1 **Title**

2 **Soil prokaryotes associated with decreasing pathogen density during anaerobic**

3 **soil disinfestation**

4

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17 **Abstract**

18 Anaerobic soil disinfestation (ASD) is a chemical-independent method that can reduce
19 pathogens. Although soil microbes play essential roles in ASD, the relationship between
20 the microbial community structure and disinfestation efficiency remains unclear. To this
21 end, we investigated changes in the microbial community and pathogen density during a
22 period of ASD under field conditions for 14 days in a greenhouse using three different
23 substrates. Soil samples were collected at 0, 3, 7, and 14 days after ASD treatment. The
24 pathogen densities were analyzed by real-time polymerase chain reactions, prokaryotic
25 community analysis was conducted using unidirectional pyrosequencing, and the factors
26 related to pathogen density were statistically analyzed. The pathogen density rapidly
27 decreased by >90% at 3 days after treatment and then slowly decreased until day 14, but
28 the rate of decrease differed among the substrates. The microbial communities became
29 altered after 3 days and recovered to their original state on day 14. The dipyridyl
30 reaction, microbial diversity, richness, and community structure were not correlated
31 with pathogen density. The most negatively correlated operational taxonomic units with
32 pathogen density were Clostridia and Bacilli, both belonging to Firmicutes. These

33 results suggested that the growth of specific microbes, but not the changes in microbial

34 community structure, might be important for ASD disinfestation efficiency.

35

36 **Keywords**

37 Bacilli, Clostridia, C/N ratio, Fusarium wilt, rank correlation

1. Introduction

Tomato (*Solanum lycopersicum*) is one of the most important vegetables worldwide, with a global yield of approximately 240 million tons in 2017 (FAO, 2019). Soil-borne pathogens cause various plant diseases, including take-all, damping-off, crown rots, and wilting. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is one of the most serious soil-borne tomato diseases (Larkin and Fravel, 1998). The control methods for this disease, such as soil amendments, crop rotation, biological control, and field sterilization, are often ineffective because they can be affected by environmental factors, such as temperature, precipitation, soil properties, etc. (Campbell, 1994). Although soil disinfestation using chemicals can decrease the pathogen, food safety requirements and the need to reduce environmental pollution have made the development of eco-friendly disinfestation methods crucial (Griffiths et al., 2000; Zhou et al., 2019).

Since 2000, the use of anaerobic soil disinfestation (ASD) to generate anaerobic conditions in soil has been studied in Japan (Momma, 2008; Shinmura, 2000), the Netherlands (Blok et al., 2000; Messiha et al., 2007), and the USA (Butler et al., 2012;

Rosskopf et al., 2014; Shennan et al., 2014). ASD involves treating the soil with labile organic carbon, irrigation to saturation using water, and covering with polyethylene mulch film for 2–5 weeks. The organic matter increases microbial respiration, and irrigation purges soil air, while the polyethylene film prevents an inflow of oxygen from the atmosphere, which collectively induce reductive soil conditions. This technique is effective for suppressing several soil-borne diseases, including bacterial wilt, Fusarium wilt, and root rot nematode (Butler et al., 2014; Shrestha et al., 2016). However, the exact mechanisms of ASD that lead to disease suppression remain unclear.

Soil microbes play an essential role in disinfestation by ASD. Momma et al. (2010) showed that sterilized soil loses its disinfestation ability. Microbial communities drastically changed following ASD treatment, especially Clostridia, a class of anaerobic bacteria, that increased significantly after ASD treatment (Mowlick et al., 2013a, 2013b; Rosskopf et al., 2014). However, Clostridia often increase in anaerobic soil regardless of ASD treatment. The relationship between the disinfestation efficiency and microbial community during ASD treatment is unelucidated. To clarify this relationship, monitoring of the changing microbial communities and pathogen densities during the

disinfestation period are required. To our knowledge, the changes in the microbial community during the course of ASD treatment have only been analyzed by Li et al. (2017) under *in vitro* conditions, and the relationship between prokaryotic communities and pathogen density was not established.

Ethanol and molasses (as labile organic substrates) and wheat residue, rice husk, and mustard residues (as recalcitrant organic substrates) are often used for ASD, and the substrate type has been found to affect the disinfestation efficiency (Strauss and Kluepfel, 2015; Testen and Miller, 2018). In our study, two labile organic substrates (sugar-contained diatoms [SCDs] and dried molasses [DM]) and one recalcitrant organic substrate (wheat bran [WB]) were used as the carbon sources. We aimed to demonstrate 1) the behavior of FOL and microbial communities over time, and 2) the effects of substrate types on FOL density and microbial community structure during the ASD period under field conditions.

2. Materials and methods

2.1 Sampling field and ASD treatment

86 The experiments were performed with soil samples from a tomato-planted greenhouse
87 located in Chiba Prefectural Agriculture and Forestry Research Center, Chiba prefecture
88 (35° 54' N, 140° 19' E). The soil pH was 5.9, and the carbon and nitrogen
89 concentrations were 47.2 and 3.38 g kg soil^{-1}, respectively. To make FOL-contaminated
90 soil, 10 FOL-infected tomato tubers were packed in a mesh bag and buried in the soil at
91 a depth of 0–15 cm. WB, SCDs (Ajinomoto Coo., Inc., Saga, Japan), and DM (Omalass
92 95; Westway Feed Products, Tomball, TX, USA) were used as substrates for the ASD
93 treatment. SCDs are discharged from food-processing facilities as by-products of the
94 filtration of saccharified liquids. The main components of such by-products are sugars
95 derived from the saccharified solution of tapioca starch and diatoms used as a filtering
96 aid. These by-products, containing 40% sugar by weight, were powdered. DM is a
97 livestock feed containing water-soluble sugar. The material comprises 33% soybean
98 husks and 67% sugarcane molasses, and they were absorbed. The chemical properties of
99 each substrate are shown in Table 1. The treatment was applied at a rate of 15 t ha^{-1} in
100 each field with a rototiller at a depth of 30 cm. Each treatment was performed in
101 duplicate on 2.5 × 2.2 m plots that were separated by a 50-cm high wave barrier. The

102 field was covered by a 0.1-mm thick transparent polyethylene film and flooded with
103 150 L of water on day 0. Each site was flooded at the time of disinfestation, and no
104 irrigation was conducted thereafter. Disinfestation was conducted from August 3, 2015,
105 for 14 days. Soil samples were collected at a depth of 0–15 cm from each treated field
106 using a core sampler (Gauge Auger DIK-106B; Daiki Rika Kogyo Co., Ltd, Saitama,
107 Japan) on day 0, 3, 7, and 14 after the ASD treatment. The reduction area was
108 visualized by spraying a bipyridyl solution (1.0 g of 2,2′-bipyridyl and 77 g of
109 ammonium acetate dissolved in 1 L of 1% acetic acid) on a freshly exposed soil face.
110 Soil samples were collected from five randomly selected locations in each plot and
111 mixed well to make a composite sample. A total of 24 soil samples (three substrates ×
112 four sampling periods × two replicates) were collected and stored at −20 °C until use.
113

114 **2.2 Quantification of *F. oxysporum* in the field**

115 Soil DNA was extracted from 0.5 g of soil with an ISOIL for Beads Beating kit
116 (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. DNA
117 quantification and integrity were measured using a Nanodrop spectrophotometer

(Thermo Fisher Scientific, Waltham, MA, USA) and gel visualization (0.8% agarose in tris-acetate-EDTA buffer), respectively. Real-time polymerase chain reactions (PCRs) were performed for quantification of *F. oxysporum* density in soil, according to (Inami et al., 2010). The reaction mixture (20 µl) contained 10 µl of TaqMan Universal Master Mix (Thermo Fisher Scientific), 0.5 µM of each primer, 0.25 nM of TaqMan probe, and 15 ng of template DNA. Real-time PCR was performed in duplicate for each sample to amplify the rDNA intergenic spacer region of *F. oxysporum* f. sp. *lycopersici* using specific primer sets SIX1f (5'-GTGCCAGCMGCCGCGGTAA-3') and SIXir (5'-GGAC-TACVSGGGTATCTAA-3') and a TaqMan probe carrying a reporter (6-carboxyfluorescein) and a quencher (minor groove binder) SIX1pr (5'-TTGACCTACACGGAATAT-3'). Standard curves were obtained by serial dilutions of linearized plasmids with cloned fragments of the specific genes and were linear (R^2 = 0.99) in the range used (data not shown).

2.3 Tag-encoded amplicon sequencing targeted by 16S rRNA for prokaryotes

PCR was performed on each sample to amplify the V4 variable region of the 16S rRNA gene using the bacterial and archaeal universal primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGAC-TACVSGGGTATCTAA-3') (Caporaso et al., 2011). Each PCR amplicon was purified twice using the Agencourt AMPure XP system (Beckman Coulter, Inc., Brea, CA, USA) to remove short DNA fragments and was quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). Following successful amplification, the PCR products were adjusted to equimolar concentrations and subjected to unidirectional pyrosequencing at Bioengineering Lab. Co., Ltd. (Kanagawa, Japan) on a MiSeq instrument (Illumina, San Diego, CA, USA). A total of 1,161,378 sequences were obtained from the 24 samples after sequencing (Supplemental Table 1). The sequencing data were deposited in the DNA Data Base of Japan Sequence Read Archive under accession number DRA006673.

2.4 Data analysis

Raw FASTQ files were pre-processed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). Data from read sequences, quality, flows, and

149 ancillary metadata were analyzed using the QIIME pipeline. Quality filtering consisted
150 of discarding reads <200 bp or >1000 bp in length, excluding homopolymer runs of
151 >six bp and >six continuous ambiguous bases, and accepting one barcode correction
152 and two primer mismatches. Moreover, reads with a mean quality score <25 were also
153 removed. Finally, singleton operational taxonomic units (OTUs) and chimeric
154 sequences were removed for statistical analysis. Denoising was performed using the
155 built-in Denoiser algorithm, and chimera removal and OTU picking were accomplished
156 with Usearch61 considering a pairwise identity percentage of 0.97. Taxonomy
157 assignment was performed using the Ribosomal Database Project Classifier, a naïve
158 Bayesian classifier, with a minimum confidence of 0.8 against the Greengenes database,
159 October 2012 release. The OTU-based analysis was performed on pyrotag-based
160 datasets to calculate richness and diversity using the phyloseq package of R 3.5.1
161 (McMurdie and Holmes, 2013). The diversity within each sample was estimated using
162 the non-parametric Shannons's diversity index and Simpson's diversity index. The
163 Chao1 estimator was calculated to estimate the species richness of each sample.
164 Multivariate analysis of community structure and diversity was performed on the

165 pyrotag-based datasets using a weighted UniFrac dissimilarity matrix calculated in
166 QIIME, jackknifing (1000 reiterations) read abundance data at the deepest level
167 possible (9601 reads), and unconstrained ordination offered by cluster analysis with
168 Ward's method. The effect of differences between substrates and the disinfestation
169 period on the microbial community were assessed by permutational multivariate
170 analysis of variance using R. The Mantel test was used to evaluate the effect of FOL
171 density on microbial communities (Anderson and Walsh, 2013). Spearman's rank
172 correlation methods were used to determine which OTUs were correlated with FOL
173 density changes common among each substrate-treated soil sample.
174

175 **3. Results**

176 **3.1 Changes of FOL density during ASD**

177 We described each sample with the substrate name and replication number, for
178 example, WB1 indicated the first sample of two replicates in soil receiving WB
179 treatment. The FOL density was 642–1832 copies g soil^{-1} on day 0, decreasing to
180 0.6%–10.6% on day 3 and then gradually decreasing up to day 7, except for sample

181 DM1 (Table 2). On day 14, the FOL densities of the DM-treated samples were

182 decreased (<1 copy g soil^{-1}), but that of the other treatments were not. A bipyridyl

183 reaction was observed in WB1, SCD1, and DM1 14 days after ASD (Supplemental

184 Table 2).

185

186 **3.2 The succession of prokaryotic soil communities during the disinfestation period**

187 The prokaryotic sequences were clustered into 130,331 OTUs at a 97% similarity.

188 Shannon's index changed from 0.78 to 1.1 times when day 0 was compared with day 14

189 (after disinfestation) across each treatment. For the SCD-treated samples, a decrease in

190 both OTU numbers (SCD1, 76% decrease; SCD 2, 32% decrease) and Chao1 (SCD1,

191 89% decrease; SCD2, 44% decrease) was observed on day 14 when compared with day

192 0 (Table 3). For the other treatments, the OTU numbers and Chao1 were mildly

193 decreased or increased after disinfestation. Our results also indicated that changes in

194 prokaryotic diversity and richness were not correlated with changes in FOL density

195 during the ASD period (Table 4).

196 The dominant classes of Bacilli, Clostridia, Alphaproteobacteria, Betaproteobacteria,
197 Deltaproteobacteria, and Gammaproteobacteria occupied >5% of relative abundance in
198 all fields (Figure 1). Bacilli and Clostridia were increased >1.5 times on day 3 in all
199 treatments. In the WB treatment, several microbes were drastically decreased between
200 days 3–7, and they increased from days 7–14. On the other hand, the microbial
201 communities were relatively stable 7 days after the SCD and DM treatments (Figure 1).
202 Prokaryotic growth stages were classified as either early (0–3 days), intermediate (3–7
203 days) or late (7–14 days), based on the period when each prokaryote increased >two-
204 fold for both duplicates. In the SCD and DM treatments, Bacilli and Clostridia were
205 increased in the early stage, and Planctomycetia were increased in the intermediate
206 stage (Table 5). In the WB-treated samples, Bacilli and Clostridia were also increased in
207 the early stage, while Betaproteobacteria, Alphaproteobacteria, Gammaproteobacteria,
208 Planctomycetia, and Saprospirae were increased in the late stage.
209 Weighted UniFrac analysis showed that prokaryotic communities were roughly
210 separated throughout the disinfestation period (Figure 2). The initial microbial
211 community was similar in all samples except for SCD1 and became separated as SCD1,

212 WB1, WB2, and DM2 on day 3, as was the case on day 0. The changes in the
213 prokaryotic community in the late stage were not different in DM1, DM2, and SCD2
214 because they were included in the same cluster on day 7 and 14. On the other hand, that
215 of the other treatments were divided into different clusters between day 7 and 14.
216 PERMANOVA analysis revealed that the microbial communities were independently
217 affected by substrates and the disinfestation period (Table 6). The Mantel test showed
218 that the microbial communities were not altered by FOL density ($F = 1.82$, $p = 0.06$).
219 These results suggested that factors other than the microbial community affected the
220 decrease in FOL density. Then, OTUs that changed along with FOL density were
221 selected using rank correlation analysis. Eleven OTUs were negatively ($\rho < -0.5$)
222 correlated with changes in FOL density that were common among each substrate-treated
223 field (Table 7). There were 7/11 OTUs belonging to Firmicutes; of which three (19544,
224 122066, and 95490) belonged to Clostridia, 2 (7428 and 109424) to Bacilli, and 1
225 (78089) to Negativicutes. The other OTUs belonged to Acidobacteria (24789),
226 Planctomycetia (9173), and Proteobacteria (69473 and 52717).
227

4. Discussion

4.1 Relationships between substrate types, microbial community, and FOL density

The C/N ratio of substrates is one of the indicators of the decomposition rate (Constantinides and Fownes, 1994; Nicolardot et al., 2001). The WB-treated samples showed a lower level of water-soluble organic carbon and a higher C/N ratio than those treated with SCD or DM because WB is composed of recalcitrant carbon fractions, such as cellulose, hemicellulose, and lignin. Following WB treatment, the microbial community was changed, even after 7 days, but this was unrelated to the disinfestation effect. During ASD, DM rapidly dissolved in the irrigation water because of a higher water-soluble organic carbon fraction and C/N ratio. Following DM treatment, FOL density decreased during days 7–14, but the structures of the prokaryotic communities were not drastically changed during these periods because of the rapid decomposition of the substrate. Previous studies have shown that organic matter with a lower C/N amended into the soil could induce the highest anaerobic conditions, and the substrate C/N ratio was negatively correlated with the disinfestation effects (Blok et al., 2000; Shrestha et al., 2016; Testen and Miller, 2018). In our study, different substrates exerted

244 different effects on the prokaryotic community, but the substrate decomposition rate,
245 especially the variables of substrate C/N ratio and the content of water-soluble organic
246 carbon, was not correlated with the disinfestation efficiency.
247
248 **4.2 Relationships between microbial diversity, community structure, and FOL**
249 **density**
250 During ASD treatment, as the microbial community shifts toward facultative and
251 obligate anaerobes, the anaerobic decomposition of labile carbon creates short-chain
252 organic acids (e.g., acetic, n-butyric, and propionic acid). The volatile fatty acids
253 (VFAs) are likely toxic to soil-borne plant pathogens, plant-parasitic nematodes, and
254 weeds (Momma, 2008; Momma et al., 2006). (Momma et al., 2013) showed that
255 bipyridyl testing (an indicator of reduction) of disinfested soil was a useful method to
256 evaluate whether ASD treatment was conducted appropriately. Liu et al. (2016)
257 showed that although Shannon's diversity index was decreased on day 4 and did not
258 change significantly thereafter, the FOL population decreased after day 4. Messiha et al.
259 (2007) indicated that microbial diversity was not different between substrate amended

and non-amended soil following ASD treatment, but their community structure was different. On the other hand, Shennan et al. (2014) showed that ASD conducted with molasses did not alter community structure. These studies suggest that microbial diversity, community structure, and soil redox potential (Eh) are not correlated with disease incidence, corresponding with our findings. Hence, different mechanisms could be critical for suppressing specific organisms, but the production of organic acids via the anaerobic decomposition of added carbon, release of VFAs, and biocontrol by microorganisms that flourish during the process are all potentially important (Momma et al., 2007; Shrestha et al., 2016). Yonemoto et al. (2006) indicated that a decreasing Eh value and FOL density were not directly correlated and concluded that it was important for the concentration of VFAs or microbial community change. Li et al. (2017) revealed the changes in the microbial community during the disinfestation period, and the presence of some anaerobic bacteria correlated with soil organic acid content. Unfortunately, we did not analyze the soil chemical properties, such as pH, VFA concentrations, and soil organic carbon concentration, in our study. Recent studies have shown that specific microbes, like Clostridia and *Zopfiella*, isolated from ASD-treated

soil could suppress disease incidence (Liu et al., 2019; Momma, 2008; Ueki et al., 2017). Therefore, specific microbes may be necessary for disease suppression by ASD.

4.3 Microbes associated with FOL dynamics

Studies have shown that Clostridia and Bacilli belonging to Firmicutes increased in number and became the dominant bacteria following ASD treatment regardless of soil and substrate types (Huang et al., 2016, 2015; Mowlick et al., 2012; Poret-Peterson et al., 2019, Testen and Miller, 2018). During ASD, decreased oxygen promotes the increased prevalence of anaerobic microbes (Momma et al., 2006; Mowlick et al., 2014, 2013a; Runia et al., 2014). In our study, the conditions induced by ASD, regardless of the carbon source, may have functioned as a habitat filter, allowing the proliferation of closely related taxa with shared physiological adaptations. Clostridia and Bacilli drastically increased and FOL decreased in the early stage of ASD. Increasing clostridial populations in ASD-treated soils might correlate with the elevated production of VFAs and is likely toxic to soil-borne plant pathogens (Momma et al., 2006; Mowlick et al., 2012). Some Clostridia can directly kill FOL (Momma, 2008; Ueki et

292 al., 2017). Bacilli, including *Bacillus* and *Paenibacillus*, are well-known biocontrol
293 agents for FOL. Some Bacilli might contribute to the rapid decrease of soil Eh at the
294 initial stage of ASD treatment via oxygen consumption (Mowlick et al., 2012).
295 Therefore, increased Clostridia and Bacilli may induce a decrease in FOL density in the
296 early stage of ASD treatment by decreasing oxygen, producing VFA, and directly
297 killing FOL. We have yet to obtain direct evidence for the suppression of FOL by
298 Clostridia and Bacilli, but these biocontrol agents may play essential roles in
299 disinfestation.
300

301 **5. Conclusion**

302 Soil microbes play important roles in ASD efficiency. In this study, we analyzed the
303 changes of FOL density and microbial community during ASD among three substrates
304 to elucidate the relationship between FOL density and the microbial community. FOL
305 density was drastically decreased for the first 3 days following ASD and slowly
306 continued decreasing until day 14. The microbial community was significantly changed
307 on day 3, but some microbes were increased until day 14 following WB treatment. The

308 microbial diversity, richness, and community structure as well as the C/N ratio of
309 substrates were not correlated with FOL density in all treatments. Notably, Clostridia
310 and Bacilli were negatively correlated with a decrease in FOL density. These results
311 suggested that specific microbes might be involved in disinfestation efficiency and not
312 changes in the entire community structure itself. Future studies will investigate the
313 usefulness of these microbes as indicators of ASD efficiency and their roles in disease
314 suppression.
315

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476

477 **Table 1. Chemical properties of each substrate used for the ASD treatment**

478

Organic matter	TC ($g\ kg^{-1}$)	TN ($g\ kg^{-1}$)	C/N ratio ($g\ kg^{-1}$)	WSOC ($g\ kg^{-1}$)
WB	400	24.0	16.4	132
SCD	250	10.3	24.2	181
DM	419	13.8	30.4	357

479 TC: total carbon, TN: total nitrogen, C/N ratio: the ratio of total carbon per total

480 nitrogen, WSOC: water-soluble organic carbon, WB: wheat bran, SCD: sugar-contained

481 diatoms, DM: dried molasses.

482 **Table 2. Changes of *F. oxysporum* density (copies g soil^{-1}) during disinfestation**

483 **period**

484

Soil treatment	Replication number	Day 0	Day 3	Day 7	Day 14
WB	1	1381	15.0	11.6	45.6
	2	642	68.1	13.4	27.4
SCD	1	696	39.1	25.9	23.0
	2	798	12.0	3.10	13.1
DM	1	1545	94.2	120	0.30
	2	1832	11.8	9.97	0.61

485 WB: wheat bran, SCD: sugar-contained diatoms, DM: dried molasses

Table 3. Changes of prokaryotic diversity and richness during the disinfestation period

		Replication number	Day 0	Day 3	Day 7	Day 14
Shannon index	WB	1	7.04	6.27	2.34	6.98
		2	6.98	5.99	6.58	6.93
	SCD	1	7.35	3.88	6.94	5.73
		2	6.81	6.93	6.40	7.47
	DM	1	7.61	6.67	6.83	7.03
		2	6.72	6.36	6.79	7.03
OTU number	WB	1	7894	6667	1663	9453
		2	5185	5776	6067	9543
	SCD	1	7374	1143	7618	1794
		2	5020	5405	2969	3401
	DM	1	10389	4193	8509	9051
		2	5419	11196	5665	9594
Chao1	WB	1	23586	18187	3239	32294
		2	10557	16486	24630	31668
	SCD	1	28449	4901	21225	3028
		2	10361	21282	6321	5766
	DM	1	25111	29846	26122	30441
		2	12531	8092	14581	30164

WB: wheat bran, SCD: sugar-contained diatoms, DM: dried molasses.

The number after substrate indicated that the sample number of two replicates

492 **Table 4. Pearson's and Spearman's correlation coefficients between FOL density**

493 **and prokaryotic diversity or richness**

494

Parameter	Pearson's correlation	Spearman's correlation
Shannon's index	0.279	0.212
OTU number	0.038	0.116
Chao1	0.164	0.048

495

497 **Table 5. Responses of bacterial classes during ASD**

498

Bacterial class	WB	SCD	DM
Bacilli	Early	Early	Early
Clostridia	Early	Early	Early
Betaproteobacteria	Late	Early	
Alphaproteobacteria	Late		
Gammaproteobacteria	Late	Intermediate	
Planctomycetia	Late	Intermediate	Intermediate
Saprospirae	Late		

499

500 The bacteria that increased >2-fold times in both duplicates were selected.

501 Early: day 0–3; intermediate: day 3–7 day; late: day 7–14.

502 WB: wheat bran, SCD: sugar-contained diatoms, DM: dried molasses.

503 **Table 6. PERMANOVA results for prokaryotic communities**

504

Variable	F. model	R^2	Pr(>F)
Substrates	1.806	0.136	0.020*
Time	1.724	0.195	0.014*
Substrates and time	0.957	0.217	0.566

505

506 *Indicates statistical significance ($p < 0.05$).

507 **Table 7. OTUs negatively correlated with FOL density**

508

OTU number	Rho value	Closest relatives	Phylum
OTU19544	−0.585	Clostridiales	Firmicutes
OTU122066	−0.563	*Clostridium*	Firmicutes
OTU7428	−0.558	Bacillales	Firmicutes
OTU109424	−0.558	*Paenibacillus*	Firmicutes
OTU78089	−0.556	Veillonellaceae	Firmicutes
OTU23993	−0.536	*Unidentified Firmicutes*	Firmicutes
OTU95490	−0.528	Peptococcaceae	Firmicutes
OTU24789	−0.527	*Candidatus Solibacter*	Acidobacteria
OTU9173	−0.508	Planctomycetaceae	Planctomycetia
OTU69473	−0.507	Unidentified Proteobacteria	Proteobacteria
OTU52717	−0.502	Comamonadaceae	Proteobacteria

509 Rho value indicates the correlation coefficient between FOL density and each OTU as

510 per Spearman's rank correlation method.

511 **Figure legends**

512 **Figure 1.** Relative abundance of prokaryotic communities at the class level during the

513 disinfestation period. (a) WB1, (b) WB2, (c) SCD1, (d) SCD2, (e) DM1, (f) DM2

514

515 **Figure 2.** Changes in prokaryotic communities during ASD treatment. WB: wheat bran,

516 SCD: sugar-contained diatoms, DM: dried molasses. The numbers at each plot show the

517 date of sampling and the sample number connected with a hyphen. The line indicates

518 the separation of each cluster based on *k*-means analysis.

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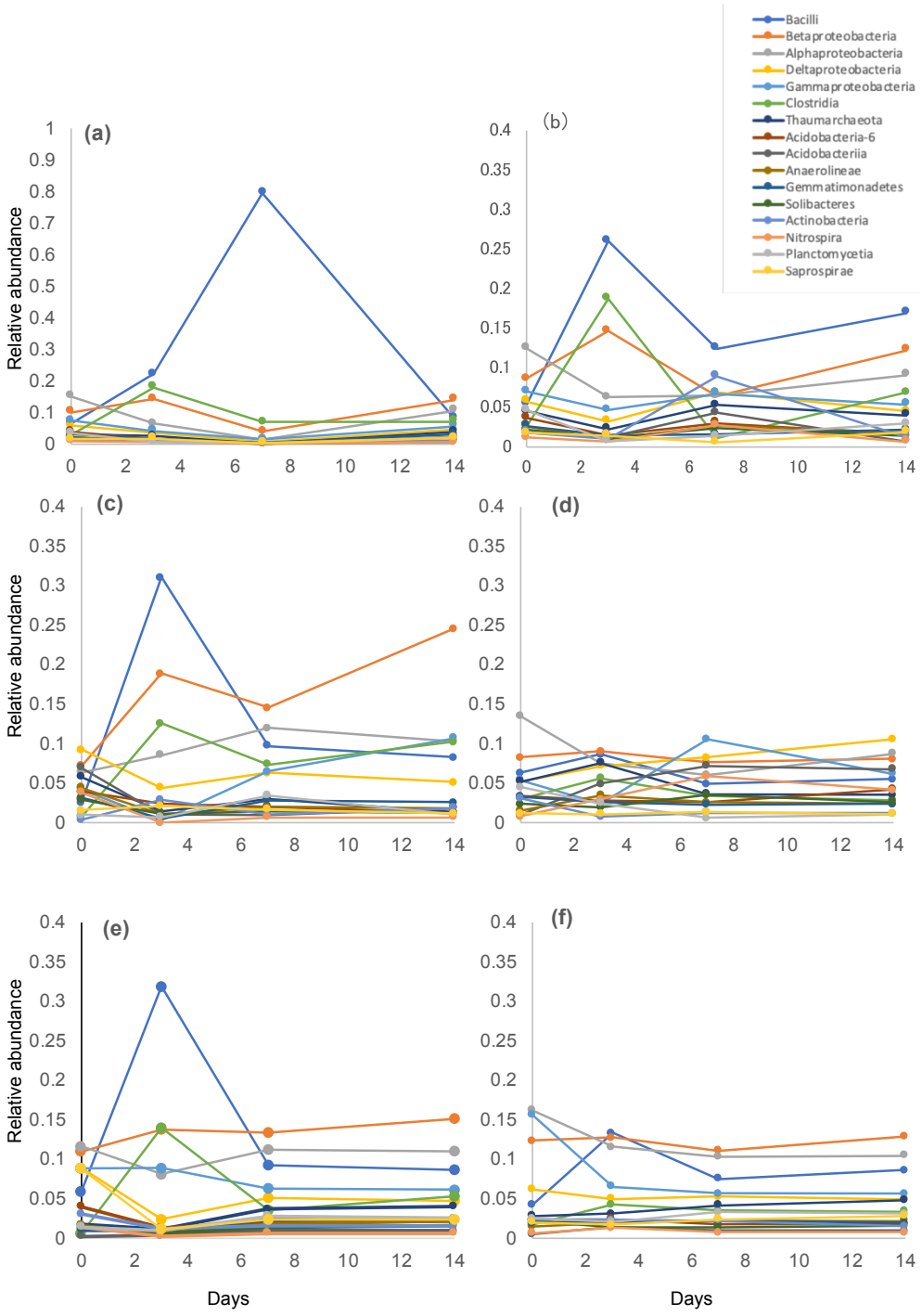
Figure 1



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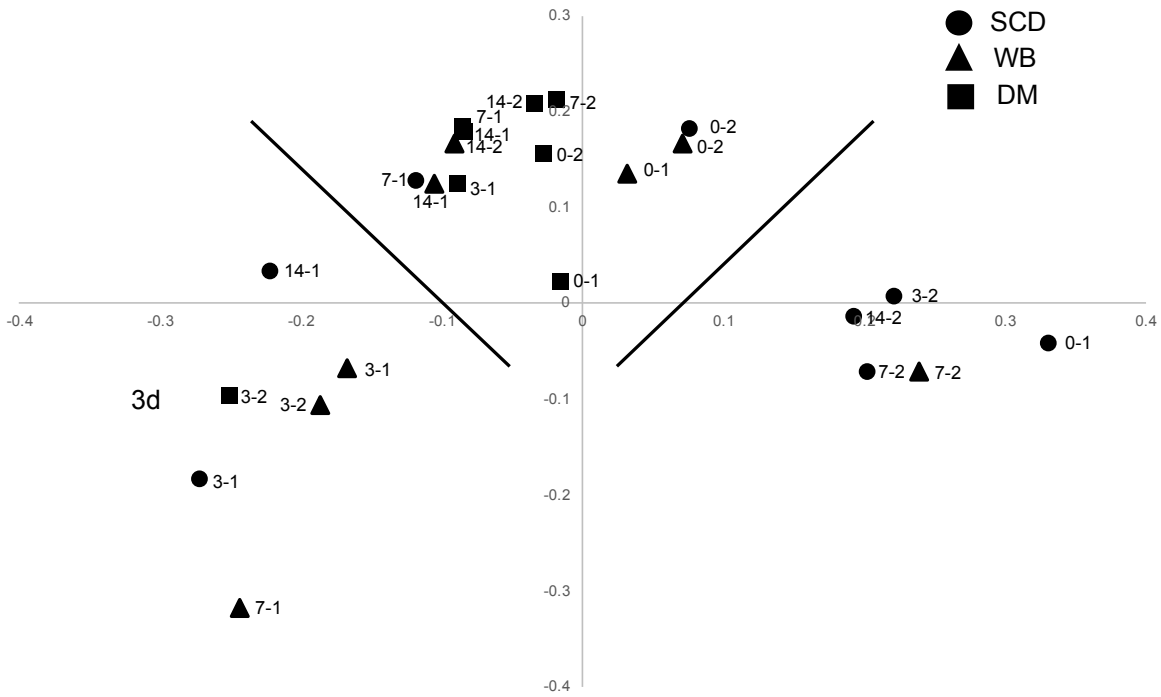
532 **Figure 2**

533



534

535

536 Supplemental Table 1. Sequencing read numbers of each sample

537

	Replication number	Day 0	Day 3	Day 7	Day 14
WB	1	67,737	53,661	32,042	57,352
	2	47,167	45,457	53,315	53,786
SCD	1	59,144	54,281	54,852	16,094
	2	44,798	45,825	52,338	9,601
DM	1	61,651	24,593	38,626	50,307
	2	40,350	89,546	56,514	55,341

538 WB: wheat bran, SCD: sugar-contained diatoms, DM: dried molasses.

539 Supplemental Table 2. The dipyridyl reaction of each soil sample during disinfestation

540 period

	Replication number	Day 0	Day 3	Day 7	Day 14
WB	1	−	−	+	+
	2	−	−	−	−
SCD	1	−	−	−	+
	2	−	−	−	−
DM	1	−	−	+	+
	2	−	−	−	−

541 −, no bipyramidal reaction was observed; +, bipyramidal reaction was observed

542 WB: wheat bran, SCD: sugar-contained diatoms, DM: dried molasses.