



## RESEARCH ARTICLE - ANTS

## Comparison of Surface Sterilization Methods for the Analysis of Insect Gut Microbiota: *Solenopsis invicta* (Formicidae) as an Example

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### Abstract

The gut microbiota of vertebrates and invertebrates has become the focus of recent research. However, current sterilizing practices need more systematic quantitative methods due to limitations caused by their minute body size. Thus, an effective sterilization process incorporating organic and inorganic methods to obtain invertebrate microbiota, particularly when evaluating smaller insects, has yet to be elucidated. This study investigated if the whole abdomen should be utilized as the material to sterilize and examined whether physical and chemical surface sterilization methods could be combined to facilitate the acquisition of gut microbiota from the imported red fire ant (*Solenopsis invicta* Buren, 1972). Eight methods were designed by incorporating three chemical reagents (sterile water, 2.0 mg/L NaClO, and 75% ethanol) and one physical treatment (250 nm UV). The length range of the amplified fragment in the red imported fire ant is 401-450 bp. According to the results of the GLM regression model and interaction effect model, none of these factors (sterile water, 2.0 mg/L NaClO, and 75% ethanol, 250 nm UV) were significant for statistical regression of the Chao index, and these factors did not significantly interact with each other. Based on Alpha and Beta diversity analysis, none of the methods significantly affected the diversity of insects' gut microbiome. Finally, we suggested that it is feasible for different species of small insects to select appropriate methods according to the current situation. Still, it is best to achieve unity in the same group.

### Introduction

Globally, invertebrate insects dominate our living ecosystems, and their diversity drives the functioning of gut microbiota. An insect's digestive tract comprises a foregut, crop, midgut, and hindgut. Previous studies have isolated insect gut microbiota samples by dissecting the abdomen under an optical microscope or stereoscope microscope. However, this method has some limitations, for example, He et al. (2014) obtained isolates from the gut microbiota of *Camponotus fragilis* via traditional dissection methods, and their results showed that the lab-raised ants yielded 12

species via the use of classical microbial culture methods. Nonetheless, polymerase chain reaction-restriction fragment length polymorphism analysis yielded 11 species. However, these methods are only sometimes reliable as most archaea and bacterial diversity remain uncultured or poorly characterized (Lewis et al., 2020). Moreover, traditional anatomical methods do not apply to tiny insects. Nowadays, with the development of deep sequencing (Urakawa et al., 1999; Koto et al., 2008; Kim et al., 2020), many methods are available for acquiring samples and cultivating bacteria, leading to more significant variations in results. Some of these variations might be because human factors influence gut dissection.



Ultraviolet (UV) is a reliable and environment-friendly sterilization method for water (Yagi et al., 2007) that can cause significant attenuation of the growth factor release kinetics (Evrova et al., 2019), leading to a sterilization effect on the microbiota (Yung et al., 2020). Yung et al. (2020) found that UV effectively inactivated the *Bacillus atrophaeus* endospores, and it is now becoming a common sterilization method. Borm et al. (2002) showed the efficacy of this treatment by utilizing UV to sterilize ants for 2 hours to complete a comprehensive screening of all the potential microorganisms inside the bodies of leafcutter ants.

Ethanol, an organic molecule, is a common body surface sterilization reagent used to eliminate pathogens in stool specimens from healthy donors. This reagent is useful for evaluating the safety and efficacy of bacterial spores and is important in preventing recurrent *Clostridium difficile* infections (Khanna et al., 2016; Afouda et al., 2020). Taylor et al. (2017) used several antimicrobials and surfactants to decrease the three stink bugs. Sodium hypochlorite (NaClO) is one of the most effective disinfectants in endodontics. It can also suppress endosymbionts in stink bugs as a pest control strategy in the field (Kashkouli et al., 2019).

To our knowledge, no method has been developed that combines physical and chemical treatments simultaneously to obtain the gut microbiota of ants. Thus, we select the chemical treatment NaClO and ethanol, which refer to the method that obtains *Camponotus fragilis* gut microbiology (He et al., 2014), and select the physical treatment UV according to Borm et al. (2002); these methods had been put into gut-associated researches. The current study combines inorganic (NaClO), organic (ethanol), and physical (UV) sterilization treatments to investigate the gut bacteria of very small insects (fire ants) in order to determine the most combined favorable efficacy of these treatments. This study also investigates if the whole abdomen can be utilized as the material required for sterilization procedures.

## Materials and methods

### Ant collection

Twenty-seven samples (540 ants in total, each sample includes 60 ants, three samples of the same prefix as a processing group) were collected from a common nest built in the soil at Guangxi Normal University in Guilin, Guangxi, in southern China. In addition to that, 200 ants were prepared to ensure a suitable number of obtaining gut microbiomes and were divided into ten groups (which was carried out before this study, these 200 ants were washed with sterile water twice, once a minute), group 1-5 represent minor workers, the specific number of each group is in order 10, 15, 20, 25, 30; group 6-10 represent large workers, specific number as same as group 1-5 (Fig 1). Here, the major workers and minor workers were placed in a glass bottle with some soil to keep them alive. Then, these ants were chosen on a clean bench (provide a local dust-free aseptic working environment with one-way flow type air purification equipment). The total length of the large workers chosen was about 5.0 mm, with the abdomen of about 1.5 mm. The total length of the minor workers was about 3.0 mm, with an abdomen of about 1.0 mm. We mixed in the two types of ants randomly. We brought their numbers up to 20 (in our preliminary experiment, both large and minor ants successfully extracted bacteria DNA from their gut).

Surface sterilization: 75% ethanol and 2.0% NaClO were prepared. Surgical blades, beakers, tweezers, 2 ml centrifuge tubes (labeled), and water were sterilized in the autoclave (vapor pressure- 103.4 kPa, 122 °C, for 30 minutes). Method GT was set as a blank control with 20 ant abdomens. The other eight methods were used to clean the surface; each included 20 ant abdomens. Specific methods are listed in Table 1. In order to ensure that the microbiome is more fully acquired in the host, all of the ants are alive; prior to the experiment, several ants were put into centrifuge tubes and monitored at each step until it was confirmed that the ants would survive all of the steps.



**Fig 1.** The pre-experimental design for detecting optimal ant populations, stripes 1-5 represent five groups of different quantitative gradients of minor workers (The specific numbers are respectively 10, 15, 20, 25, 30), stripes 6-10 represent the same situation but of minor workers.

**Table 1.** Methods design of surface sterilization. There are 540 ants divided into 27 samples (nine groups as nine methods). The three samples of method GT (Group GT\_1, GT\_2, GT\_3) were not subject to sterilization and were used as controls. All 27 samples included the same amount of workers (n = 20). Each method involved three repetitions (1-3), and each sample was processed following the table from left to right. "+" represent perform this operation, "-" represent not.

Number	Sterile water clean 1 min	250 nm UV sterilize 20 min	2.0 mg/L NaClO sterilize 2 min	75% Ethanol sterilize 1 min	Sterile water washing twice (once a minute)
GT (1-3)	-	-	-	-	-
M1 (1-3)	+	-	-	-	+
M2 (1-3)	+	+	-	-	+
M3 (1-3)	+	-	+	-	+
M4 (1-3)	+	-	-	+	+
M5 (1-3)	+	+	+	-	+
M6 (1-3)	+	+	-	+	+
M7 (1-3)	+	-	+	+	+
M8 (1-3)	+	+	+	+	+

#### *DNA extracted and PCR amplification*

According to the steps of the test kit from TIANGEN biotech company, DNA was extracted from 540 ants and stored in dry ice before sending to the Shanghai Magi Biological Company for sequencing.

The sequenced primer used for amplification was 338F-806R (used for testing 16 S rRNA), the corresponding primer was 338F (5'-ACTCCTACGGGAGGCGCAG-3'), and 806R (5'-GGACTACNNGGGTATCTAAT-3'). PCR amplification was performed using TransStart FastPfu DNA Polymerase in a 20  $\mu$ l reaction system. 5 $\times$ FastPfu Buffer 4  $\mu$ l, 2.5 mM dNTPs 2  $\mu$ l, forward Primer (5  $\mu$ M) 0.8  $\mu$ l, reverse Primer (5  $\mu$ M) 0.8  $\mu$ l, FastPfu Polymerase 0.4  $\mu$ l, BSA 0.2  $\mu$ l, template DNA 10 ng were prepared and enough ddH<sub>2</sub>O was added to reach 20  $\mu$ l. The PCR instrument was ABI GeneAmp® 9700; reaction parameters were 1 $\times$  (5 minutes at 95 °C), cycles  $\times$  (30 seconds at 9530 seconds at T<sub>m</sub> °C; 45 seconds at 72 °C), 10 minutes at 72 °C, and 10 °C until halted by the user.

#### *Establishing Illumina library and sequencing*

The "Y" shaped joint was connected, then magnetic bead screening was used to remove the connector self-continuous segment, and the library templates were enriched by PCR amplification; finally, these templates were degenerated with NaOH, resulting in single-stranded fragments of DNA.

One endpoint of DNA fragments was complemented with the primer's basic groups and then fixed into the chip.

Another endpoint of DNA fragments was matched with a nearby different primer to be fixed and form a bridge. Then, PCR amplification was conducted again, which produced DNA clusters. The DNAs were linearized and divided into single strands. DNA polymerases were added along with four dNTPs with fluorescence. The surface of the reaction plate was scanned with a laser, cutting the fluorescence and termination genes chemically, restoring the viscosity of the 3' endpoint, and continuing to aggregate the second nucleotide. Finally, the results of fluorescent signals were collected, and the sequences of the DNA fragments were obtained.

#### *Community richness*

The Mothur software was utilized for all the diversity analyses (version v.1.30.1 [http://www.mothur.org/wiki/Schloss\\_SOP#Alpha\\_diversity](http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity)) (Schloss et al., 2009). The Chao index was used to estimate the species abundance. In addition, we also calculated the Ace, Coverage, Shannon, and Simpson estimators, which enriched the uniformity and sequencing depth indexes of the samples, respectively.

#### *Statistical model comparison*

The GLM regression model was designed to assess which factor is significant for the regression of results, and for further exploring these factors and whether they react with each other, the interaction effect model was utilized to verify that. These analyses were completed on the software R 4.0.4.

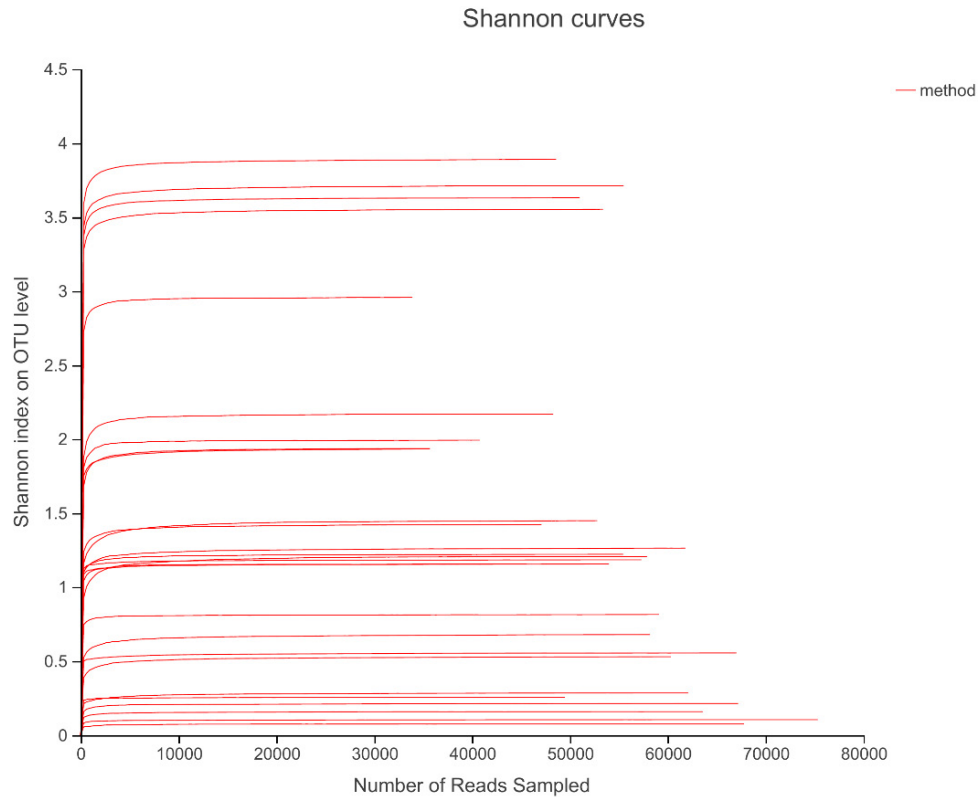
## Results

### *Sequencing depth and average sequencing length*

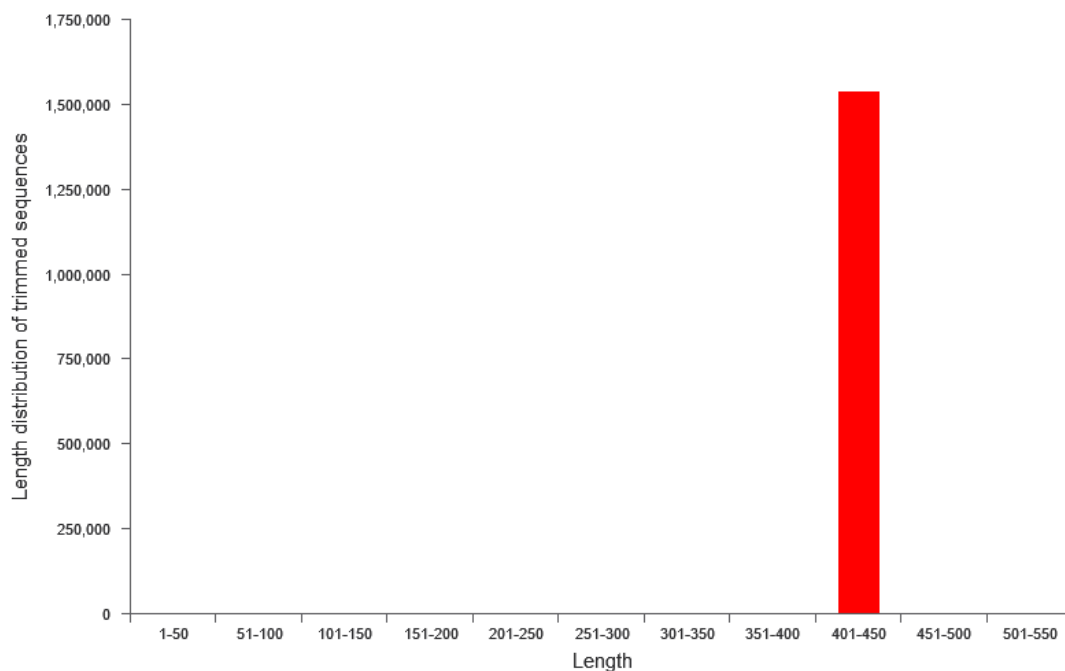
The dilution curve (Fig 2) randomly extracts a certain number of sequences from the sample and makes statistics on the alpha diversity index of the corresponding samples of these sequences. In our results, we choose the Shannon

estimator as an indicator to detect the depth of sequencing; the curve is gentle, which means the amount of sequencing data is sufficient.

We analyzed the 16S rDNA gene sequences of the imported red fire ant gut microbiota from 540 ants treated by these nine methods, the total number of bases reached 1539082, and the average length was about 401-450 bp (Fig 3).



**Fig 2.** Dilution curve of Shannon estimator. The abscissa represents the amount of data extracted; the y-coordinate represents the value of the Shannon estimator.



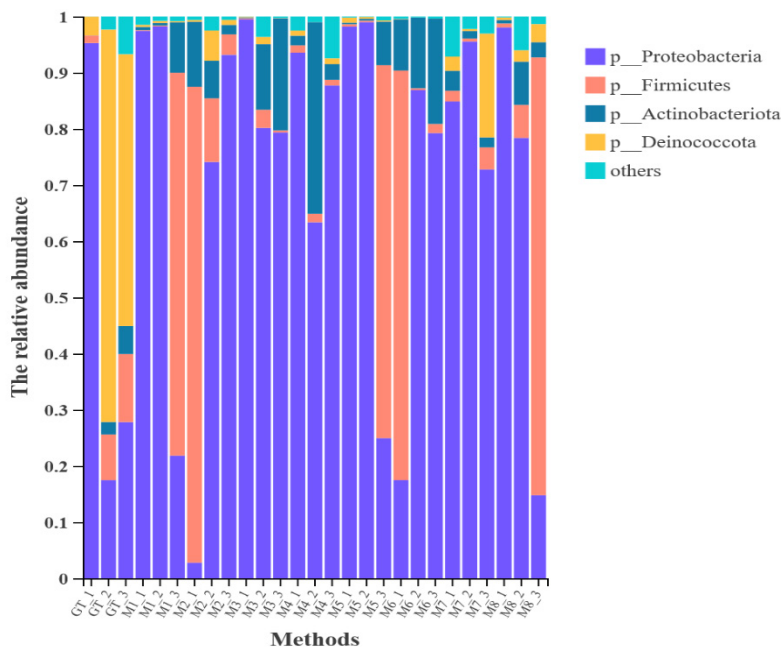
**Fig 3.** Mean sequencing length (bp) of 16S rDNA gene in these imported red fire ants.

*Distribution of phylum taxa level through different methods*

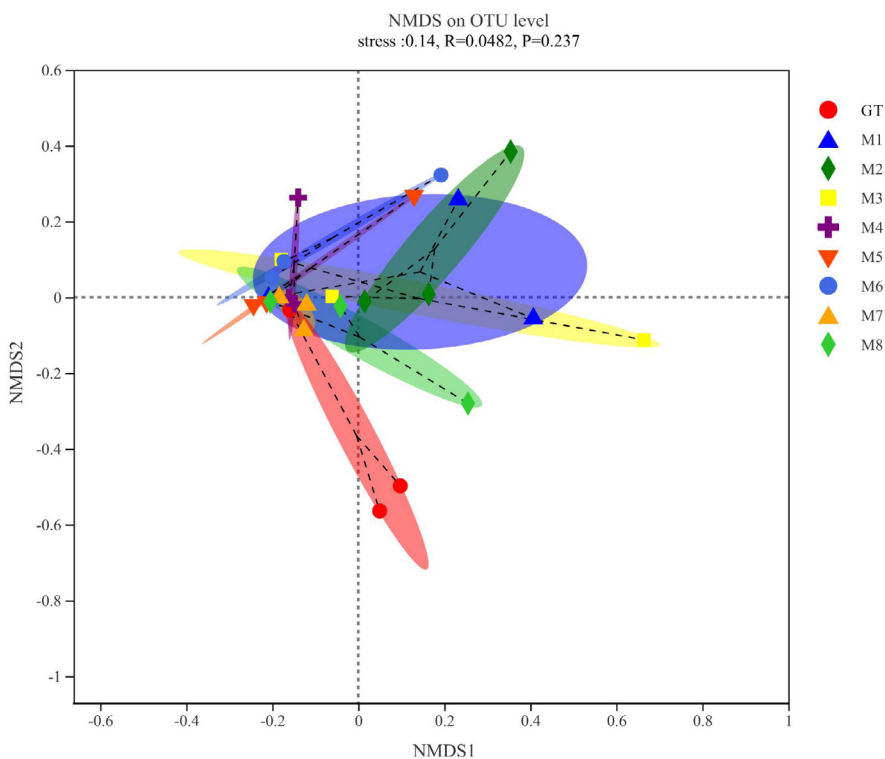
*Alpha diversity analysis*

Results from all the methods showed that the dominant phyla are p\_Proteobacteria and p\_Firmicutes. In more than ten groups, the first dominant phylum takes part in more than 80%. The phylum p\_Firmicutes occurred in 5 groups, and more than a half. It is confusing that p\_Deinococcota occupy groups GT\_2 and GT\_3 by a significantly greater number of OTUs. The detailed distribution is depicted in Fig 4.

Based on the 97% similarity level of OTU, the OTUs (operational taxonomic unit) were used to calculate all these alpha diversity indexes (Table 2). The largest number of OTU (75340) exists in group M5\_2, the smallest OTU exists in group GT\_3 (33813); the highest Ace index and the Chao index both exist in group M1\_3 (803.30, 798.01); the maximum value of the Shannon index and the minimum value



**Fig 4.** The composition percent of red imported fire ants gut microbiota in the phylum taxa. Abscissa represents these sterilization methods; ordinate shows the percent of each phylum.



**Fig 5.** NMDS analysis of all the surface sterilization methods. The confidence ellipse of the same shape and color represents a method.

of Simpson index exist in group M2\_1 (3.891, 0.042); and all of the coverages were more than 0.99.

#### *Beta diversity analysis of surface sterilization methods*

NMDS (Nonmetric multidimensional scaling) analysis on the OTU level showed the comparison of differences between groups of these methods. Stress (0.14) less than 0.2 means high reliability. In Fig 5, all the groups are not completely separated,  $R = 0.0482$ ,  $p = 0.237$ , which means these nine surface sterilization methods' influence on the OTU level results was insignificant.

#### *Statistical analysis for the GLM regression model and interaction effect model*

The results of the GLM regression model showed that none of the three factors contributed significantly to the Chao index's regression; all of the p values were greater than 0.05 (Table 3). In order to explore whether these factors sufficiently interacted with each other to impact the results, the interaction effect model was evaluated (Table 4). This model could assess the influence induced by any two factors; the results proved that no significant interaction effect was found between any two factors ( $p > 0.05$ ).

**Table 2.** The sequencing information of the eight samples. Number of OTUs, Alpha diversity index: Ace index, Chao index, Coverage index, Shannon index, and Simpson index are included.

Sample ID	0.97					
	OTU	Ace index	Chao index	Coverage index	Shannon index	Simpson index
GT_1	49234	88.52	90.00	0.999	0.255	0.904
GT_2	40658	189.41	196.011	1.000	1.993	0.436
GT_3	33813	227.10	233.14	1.000	2.957	0.214
M1_1	62049	516.89	506.52	0.998	0.285	0.938
M1_2	67008	727.64	511.98	0.998	0.556	0.760
M1_3	55456	803.30	798.01	0.997	3.714	0.054
M2_1	48516	589.76	575.90	0.998	3.891	0.042
M2_2	35668	369.17	372.56	0.999	1.936	0.484
M2_3	55337	269.09	273.03	0.999	1.222	0.433
M3_1	67628	146.11	120.62	1.000	0.075	0.983
M3_2	46800	442.10	452.40	0.998	1.423	0.543
M3_3	57198	305.48	292.50	0.999	1.185	0.479
M4_1	57978	554.06	555.89	0.998	0.679	0.844
M4_2	35648	619.69	602.49	0.996	1.934	0.260
M4_3	57696	615.54	625.92	0.999	1.207	0.714
M5_1	63564	131.46	133.59	1.000	0.158	0.959
M5_2	75340	202.96	198.71	0.999	0.104	0.976
M5_3	53208	687.67	661.11	0.998	3.553	0.081
M6_1	50996	504.01	494.81	0.998	3.632	0.058
M6_2	59006	333.15	304.87	0.998	0.815	0.654
M6_3	53837	312.90	302.22	0.998	1.156	0.499
M7_1	52783	752.34	750.15	0.998	1.449	0.647
M7_2	60246	520.84	515.99	0.998	0.529	0.877
M7_3	61608	715.29	688.78	0.997	1.263	0.537
M8_1	67023	191.71	159.04	0.999	0.214	0.950
M8_2	48135	405.79	408.61	0.999	2.170	0.434
M8_3	45312	162.76	165.15	1.000	1.156	0.580

**Table 3.** Effect of three factors on the Chao index in the GLM regression model.

Factors	Estimate	SE	t value	p value
250nm UV	3.375	17.209	0.196	0.852
2.0mg/L NaClO	2.080	17.209	0.121	0.909
75% Ethanol	-5.744	17.209	-0.334	0.752

**Table 4.** The interaction effect model of any two factors on the Chao index. Three models are designed to detect whether any two of the three factors interact with each other or not.

Result	Df	RSS	Df Sum of Sq	F	Pr (>F)
Model 1: Chao ~UV * NaClO	1 156.06	1	51.988	0.3331	0.6667
Model 2: Chao ~NaClO * Ethanol	1 156.06	1	2107.9	13.508	0.1691
Model 3: Chao ~UV * Ethanol	1 156.06	1	55.099	0.3531	0.6587

## Discussion

The PCR amplification results showed that the amplification fragment was about 401-450 bp (Fig 3), and the primer 338F-806R amplified the DNA fragments successfully. In this study, the suitable number of ants required to be sequenced was successfully determined; the design of each group with 20 ants is believable for Shannon curves are gentle and electrophoresis bandings are clear; this provided us a view of sample number for acquiring gut microbiome in this tiny insect (Fig 1, Fig 2). It is worth noting that though the electrophoresis banding “1” (Fig 1, which represents the ten ants) is as clear and bright as banding “3” (which represent the 20 ants), according to the conclusion of Lee et al. (2008), we speculated it might be due to their own rare and unstable bacterial composition. Our results supported it (20 ants) as a more stable and accurate value because each method was designed with 20 ants in this study, and the intestinal flora was successfully extracted just once.

Our sequenced bacterial clones from the guts were determined to be predominantly from the phyla p\_Proteobacteria and p\_Firmicutes (Fig 4), which is consistent with Lee et al. (2008). They provided powerful evidence to confirm it was possible to use the whole abdomen as the material to sterilize.

It seems the Alpha diversity index cannot reveal the effect of sterilization. The large Shannon index difference of group M5\_1 (0.158) (or group M5\_2, 0.104) and group M5\_3 (3.53) treated by UV may suggest the limitation of using the alpha diversity index to evaluate the surface sterilization intuitively. Because they are collected from the same nest and treated under UV at the same time. In addition to the results of Alpha diversity, NMDS analysis further confirms that the effect of surface sterilization is not obvious between different methods (Fig 5). Statistical analysis (GLM regression model and interaction effect model) approves the above results by more intuitive data ( $p > 0.05$ ). In other words, our results seem to fit well with the experimental results of Hammer et al. (2015). The different surface sterilization methods did not significantly affect the diversity of the insect gut microbiome, suggesting that no single method or combination of methods is best. However, we believe that the process of body surface sterilization cannot be ignored; a strong piece of evidence is that ethanol and bleach-treated specimens showed major differences in internal microbial diversity in ticks (Binetruy

et al., 2019). Long-term field collection experience tells us that most ants live in the soil with a wide variety of bacteria. Although cleaning the body surface for studying the gut microbiota is important, unifying the sterilization method within an insect taxa is more important than selecting different sterilization methods for the body surface. For example, aphid surface sterilization is almost always a combination of 70% ethanol and sterile water (Qin et al., 2021). At the same time, we should pay attention to insects' different growth and development stages or the material sources of different parts.

Some outstanding research has been reported recently, which has confirmed that a primate's gut microbiota could change with seasons (Li et al., 2020); they also determined the dominant microbial community and predicted its function. As for *Solenopsis invicta*, a recent study by Xiao et al. (2023) demonstrated that gut microbiota composition and diversity varied among populations due to differences in host genotype and geographical distribution. They pointed out that population-level differences in *S. invicta* gut microbiota may depend more on environmental factors than host genotypes. Previous studies have reviewed the gut microbiota of bees (Voulgari-Kokota et al., 2019). However, the testing methods available could not completely define the core microbial community or determine the impact on the host. Therefore, since more extensive testing methods are currently available, future research should focus on the microbial community, the impact on the host, seasonal variation, living habitats, and feeding habits.

## Conclusion

Regarding the ideal material to obtain gut microbiome from ants, the whole abdomen is worthy of adoption, and the number of extraction units (20 ants) could be used as a numerical reference for other tiny insects (species similar in size to the red fire ant). Different surface sterilization methods did not significantly affect the diversity of insect gut microbiome, but choosing a suitable method for better comparison of published research is indispensable. The scientific community working with ant gut microbiota must unify sterilization methods as best as possible within the same species.

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## Authors' Contributions

C-ZL: conceptualization, methodology, writing-original draft, writing-review & editing.

Y-BL: writing-review & editing.

Z-HH: formal analysis, writing-review & editing.

D-FC: investigation, writing-review & editing.

## References

- Afouda, P., Hocquart, M., Pham, T.P.T., Kuete, E., Ngom, I.I., Dione, N., Valles, C., Bellali, S., Lagier, J.C., Dubourg, G. & Raoult, D. (2020). Alcohol pretreatment of stools effect on culturomics. *Scientific Reports*, 10: 5190. <https://doi.org/10.1038/s41598-020-62068-x>
- Binetruy, F., Dupraz, M., Buysse, M. & Olivier, D. (2019). Surface sterilization methods impact measures of internal microbial diversity in ticks. *Parasites Vectors*, 12: 268. <https://doi.org/10.1186/s13071-019-3517-5>
- Borm, S.V., Billen, J. & Boomsma, J.J. (2002). The diversity of microorganisms associated with *Acromyrmex* leafcutter ants. *BMC Evolutionary Biology*, 2: 221-271. <https://doi.org/10.1186/1471-2148-2-9>
- Evrova, O., Kellenberger, D., Scalera, C., Calcagni, M., Giovanoli, P., Vogel, V. & Buschmann, J. (2019). Impact of UV sterilization and short-term storage on the in vitro release kinetics and bioactivity of biomolecules from electrospun scaffolds. *Scientific Reports*, 9: 15117. <https://doi.org/10.1038/s41598-019-51513-1>
- Hammer, T.J., Dickerson, J.C. & Fierer, N. (2015). Evidence-based recommendations on storing and handling specimens for analyses of insect microbiota. *PeerJ*, 3: e1190. <https://doi.org/10.7717/peerj.1190>
- He, H., Wei, C. & Wheeler, D.E. (2014). The gut bacterial communities associated with lab-raised and field-collected ants of *Camponotus fragilis* (Formicidae: Formicinae). *Current Microbiology*, 69: 292-302. <https://doi.org/10.1007/s00284-014-0586-8>
- Khanna, S., Pardi, D.S., Kelly, C.R., Kraft, C.S., Dhere, T., Henn, M.R., Lombardo, M.J., Vulic, M., Ohsumi, T., Winkler, J., Pindar, C., McGovern, B.H., Pomerantz, R.J., Aunins, J.G., Cook, D.N. & Hohmann, E.L. (2016). A novel microbiome therapeutic increases gut microbial diversity and prevents recurrent *Clostridium difficile* infection. *Journal of Infectious Diseases*, 214: 173-181. <https://doi.org/10.1093/infdis/jiv766>
- Kashkouli, M., Fathipour, Y. & Mehrabadi, M. (2019). Potential management tactics for pistachio stink bugs, *Brachynema germari*, *Acrosternum heegeri* and *Acrosternum arabicum* (Hemiptera: Pentatomidae): high temperature and chemical surface sterilants leading to symbiont suppression. *Journal of Economic Entomology*, 112: 244-254. <https://doi.org/10.1093/jee/toy324>
- Kim, E., Cho, E.J., Yang, S.M., Kim, M.J. & Kim, H.Y. (2020). Novel approaches for the identification of microbial communities in kimchi: MALDI-TOF MS analysis and high-throughput sequencing. *Food Microbiology*, 94: 103641. <https://doi.org/10.1016/j.fm.2020.103641>
- Koto, A., Nobu, M.K. & Miyazaki, R. (2020). Deep sequencing uncovers caste-associated diversity of symbionts in the social ant *Camponotus japonicus*. *mBio*, 11: e00408-20. <https://doi.org/10.1128/mbio.00408-20>
- Lee, A.H., Husseneder, C. & Hooper-Bùi, L. (2008). Culture-independent identification of gut bacteria in the fourth-instar red imported fire ant, *Solenopsis invicta*, Buren, larvae. *Journal of Invertebrate Pathology*, 98: 20-33. <https://doi.org/10.1016/j.jip.2007.10.008>
- Lewis, W.H., Tahon, G., Geesink, P., Sousa, D.Z. & Ettema, T.J.G. (2021). Innovations to culturing the uncultured microbial majority. *Nature Reviews Microbiology*, 19: 225-240. <https://doi.org/10.1038/s41579-020-00458-8>
- Li, Y.H., Chen, T., Liang, J.P., Li, Y.B. & Huang, Z.H. (2021). Seasonal variation in the gut microbiota of rhesus macaques inhabiting limestone forests of southwest Guangxi, China. *Archives of Microbiology*, 203: 787-798. <https://doi.org/10.1007/s00203-020-02069-6>
- Qin, M., Chen, J., Xu, S.F., Jiang, L.Y. & Qiao, G.X. (2021). Microbiota associated with *Mollitrichosiphum* aphids (Hemiptera: Aphididae: Greenideinae): diversity, host species specificity and phylosymbiosis. *Environmental Microbiology*, 23: 2184-2198. <https://doi.org/10.1111/1462-2920.15391>
- Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J. & Weber, C.F. (2009). Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75: 7537-7541. <https://doi.org/10.1128/AEM.01541-09>
- Stawarz-Janeczek, M., Kryczyk-Poprawa, A., Muszyńska, B., Opoka, W. & Pytko-Polończyk, J. (2021). Disinfectants used in stomatology and SARS-CoV-2 infection. *European Journal of Dentistry*, 15: 388-400. <https://doi.org/10.1055/s-0041-1724154>

- Taylor, C., Johnson, V. & Dively, G. (2017). Assessing the use of antimicrobials to sterilize brown marmorated stink bug egg masses and prevent symbiont acquisition. *Journal of Pest Science*, 90: 1287-1294. <https://doi.org/10.1007/s10340-016-0814-z>
- Urakawa, H., Kita-Tsukamoto, K. & Ohwada, K. (1999). Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology*, 145: 3305-3315. <https://doi.org/10.1099/00221287-145-11-3305>.
- Voulgari-Kokota, A., McFrederick, Q.S., Steffan-Dewenter, I. & Keller, A. (2019). Drivers, diversity, and functions of the solitary-bee microbiota. *Trends in Microbiology*, 27: 1034-1044. <https://doi.org/10.1016/j.tim.2019.07.011>
- Xiao, Q., Wang, L., Chen, S.Q., Zheng, C.Y., Lu, Y.Y. & Xu, Y.J. (2023). Gut microbiome composition of the Fire Ant *Solenopsis invicta*: an integrated analysis of host genotype and geographical distribution. *Microbiology Spectrum*, 11: e0358522. <https://doi.org/10.1128/spectrum.03585-22>.
- Yagi, N., Mori, M., Hamamoto, A., Nakano, M., Akutagawa, M., Tachibana, S., Takahashi, A., Ikehara, T. & Kinouchi, Y. (2007). Sterilization using 365nm UV-LED. *IEEE Engineering in Medicine and Biology Society, Conference, 2007*: 5842-5845. <https://doi.org/10.1109/IEMBS.2007.4353676>
- Yung, P.T., Lester, E. & Ponce, A. (2020). Quantitative and fast sterility assurance testing of surfaces by enumeration of germinable endospores. *Scientific Reports*, 10: 431. <https://doi.org/10.1038/s41598-019-57175-3>

