for corn cob to have an
391 influence on the GM. Previous studies have demonstrated that different bedding materials do
392 not alter the microenvironment parameters (ammonia levels, temperature, and humidity) in
393 ventilated cages (34, 35), but there are other factors that could be influencing the changes in
394 GM. It has been shown that mice and rats prefer alternative wood based beddings over corn
395 cob bedding, and that corn cob can influence their sleeping habits (36-38). In a pre-diabetic
396 mouse model, corn cob bedding reduced the efficiency of feed conversion when fed a high-fat
397 diet (20). Corn cob bedding also contains endocrine-disrupting agents that can disrupt breeding
398 behaviors in rats (39) and decrease aggressive behavior in the California mouse (*Peromyscus*
399 *californicus*) (40). Other potential factors can be the greater amount of endotoxins and coliform
400 levels present in corn cob bedding as compared to paper bedding (41). In a previous study
401 evaluating the effects of changing husbandry conditions, mice switched from corn cob to a
402 paper-based bedding demonstrated changes in microbiota composition at day 1 following the
403 switch. However, at day 5 there were no detectable differences (21), suggesting a transient
404 effect. While this husbandry change did not have a long-lasting effect on the FM, sampling of
405 the cecum revealed readily detected changes.
406
407 When comparing beta diversity with PCoA, the plots with the most separation involved a
408 group that was offered RO water (Fig 2), reflecting this treatment regimen's capacity to influence
409 the GM. No clear explanation can be given as to why this was the case. However, when
410 considering the mechanistic process of reverse osmosis, it is the only decontamination method

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411 out of the four used that filters the water. The water passes through a membrane that is able to
412 filter many compounds such as disinfectant byproducts, pesticides, endocrine-disrupting
413 compounds, and pharmaceutical residues (42) that can potentially have a physiological
414 influence on our rodents. This water filtration process can also filter out other compounds and
415 minerals that would normally be available to the GM and therefore can directly influence the
416 microbial content. Another interesting finding was that the autoclaved groups had a decreased
417 richness of the CM when compared to all other groups. The fact that the water bottles for all
418 groups were autoclaved in addition to other treatment in six of the groups (i.e. filtration or
419 acidification), suggests that those additional treatments may inadvertently serve as a nidus for
420 other bacteria or provide an environment fostering changes in the community composition.
421 When evaluating α-diversity, significant differences were limited to the CM and were only
422 demonstrated in the Shannon diversity index (S3 Table). The Simpson diversity index is more
423 sensitive to abundant species (43), and therefore taxonomic units of low abundance have a
424 smaller impact on this index. Collectively, these findings suggest that lower abundance taxa
425 played a lesser role in the differences seen among husbandry factor combinations.
426
427 Regarding the taxa putatively susceptible to the husbandry factors under investigation,
428 RF and two-way ANOVA identified many of the same OTUs. A total of 26 OTUs were
429 significantly different based on two-way ANOVA with *p* values corrected to account for multiple
430 testing, all with a significant main effect of water. Fourteen of those 26 were identified in the RF
431 analysis as significant classifiers for the specific water and bedding combination. Several of the
432 OTUs represented as important classifiers were OTUs that have been associated with health
433 and disease. The genus *Akkermansia,* known to modulate the immune system and associated
434 with metabolic diseases such as obesity (44-47), was increased in groups receiving autoclaved
435 water. An unclassified species of *Lactobacillus* was also selected as a classifier and the relative
436 abundance was significantly different between groups, likely due to its low relative abundance in

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437 samples from mice receiving RO-treated water. *Lactobacillus* is a genus that has gained
438 attention through the years for its potential probiotic applications (48). In mice, certain
439 *Lactobacillus* species have demonstrated the ability to stimulate an immunoregulatory response
440 that allows the bacteria to persist in the bowel (49). Moreover, several *Lactobacillus* spp. have
441 repeatedly been implicated as microbial determinants of cognitive function and behavior in
442 mouse models (50-53) suggesting that husbandry factors affecting the GM are a critical
443 consideration for investigators in the field of neuroscience and ethology.
444
445 In summary, water decontamination methods and bedding material used in rodent
446 facilities can alter the GM and therefore must be considered when designing a study. Significant
447 changes were primarily noted in cecal samples, confirming observations in previous studies (14)
448 and suggesting that fecal sampling alone may be insufficient to unearth subtle changes in GM.
449 Water decontamination methods vary within rodent facilities and can alter the GM composition,
450 adding a potential variable to experimental outcomes. Accounting for and documenting these
451 factors will aid in efforts to optimize reproducibility. It is therefore essential for investigators to
452 provide full details as described in the ARRIVE guidelines (54) when writing a manuscript in
453 order to increase reproducibility and ultimately translatability of our animal studies.

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457 # Author Contributions

458 Conceived and designed the experiments: WAB ACE CF. Performed the experiments: WAB.
459 Analyzed the data: WAB. Contributed reagents/materials/analysis tools: ACE CF. Wrote the
460 paper: WAB ACE CF.

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592

593 Supporting information

594 **S1 Table. Water decontamination methods survey.** Survey sent via e-mail through
595 Compmed listserv on December 15, 2015.

596 **S1 Fig. Average relative abundance of operational taxonomic units (OTUs) in each group.**
597 Bar graphs representing the average relative abundance of OTUs in each group for (A) fecal
598 microbiota and (B) cecal microbiota. Each color represents a different OTU. Legend on right
599 represents OTUs with high (>1%) relative abundance.

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600 **S2 Table. Fecal richness and diversity analysis.** Two-way ANOVA results of richness and
601 diversity (Shannon and Simpson diversity indices) from fecal endpoint (Day 28) samples.
602 Considered $p<0.05$ significant.

603 **S3 Table. Cecal richness and diversity analysis.** Two-way ANOVA results of richness and
604 diversity (Shannon and Simpson diversity indices) from cecal samples. Considered $p<0.05$
605 significant.

606 **S2 Fig. Hierarchical clustering of cecal samples using the most variable operational**
607 **taxonomic units (OTUs).** Hierarchical clustering of the top 25 (lowest *p*-values corrected by
608 false discovery rate) OTUs by one-way ANOVA of all cecal samples. Color intensity shows cube
609 root transformed normalized abundance of OTUs in each sample. Color-coded bars at top
610 represent bedding/water group (legend on right).

611 **S4 Table. Significantly different operational taxonomic units (OTUs)**. Two-way ANOVA
612 results of significantly different ($p<0.05$) OTUs. Calculated using cube root transformed
613 normalized abundance of OTUs in each sample. Numbers represent false discovery rate
614 adjusted *p*-values.

615

616 **S3 Fig. Changes of fecal microbiota from arrival to endpoint.** Principal coordinate analysis
617 of all fecal samples at arrival (black circles) and all different endpoint groups (legend on right).
618 One-way PERMANOVA of ranked Bray-Curtis similarity indices results shown.

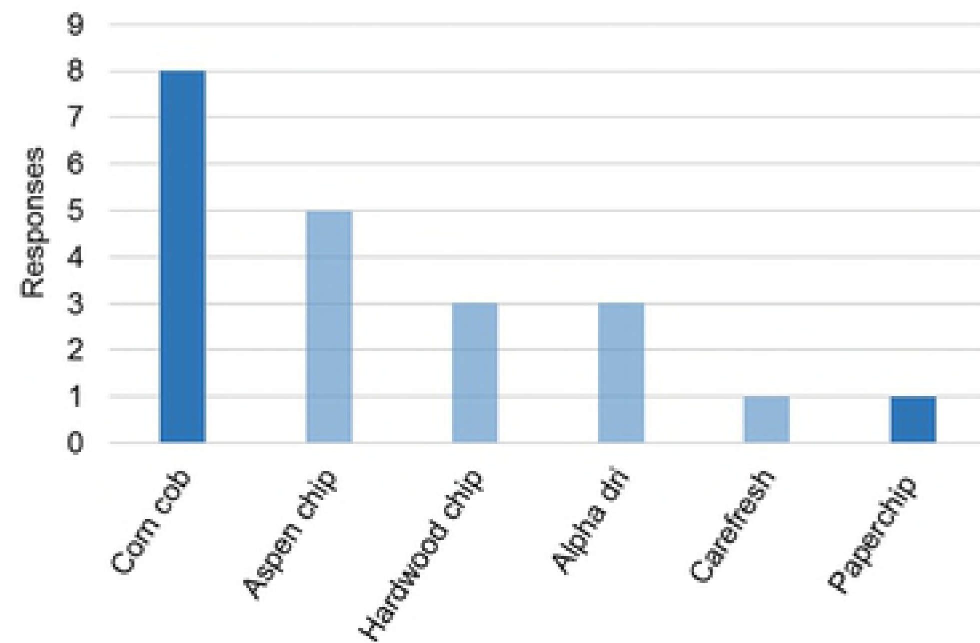
A

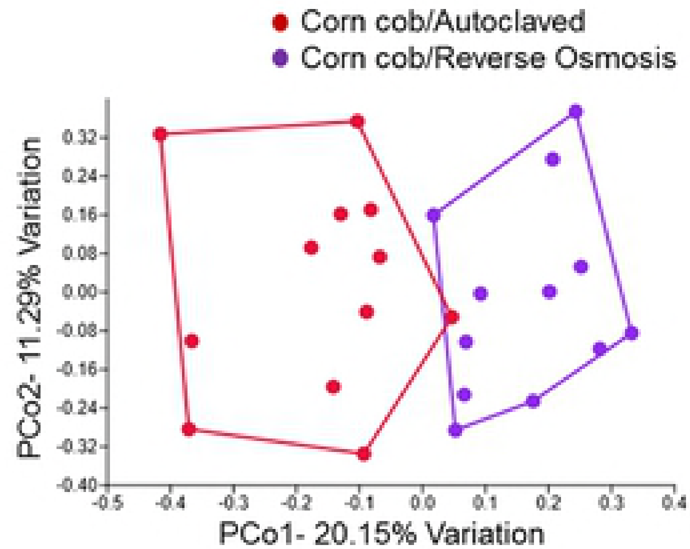
Water Decontamination Technique Survey

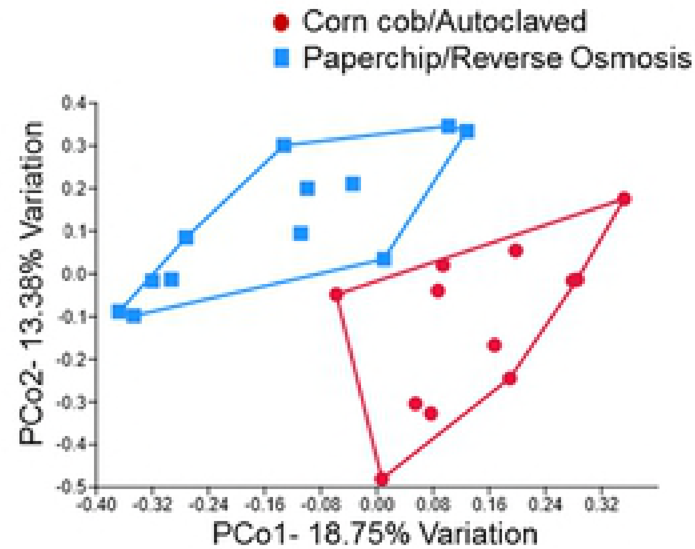
Responses

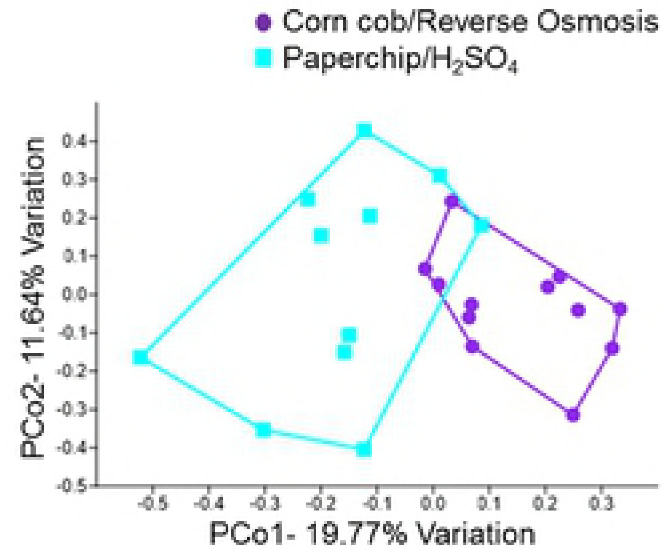
10
9
8
7
6
5
4
3
2
1
0

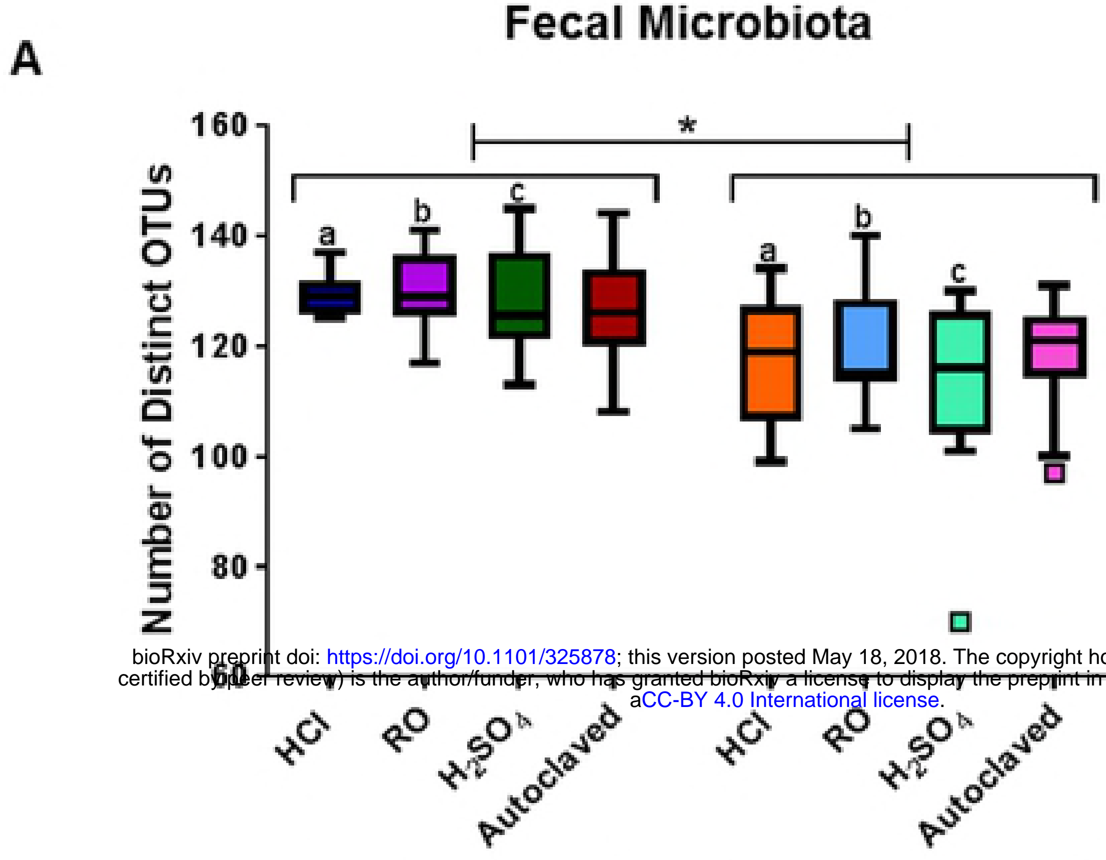
Reverse osmosis
Hydrochloric acid
Autoclave
Chlorinate
UV light
Tetracycline
Tap water
RO and Chlorinate
Sulfuric acid
Dechlorination
Hydropac
Phosphoric acid
RO and UV




A
Corn cob/Autoclaved
Corn cob/Reverse Osmosis
PCo2- 11.29% Variation
PCo1- 20.15% Variation


B
Corn cob/Autoclaved
Paperchip/Reverse Osmosis
PCo2- 13.38% Variation
PCo1- 18.75% Variation


C
Corn cob/Reverse Osmosis
Paperchip/H_2SO_4
PCo2- 11.64% Variation
PCo1- 19.77% Variation



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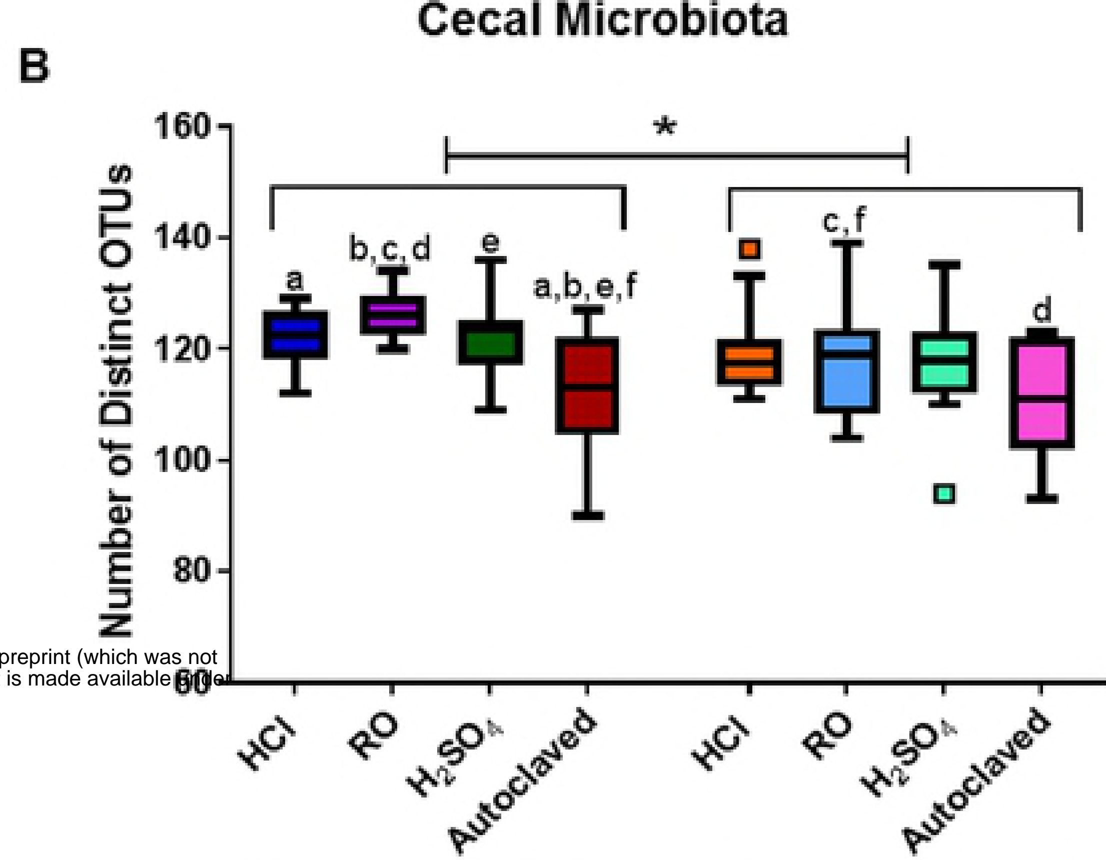

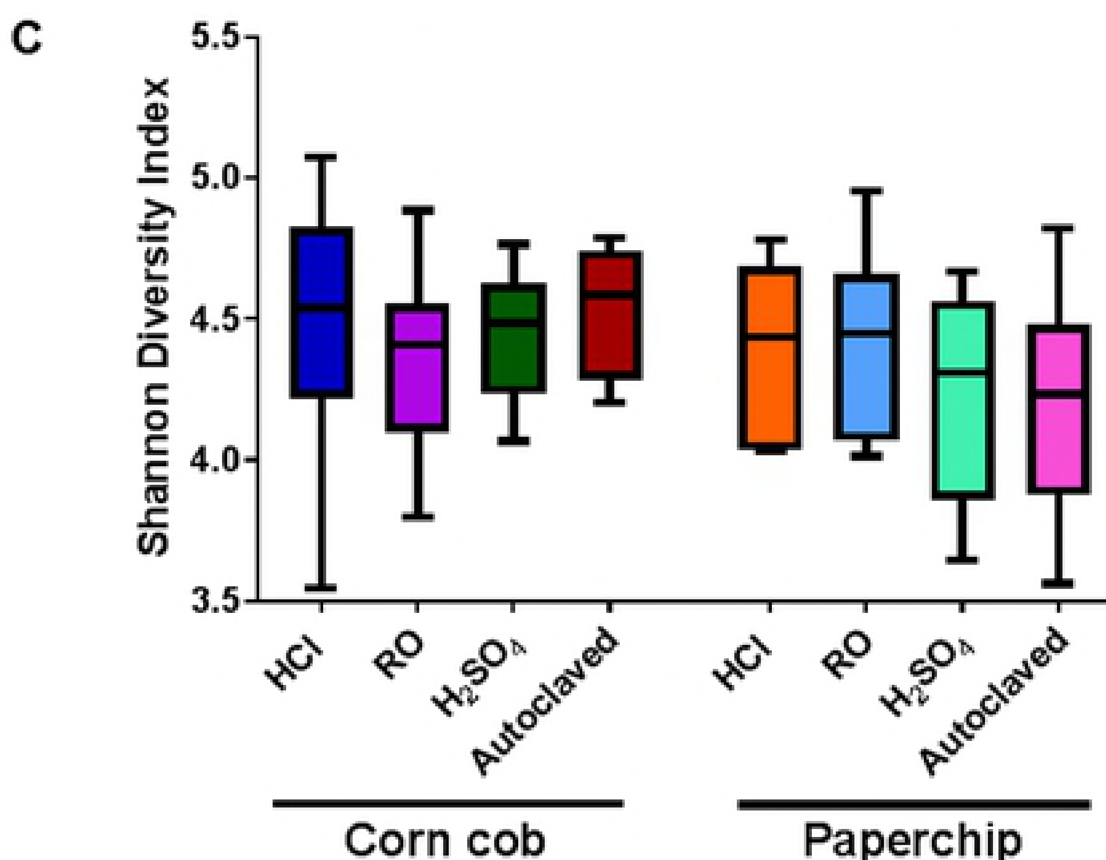

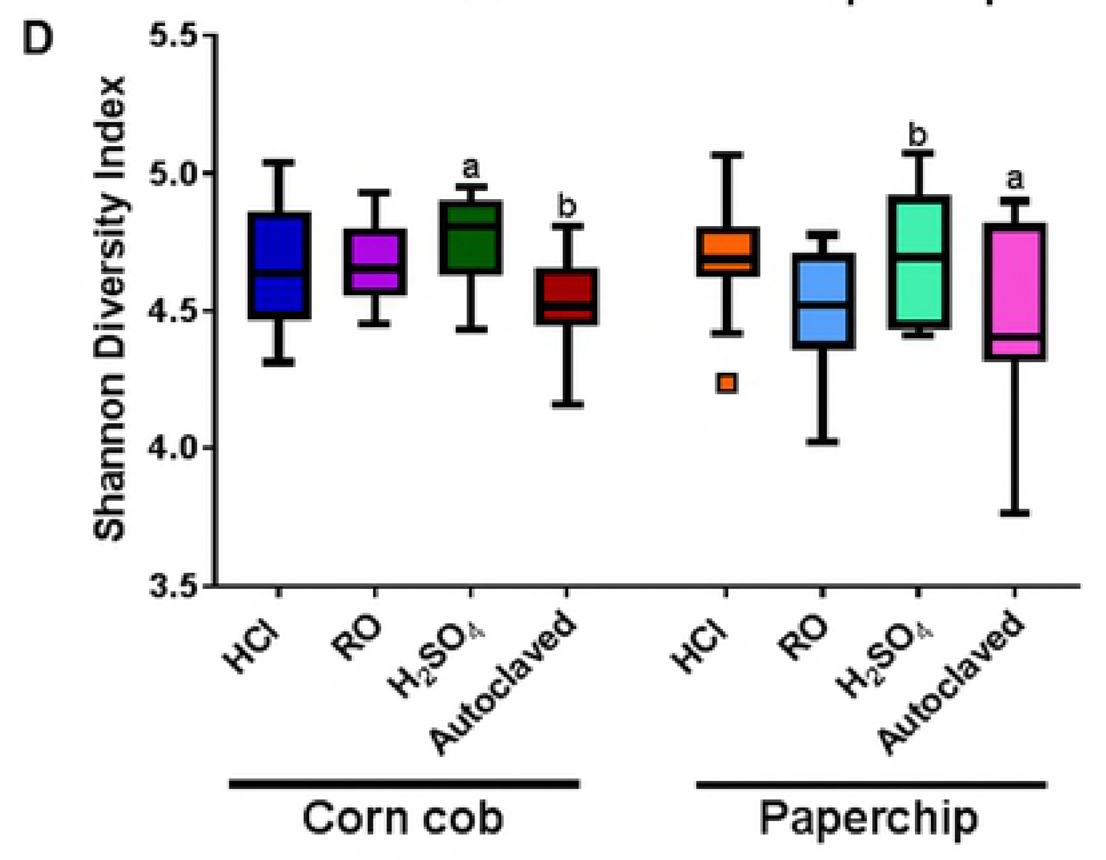

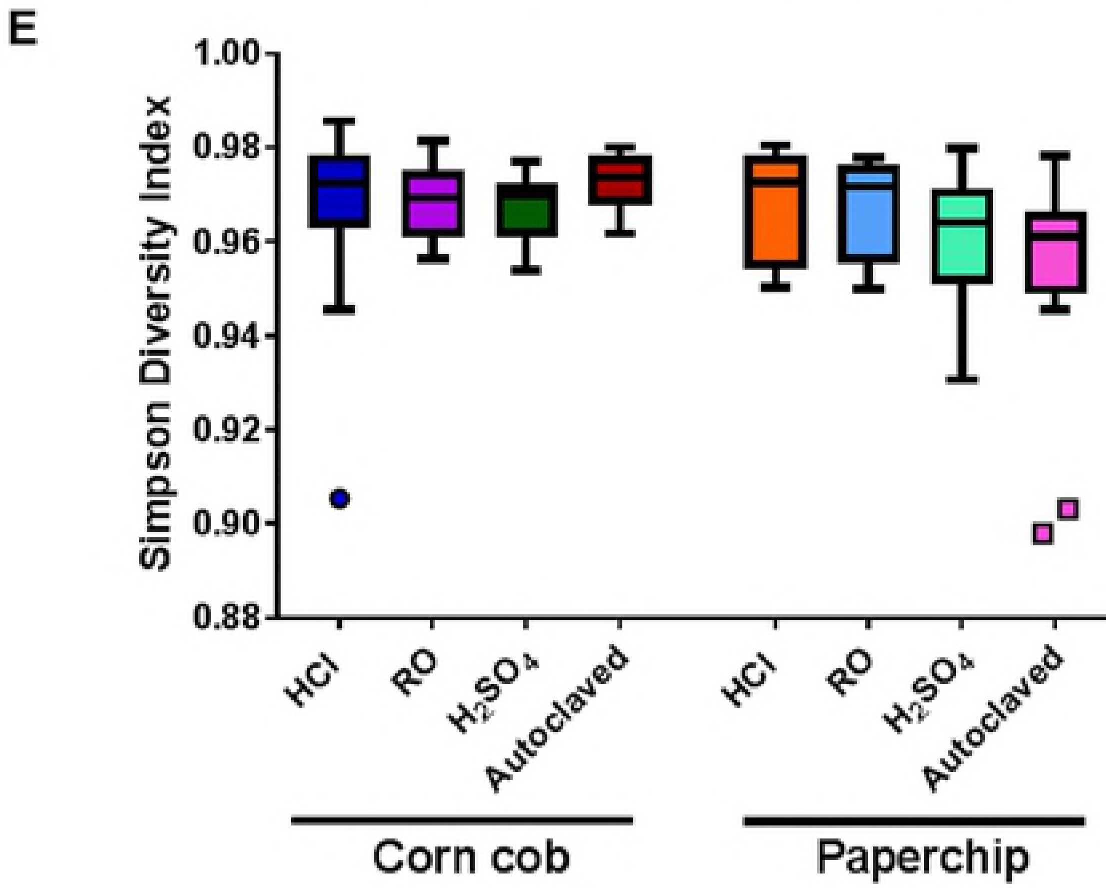

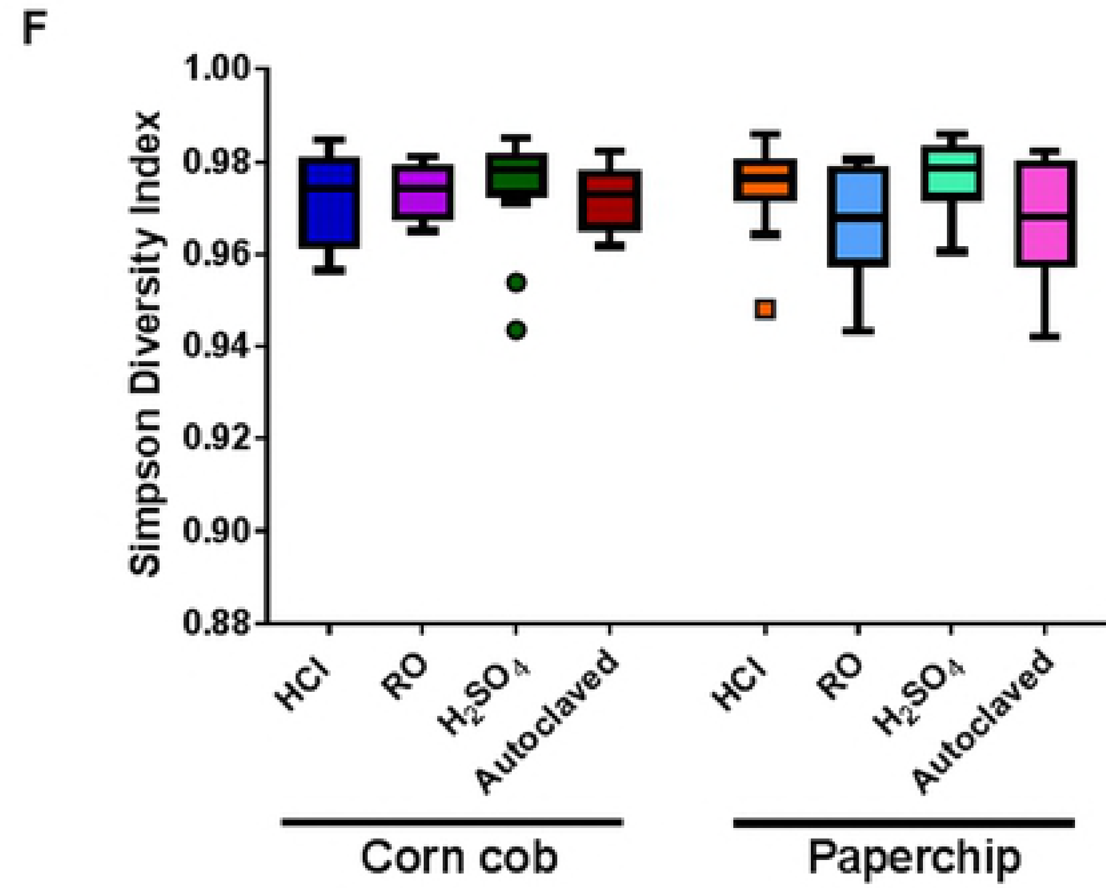

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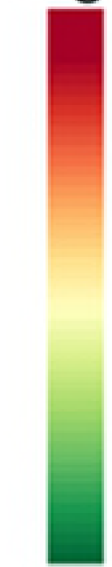

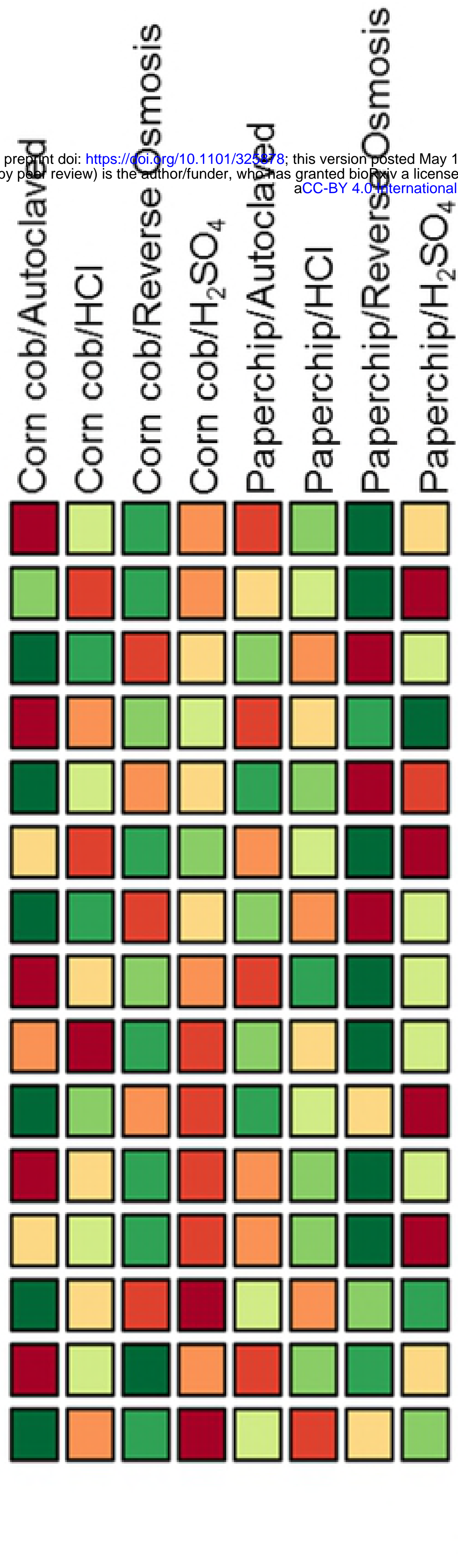

OTU #	Name
OTU 73*	Family XIII UCG-001 sp.
OTU 96*	*Lachnoclostridium* sp. 1
OTU 65*	UC Family *Clostridiales* vadinBB60 group 1
OTU 265*	*Akkermansia sp.*
OTU 174*	*Ruminococcaceae* UCG-009 sp.
OTU 162*	*Ruminiclostridium* 5 sp. 4
OTU 67*	UC Family *Clostridiales* vadinBB60 group 3
OTU 144*	UC Family *Peptococcaceae* 1
OTU 160*	*Ruminiclostridium* 5 sp. 2
OTU 129*	*Shuttleworthia* sp.
OTU 48*	*Lactobacillus* sp.
OTU 8*	*Enterorhabdus* sp.
OTU 260*	UC Order *Mollicutes* RF9 1
OTU 159*	*Anaerostipes* sp.
OTU 262	*Oryza meyeriana* sp.