Full title:

The urinary microbiota composition remains stable over time and under various storage conditions

Short title:

Effect of storage and time on urine microbiota

Authors:

Caspar Bundgaard-Nielsen[1,2¶], Nadia Ammitzbøll[1,2¶], Yusuf Abdi Isse[1,3], Abdisalam Muqtar[1,3], Ann-Maria Jensen[1], Peter Leutscher[1,2], Louise Thomsen Schmidt Arenholt[1,2,4], Søren Hagstrøm[1,2,5], Suzette Sørensen[1,2*]

[1]Centre for Clinical Research, North Denmark Regional Hospital, Denmark

[2]Department of Clinical Medicine, Aalborg University, Denmark

[3]Department of Health Science and Technology, Aalborg University, Denmark

[4]Department of Gynecology and Obstetrics, North Denmark Regional Hospital, Denmark

[5]Department of Pediatrics, Aalborg University Hospital, Denmark

* Corresponding author

E-mail: Suzette.soerensen@rn.dk (SES)

¶These authors have contributed equally to this study

Keywords: Microbiota, Microbiome, Urinary Microbiome, Urinary Microbiota, Bacteria, Urine Storage, Microbiota Storage, Bacteria DNA Extraction

Abstract:

Background: New sensitive techniques have revealed a large population of bacteria in the human urinary tract, challenging the perception of the urine of healthy humans being sterile. While the role of this urinary microbiota is unknown, dysbiosis has been linked to disorders like urgency urinary incontinence and interstitial cystitis. When comparing studies it is crucial to account for possible confounders introduced due to methodological differences. Here we investigated whether storage condition or time of collection, had any impact on the urinary microbial composition.

Results: For comparison of different storage conditions, urine was collected from five healthy adult female donors, and analyzed by 16S rRNA gene sequencing. Using the same methods, the daily or day-to-day variation in urinary microbiota was investigated in nineteen healthy donors, including four women, five men, five girls, and five boys. With the exception of two male adult donors, none of the tested conditions gave rise to significant differences in alpha and beta diversities between individuals. *Conclusion*: The composition of the urinary microbiota was found to be highly resilient to changes introduced by storage temperature and duration. In addition, we did not observe any intrapersonal daily or day-to-day variations in microbiota composition in women, girls or boys.

Together our study supports flexibility in study design, when conducting urinary microbiota studies.

Author summary

The discovery of bacteria native to the urinary tract in healthy people, a location previously believed to be sterile, has prompted research into the clinical potential of these bacteria. However, methodological weaknesses can significantly influence such studies, and thus development of robust techniques for investigating these bacteria are needed. In the present study, we investigated whether differences in storage following collection, could

affect the bacterial composition of urine samples. Next, we investigated if this composition exhibited daily or day-to-day variations.

Firstly, we found, that the bacterial composition of urine could be maintained by storage at -80 °C, -20 °C, or refrigerated at 4 °C. Secondly, the bacterial composition of urine remained stable over time. Overall, the results of this study provide information important to study design in future investigations into the clinical implications of urinary bacteria.

Background

It has been established that the human body has a symbiotic relationship with an abundance of microorganisms, which play a role in maintenance of health. In particular, microorganisms present in the gut have received much attention, and many studies have described their beneficial functions in immune regulation[1,2] and metabolic processes[3]. However, when brought out of balance (dysbiosis), the same microorganisms have been associated with several pathological states including infections[4], autoimmune diseases[5], obesity[6], and psychiatric or neurodevelopmental disorders[7–9].

Until recently, it was believed that urine under normal conditions was sterile. This has now been challenged by sensitive PCR-based techniques including 16S rRNA gene sequencing and expanded quantitative urine culture (EQUC). Studies are now emerging, investigating the urinary microbiota in various patient groups and healthy participants, showing that urine contains a plethora of bacteria with yet unknown function[10–12]. Most studies published on urinary microbiota are primarily on women, fewer on men[13] and only a single on young children[14]. In general, the core microbiota composition is very different between women and men. The urine of women is mainly dominated by *Lactobacillus* followed by *Gardnerella* genera[11,13,15–17]. Men have a more diverse bacterial composition consisting of broader representation of different genera including *Lactobacillus*, *Corynebacterium*, *Staphylococcus*, and *Prevotella*[13,18–23]. It is assumed that these bacteria provide healthy

functioning of the lower urinary tract. Notably, several studies have recently documented that an alteration in urinary microbiota correlates to diseases in the lower urinary tract, including urgency urinary incontinence[10–12,24] and interstitial cystitis[15,25]. More knowledge on the urinary microbiota may therefore help us to understand the etiology behind diseases of the lower urinary tract.

Despite the growing interest for urinary microbiota research, it appears that the methodologies and study designs, used in different studies, are highly heterogeneous, which makes it difficult to interpret and compare observed findings. Several protocol optimization studies have been conducted on fecal samples, providing valuable guidelines on how to store and process samples for gut microbiota studies[26–31]. Importantly, the chemical content and structure of urine is very different from feces, leaving urinary microbiota research as a bare and unexplored field regarding protocol recommendations. In fact, few studies have investigated the technical and methodological aspects of urine microbiota research[19,32–34]. These mainly focused on the urine collection method. e.g., suprapubic aspiration, clean-catch midstream, or transurethral catheterization sample collection. However, only a single study investigated how different temperatures and use of a stabilization buffer could affect the urinary microbiota in healthy women[34]. None of the studies has taken into consideration whether the microbiota remains stable over time.

We aimed to determine if different urine storage temperatures could influence microbiota composition in healthy women. Furthermore, we investigated if the urinary microbiota remained stable throughout the day or between two different days in healthy women, men, and children.

Results

Different storage conditions do not critically affect bacterial composition

Due to the risk of DNA degradation or bacterial growth, the ideal sampling strategy for urine microbiota analyses would be to purify DNA immediately following urination, or to transfer the urine samples directly to -80 °C or

colder. This is however not always possible or practical in a clinical setting, or when utilizing self-sampling at the home of the study participants. We therefore tested if storage of urine at different sub-optimal temperatures, altered the microbiota composition compared to a freshly processed sample. For this purpose, urine was collected from five healthy donors. Each urine sample was subsequently divided and stored according to one of the seven combinations of temperatures and times (Fig 1). Since each condition was tested in duplicate experiments, we reached a total of 70 samples. After the allocated storage period, total DNA was purified, and DNA concentrations obtained ranged from 16.2-248.0 ng/mL urine with minor variations between donors (Fig 2). With the exception of donor E, the highest DNA concentration was observed in the freshly processed sample, followed by samples stored directly at -80 °C.

16S rRNA gene sequencing of the V4 region resulted in a total of 2,097,325 reads (median 29,334, range 1299 - 132,881 reads per sample). 933 unique Operational taxonomic units (OTUs) were identified (median 106, range 33 - 502 OTUs per sample), with taxonomy assigned on the phylum level for 96.1 % of OTUs and genus level for 53.2 %. A rarefaction curve was generated, and used to deselect samples that did not adequately cover all unique OTUs and therefore showed insufficient sequencing coverage (Fig 3A). Consequently, three samples were removed. Four samples were furthermore discarded based on poor duplicate comparison (Fig 3B and 3C), probably caused by background contamination due to low-biomass samples. This led to a total of 63 samples being included in the following analyses.

Different storage conditions did not result in significant differences in alpha diversity metrics, including OTU richness (Fig 4A) and Shannon diversity (Fig 4B). This indicated that bacterial growth was limited. Interestingly, when looking at beta diversity it appears that variations between storage conditions are minor compared to interpersonal variations (Fig 5). This supports, therefore the validity of using other storage conditions, for urine microbiota analyses, than normal gold standard conditions.

Urine microbiota composition is independent of daily and day-to-day variation

First morning urine is often more concentrated than subsequent urine samples throughout the day, while differences in daily routines (e.g. sleep rhythm, diet, sexual activity or exercise) may introduce variations during the day. We therefore speculated that morning urine could contain higher bacterial loads, and possibly a different bacterial composition than urine collected in the evening. To test this hypothesis, we compared urine samples collected in the morning and evening on two independent days from 19 healthy donors (4 women, 5 men, 5 girls, and 5 boys). Following DNA extraction, the resulting DNA yield ranged from <2 to 218.25 ng per mL urine. Importantly, DNA yield did not differ based on within day or day-to-day (data not shown).

Due to the collection method being performed under less controlled conditions (self-sampling by study participants), we expected a higher risk of contamination. In order to avoid false positive samples, we excluded samples that yielded less DNA following the initial PCR amplification for library preparation, compared to the negative controls (0.0538 ng/µL)). 54 of the original 152 samples were below cut-off levels for 1st PCR library amplification. Deselected samples were distributed unevenly as none were from women, 16 (40 %) from girls, 26 (65 %) from men, and 12 (30 %) from boys. The remaining 98 samples (representing 17 participants: 4 women, 4 girls, 4 men and 5 boys) were available for microbiota comparisons. These all showed good sequencing coverage based on a rarefaction curve (data not shown). 16S rRNA gene sequencing resulted in a total of 5,575,050 reads (median 52,232, range 7529 - 122,101 reads per sample) and 2,538 unique OTUs (median 216, range 109 – 469 OTUs per sample) were identified. 93.1 % were assigned to the phylum level and 50.9 % to the genus level.

Mapping of microbiota composition by 16S rRNA gene sequencing did not show any significant difference in OTU richness or Shannon diversity between urine samples collected in the morning or in the evening (Fig 6A and 6B) or on two independent days (Fig 8A and 8B). For beta diversity, we observed that, with the exception of the adult male participants 12 and 15, urine samples maintained similar bacterial compositions regardless of collection

time point (Fig 7 and 9). For participant 12, a marked bacterial difference between morning and evening samples was observed, and between morning and evening and weekend and weekdays for participant 15.

Discussion

One of the great challenges, when performing microbiota studies on biological specimens, is always the risk of introducing bias due to methodological vulnerabilities. Studies investigating potential pitfalls are therefore essential to identify confounding factors. Here we show that the urinary microbiota is remarkably stable under different storage conditions and with respect to time.

One major challenge, when investigating the urinary microbiota, is that only low amounts of DNA, and even lower amounts of bacterial DNA, can be extracted from urine[32]. A study by El Bali et al.[32] investigated DNA extraction from urine samples and found that storage temperature had a major impact on DNA output levels. In particular, they showed that storage at -20 °C gave rise to dramatically lower DNA yields compared to samples that were either stored directly at -80 °C or where DNA was immediately extracted. We did not observe the same level of DNA loss in samples stored at -20 °C compared to -80 °C, which may be explained by the longer storage time (15 days) used in El Bali et al. Storage at -20 °C for more than 72 hours may therefore compromise urinary DNA integrity. For gut microbiota studies, maintaining bacterial composition is essential. A previous study by Jung et al.[34] reported, that storage of urine samples without stabilizing buffer at -20 °C or 4 °C, maintained bacterial composition for up to 24 days. This matches the results of our study, allowing collection of urine samples at locations at distance from laboratory facilities.

For samples collected in the home of participants, we observed that a relatively high number of these were excluded due to low DNA yield following the initial PCR during library preparation. Importantly, the samples were not excluded due to lack of high quality sequencing reads, but merely because background contamination could not be ruled out. We have not encountered any urinary microbiota studies that describe exclusion of samples

based on background cut-off levels, which leaves the risk of misinterpretation of urinary bacterial loads or profiles.

Importantly, we found that the urinary microbiota composition remained stable across different time points. While microbiota resilience over time has previously been reported in the gut and saliva microbiota[35], this is, to our knowledge, the first study to demonstrate this for the urinary microbiota. This resilience alleviates concerns about timing of urine collection in future studies investigating the urinary microbiota.

Our study suffers from certain limitations. The experiments on the day-to-day and daily variations depended on self-sampling by the study participants in their homes, and are thereby performed in a less controlled environment. This can also be considered as a study strength, since home sampling is an often-used collection method in clinical experiments[36,37]. In fact, we show that the urinary microbiota is stable despite the use of home sampling. Only in two men did we observe inconsistency between morning/evening and weekday/weekend samples. This could be due to biologically relevant fluctuations in microbiota compositions or, more likely, due to contamination from vaginal microbiota of their partner through intercourse prior to urine sampling. In particular, we observe that aberrant microbiota profiles of these men showed similarities to the microbiota profiles of women (e.g. higher relative abundances of *Lactobacillus*). We did not collect data on sexual activity of the participants, which may be considered a potential confounding factor. Another limitation is the large amount of samples that were excluded due to first PCR DNA levels below cut-off values. Whether this fall-out of samples is due to technical issues, e.g. DNA degradation or presence of PCR inhibitors, or due to biological differences in urinary bacterial loads between individuals, is unknown. The latter may be the case since we observed that a very high proportion of samples from men (65 %) did not reach above cut-off levels, indicating that only very little bacterial DNA can be isolated from adult male urine samples. In comparison, none of the

183 samples from women were discarded, 40 % from girls, and 30 % from boys. Our sample size is however too small
184 to make any solid conclusions on age and gender differences in bacterial loads.
185
186 The strengths of this study are numerous. We have used a very systematic and structured approach with a
187 relatively large number of participants. Importantly, when evaluating the day-to-day or daily variation, we
188 included study participants of different gender and age. This was to take into account whether there could be
189 differences in time-dependent stability of different core microbiotas. Our data showed that, with the exception
190 of two men, the urinary microbiota was stable regardless of gender and age. Finally, we take into account that
191 very low levels of bacterial contamination may result in false positive samples, leading to misinterpretation of
192 true microbiota profiles.
193

194 Conclusion

195 In conclusion, we showed that the urinary microbiota is stable over time, and that sub-optimal temperatures for
196 urine storage may be used. We recommend, however, that samples be transferred to -80 °C as quickly as possible
197 after collection, to avoid loss of the already limited DNA in urine samples. In addition, we highly recommend that
198 besides including important clinical parameters such as diagnoses and medication, it is important to consider
199 choice of storage condition and to implement good negative controls and use of background cut-off levels. The
200 latter is especially important when working with low biomass samples. Finally, we recommend studies
201 investigating the effects of sexual activity on the microbiota composition in urine, to determine if this may be a
202 confounding factor. We encourage further studies on the methodological and technical aspects of urinary
203 microbiota research with the aim of providing strong evidence based guidelines.
204

205 Methods

Study participants and urine collection

In total, 25 healthy volunteers, without symptoms from the bladder (based on self-reporting in a questionnaire prior to study participation) or intake of any antibiotics within the past 3 months, were included into this study. Furthermore, in cases where medicine or hormonal contraceptives were used, these should be taken within the same period on all study days. Use of non-prescription painkillers was not accepted for up to 24 hours prior to urine collection. In addition, the study participants were instructed to avoid urine collection during menstruation, and pregnant women were not included into the study. The identity of all donors was anonymous and no personal data was registered, besides the sex and age interval of which they belonged to. For the initial study on different storage conditions, 5 women were recruited, and for the following study on daily or day-to-day variations, 20 participants were recruited encompassing 5 men (18-50 years), 5 women (18-50 years), 5 boys (5-10 years), and 5 girls (5-10 years). One woman was however excluded from the latter of the two studies, due to incorrect storage of the collected urine sample, leaving 19 participants in total.

For the study on storage conditions, urine was collected at the laboratory by the clean catch method. Samples were immediately aliquoted in tubes with 10 mL urine and transferred to the specified storage conditions as summarized in Fig 1, or subjected directly to DNA extraction (RT sample). All conditions were tested in duplicates (two aliquots from each urine sample). For samples stored at -20 °C, a freezer corresponding to a domestic freezer was used to mimic a home collection situation. All samples were finally collectively stored at -80 °C, to rule out bias due to differences in low temperature exposure, until further processing.

For the study on daily or day-to-day variations, the participants collected urine at home by the clean catch method, and urine samples were immediately transferred to -20 °C domestic freezers. All participants delivered two first morning samples (weekday and weekend) and two evening samples (weekday and weekend) and likewise, collections on a weekday or weekend day were represented by two independent samples (morning and evening). This gave rise to two independent samples per time point from each participant. Each independent

sample was furthermore divided into two aliquots for duplicate DNA purification. Children were assisted by a parent to ensure correct sampling. Samples were subsequently, within 24 hours, transported on ice to the laboratory. Upon arrival to the laboratory, the samples were stored at -80 °C until further processing.

Ethics statement

Oral consent was obtained from all study participants, or from parents or other legal guardians if participants were below 18 years of age. The Regional Ethical Committee of Northern Denmark reviewed the study protocol. Since no personal information were collected from study participants and no intervention was performed, the Ethical Committee judged that no further approval was required.

DNA purification

Bacterial DNA was isolated from 10 mL of urine using the QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer´s recommendations. Prior to DNA extraction, urine samples were centrifuged at 3,000x*g* for 20 minutes. Pellets were resuspended in PBS, lysis buffer added, and a bead beating step was included using the TissueLyser LT (Qiagen) for 2 minutes at 30 Hz with a 5 mm stainless steel bead. DNA yield was measured by the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific) or by fluorometric quantification using the Qubit 4 Fluorometer (Thermo Fisher Scientific) together with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

16S rRNA gene sequencing

Bacterial 16S rRNA gene sequencing targeting the V4 region, was performed by DNAsense (Denmark), and followed a modified version of an Illumina protocol[38], as described by Albertsen et al[39], with an initial amplicon PCR. Due to upgrades in primer design during the experiment, different versions of reverse primers targeting the V4 region of the 16S rRNA gene were utilized in the different parts of this study. For the study on

storage conditions, the following primer sequences were utilized (Forward: 5'-GTGCCAGCMGCCGCGGTAA-3', reverse: GGACTAC**H**VGGGTWTCTAAT), while for the study investigating variations between evening and morning and day-to-day, a slightly modified reverse primer was used (5'-GGACTAC**N**VGGGTWTCTAAT-3'). Samples were pooled and sequencing was performed on a MISeq (Illumina, USA), as previously described[38]. To measure error rate during sequencing and batch effects, a 20 % PhiX control library was added. As negative control, nuclease-free water was used, while a complex sample obtained from an anaerobic digester system was utilized as a positive control.

Bioinformatics and statistics

Quality of sequencing reads was analyzed using FastQC (Babraham Bioinformatics, UK). Forward reads were quality trimmed using Trimmomatic v 0.32[40] to produce reads with a Phred score of at least 20 and a length of 250 bp. Subsequent bioinformatics followed the UPARSE workflow[41] to remove chimeras, cluster OTUs based on 97 % identity and assign taxonomy using the RDP classifier as previously described[26].

Data analysis was performed in R version 3.5.3[42] through the Rstudio IDE (http://www.rstudio.com/) using the ampvis2 package v.2.4.5[39], as well as Microsoft Office Excel 2013. To evaluate sequencing coverage, a rarefaction curve was generated using the amp_rarecurve command, while DNA quantity following the initial PCR amplification was compared to the corresponding negative control to rule out background contamination. Alpha-diversity was determined using OTU richness and Shannon Diversity Index, as implemented in the amp_alphadiv command. Beta-diversity was determined using PCA clustering and heat maps. PCA of variance in Hellinger transformed OTU abundance between samples and storage conditions were determined using the amp_ordinate function, while heat maps displaying the most commonly found OTUs in differing conditions, were produced using the amp_heatmap function. For continuous data like DNA concentration, distribution was tested using Shapiro-Wilks test while variance was tested using Bartlett's test. Normal distributed data was expressed

by mean values and analyzed using ANOVA followed by Bonferroni post-hoc test, while data that was not normal distributed or did not have equal variances, was expressed as median values and analyzed using Kruskal-Wallis Test followed by Dunn's post hoc test. Differences were considered statistical significant for $p<0.05$.

References

1. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, et al. Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 2012;149: 1578–93. doi:10.1016/j.cell.2012.04.037
2. Rosser EC, Oleinika K, Tonon S, Doyle R, Bosma A, Carter NA, et al. Regulatory B cells are induced by gut microbiota-driven interleukin-1β and interleukin-6 production. Nat Med. 2014;20: 1334–1339. doi:10.1038/nm.3680
3. Velagapudi VR, Hezaveh R, Reigstad CS, Gopalacharyulu P, Yetukuri L, Islam S, et al. The gut microbiota modulates host energy and lipid metabolism in mice. J Lipid Res. 2010;51: 1101–1112. doi:10.1194/jlr.M002774
4. Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, et al. Decreased Diversity of the Fecal Microbiome in Recurrent *Clostridium difficile* –Associated Diarrhea. J Infect Dis. 2008;197: 435–438. doi:10.1086/525047
5. Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BAH, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature. 2016;535: 376–381. doi:10.1038/nature18646
6. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457: 480–4. doi:10.1038/nature07540
7. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. Brain Behav Immun. 2015;48: 186–94. doi:10.1016/j.bbi.2015.03.016

8. Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD, et al. Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe. 2010;16: 444–53. doi:10.1016/j.anaerobe.2010.06.008

9. Aarts E, Ederveen THA, Naaijen J, Zwiers MP, Boekhorst J, Timmerman HM, et al. Gut microbiome in ADHD and its relation to neural reward anticipation. PLoS One. 2017;12: e0183509. doi:10.1371/journal.pone.0183509

10. Pearce MM, Zilliox MJ, Rosenfeld AB, Thomas-White KJ, Richter HE, Nager CW, et al. The female urinary microbiome in urgency urinary incontinence. Am J Obstet Gynecol. 2015;213: 347e1-347e11. doi:10.1016/j.ajog.2015.07.009

11. Thomas-White KJ, Hilt EE, Fok C, Pearce MM, Mueller ER, Kliethermes S, et al. Incontinence medication response relates to the female urinary microbiota. Int Urogynecol J. 2016;27: 723–733. doi:10.1007/s00192-015-2847-x

12. Hilt EE, McKinley K, Pearce MM, Rosenfeld AB, Zilliox MJ, Mueller ER, et al. Urine is not sterile: Use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. J Clin Microbiol. 2014;52: 871–876. doi:10.1128/JCM.02876-13

13. Lewis DA, Brown R, Williams J, White P, Jacobson SK, Marchesi JR, et al. The human urinary microbiome; bacterial DNA in voided urine of asymptomatic adults. Front Cell Infect Microbiol. 2013;3: 41. doi:10.3389/fcimb.2013.00041

14. Kassiri B, Shrestha E, Kasprenski M, Antonescu C, Florea LD, Sfanos KS, et al. A Prospective Study of the Urinary and Gastrointestinal Microbiome in Prepubertal Males. Urology. 2019;131: 204–210. doi:10.1016/j.urology.2019.05.031

15. Abernethy MG, Rosenfeld A, White JR, Mueller MG, Lewicky-Gaupp C, Kenton K. Urinary microbiome and cytokine levels in women with interstitial cystitis. Obstet Gynecol. 2017;129: 500–506.

doi:10.1097/AOG.0000000000001892

16. Pearce MM, Hilt EE, Rosenfeld AB, Zilliox MJ, Thomas-White K, Fok C, et al. The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. MBio. 2014;5: e01283-14. doi:10.1128/mBio.01283-14

17. Siddiqui H, Nederbragt AJ, Lagesen K, Jeansson SL, Jakobsen KS. Assessing diversity of the female urine microbiota by high throughput sequencing of 16S rDNA amplicons. BMC Microbiol. 2011;11: 244. doi:10.1186/1471-2180-11-244

18. Fouts DE, Pieper R, Szpakowski S, Pohl H, Knoblach S, Suh MJ, et al. Integrated next-generation sequencing of 16S rDNA and metaproteomics differentiate the healthy urine microbiome from asymptomatic bacteriuria in neuropathic bladder associated with spinal cord injury. J Transl Med. 2012;10. doi:10.1186/1479-5876-10-174

19. Dong Q, Nelson DE, Toh E, Diao L, Gao X, Fortenberry JD, et al. The microbial communities in male first catch urine are highly similar to those in paired urethral swab specimens. PLoS One. 2011;6. doi:10.1371/journal.pone.0019709

20. Nelson DE, Dong Q, van der Pol B, Toh E, Fan B, Katz BP, et al. Bacterial communities of the coronal sulcus and distal urethra of adolescent males. PLoS One. 2012;7. doi:10.1371/journal.pone.0036298

21. Nelson DE, van der Pol B, Dong Q, Revanna K V., Fan B, Easwaran S, et al. Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. PLoS One. 2010;5. doi:10.1371/journal.pone.0014116

22. Gottschick C, Deng ZL, Vital M, Masur C, Abels C, Pieper DH, et al. The urinary microbiota of men and women and its changes in women during bacterial vaginosis and antibiotic treatment. Microbiome. 2017;5: 99. doi:10.1186/s40168-017-0305-3

23. Shrestha E, White JR, Yu SH, Kulac I, Ertunc O, De Marzo AM, et al. Profiling the Urinary Microbiome in

Men with Positive versus Negative Biopsies for Prostate Cancer. J Urol. 2018;199: 161–171. doi:10.1016/j.juro.2017.08.001

24. Karstens L, Asquith M, Davin S, Stauffer P, Fair D, Gregory WT, et al. Does the Urinary Microbiome Play a Role in Urgency Urinary Incontinence and Its Severity? Front Cell Infect Microbiol. 2016;6. doi:10.3389/fcimb.2016.00078

25. Siddiqui H, Lagesen K, Nederbragt AJ, Jeansson SL, Jakobsen KS. Alterations of microbiota in urine from women with interstitial cystitis. BMC Microbiol. 2012;12. doi:10.1186/1471-2180-12-205

26. Bundgaard-Nielsen C, Hagstrøm S, Sørensen S. Interpersonal Variations in Gut Microbiota Profiles Supersedes the Effects of Differing Fecal Storage Conditions. Sci Rep. 2018; 1–9. doi:10.1038/s41598-018-35843-0

27. Wu GD, Lewis JD, Hoffmann C, Chen Y-Y, Knight R, Bittinger K, et al. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol. 2010;10: 206. doi:10.1186/1471-2180-10-206

28. Mackenzie BW, Waite DW, Taylor MW. Evaluating variation in human gut microbiota profiles due to DNA extraction method and inter-subject differences. Front Microbiol. 2015;6: 1–11. doi:10.3389/fmicb.2015.00130

29. Kia E, MacKenzie BW, Middleton D, Lau A, Waite DW, Lewis G, et al. Integrity of the human faecal microbiota following long-term sample storage. PLoS One. 2016;11: 1–8. doi:10.1371/journal.pone.0163666

30. Costea PI, Zeller G, Sunagawa S, Pelletier E, Alberti A, Levenez F, et al. Towards standards for human fecal sample processing in metagenomic studies. Nat Biotechnol. 2017;35: 1069–1076. doi:10.1038/nbt.3960

31. Panek M, Čipčić Paljetak H, Barešić A, Perić M, Matijašić M, Lojkić I, et al. Methodology challenges in studying human gut microbiota-Effects of collection, storage, DNA extraction and next generation

sequencing technologies. Sci Rep. 2018;8: 1–13. doi:10.1038/s41598-018-23296-4

32. El Bali L, Diman A, Bernard A, Roosens NHC, Dekeersmaecker SCJ. Comparative study of seven commercial kits for human dna extraction from urine samples suitable for dna biomarker-based public health studies. J Biomol Tech. 2014;25: 96–110. doi:10.7171/jbt.14-2504-002

33. Wolfe AJ, Toh E, Shibata N, Rong R, Kenton K, FitzGerald MP, et al. Evidence of uncultivated bacteria in the adult female bladder. J Clin Microbiol. 2012;50: 1376–1383. doi:10.1128/JCM.05852-11

34. Jung CE, Chopyk J, Shin JH, Lukacz ES, Brubaker L, Schwanemann LK, et al. Benchmarking urine storage and collection conditions for evaluating the female urinary microbiome. Sci Rep. 2019;9: 13409. doi:10.1038/s41598-019-49823-5

35. David LA, Materna AC, Friedman J, Campos-Baptista MI, Blackburn MC, Perrotta A, et al. Host lifestyle affects human microbiota on daily timescales. Genome Biol. 2014;15: R89. doi:10.1186/gb-2014-15-7-r89

36. Ott SJ, Musfeldt M, Timmis KN, Hampe J, Wenderoth DF, Schreiber S. In vitro alterations of intestinal bacterial microbiota in fecal samples during storage. Diagn Microbiol Infect Dis. 2004;50: 237–245. doi:10.1016/j.diagmicrobio.2004.08.012

37. McDonald D, Hyde ER, Debelius JW, Morton JT, Gonzalez A, Ackermann G, et al. American Gut: an Open Platform for Citizen-Science Microbiome Research. bioRxiv. 2018;3: 277970. doi:10.1101/277970

38. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 2012;6: 1621–1624. doi:10.1038/ismej.2012.8

39. Albertsen M, Karst SM, Ziegler AS, Kirkegaard RH, Nielsen PH. Back to basics - The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. PLoS One. 2015;10: 1–15. doi:10.1371/journal.pone.0132783

40. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data.

Bioinformatics. 2014;30: 2114–20. doi:10.1093/bioinformatics/btu170

41. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10: 996–8. doi:10.1038/nmeth.2604

42. R Development Core Team. R: A Language and Enviroment for Statistical Computing. In: the R Foundation for Statistical Computing [Internet]. [cited 3 Apr 2019]. Available: https://www.r-project.org/

Figure legends

Fig 1: Urine storage conditions. Urine samples were either processed directly (room temperature, 0 hours) or at different temperatures for different time periods. Duplicate experiments were performed.

Fig 2: DNA yield. Quantity of total DNA purified from urine shown for each donor (A-E) and storage condition. Each sample is named as "temperature"_"duration". *P<0.05. RT = room temperature, >72 = sample are stored for at least 72 hours.

Fig 3: Quality control used for removing low quality samples. A. Rarefaction Curve, showing number of unique OTUs generated based on quantity of reads. The red dotted line indicates cutoff value of 10.000 reads. Three samples were excluded due to values below cutoff. B. Principal Component Analysis (PCA) clustering of samples using Hellinger transformed OTU abundances, in order to identify possible outliers. C. Heatmap depicting the 20 most common OTUs for each separate duplicate. Each name consists of phylum followed by genus name. If no genus could be identified, the best taxonomic assignment is listed. The heatmap was used to exclude potential outliers. The following four samples were excluded: A -20 72h_2, D -20 72h_2, D 4 24h_1 and E -80 >72h_2. These has been circled in B. and marked with a red * in C.

Fig 4: Effects of storage temperature and duration on urinary microbiota alpha diversity. A. OTU richness showing numbers of unique OTUs observed in different storage conditions and durations. B. Shannon Diversity Index visualizing differences and similarities in diversity of OTU composition within samples.

Fig 5: Effects of storage temperature and duration on urinary microbiota beta-diversity. A. Clustering of samples based on PCA of Hellinger transformed OTU abundances. B. Heat map depicting the 20 most common OTUs in different storage conditions. Each name consist of phylum followed by genus name. If no genus could be identified, the best taxonomic assignment is listed.

Fig 6: Effects of sampling at evening compared to the following morning on alpha diversity. A. OTU richness depicting number of unique OTUs observed at evening compared to morning. B. Shannon diversity index visualizing similarities in diversity between evening and morning samples. Two donors, one girl (donor 8) and one man (donor 13), were excluded from this part of the analysis, since matching morning-evening sample pairs were not available due to quality control.

Fig 7: Beta diversity between samples collected in the evening compared to the following morning. A. Relatedness in bacterial composition of samples, as visualized using PCA of Hellinger transformed OTU abundances. Colors indicate donor while shapes indicate time. B. Heat maps listing the 20 most common OTUs for each donor at morning and evening. Each name consists of phylum followed by genus name. If no genus could be identified, the best taxonomic assignment is given. As for figure 7, two donors, one girl (donor 8) and one man (donor 13), were excluded from this part of the analysis, since they did not contain matching morning-evening samples.

Fig 8: Alpha diversity of urinary microbiota on weekdays compared to weekends. A. OTU richness depicting number of unique OTUs in samples collected during weekdays compared to weekends. B. Shannon diversity index visualizing similarities in diversity of samples collected during weekdays compared to weekends. Three donors, one girl (donor 8) and two men (donors 13 and 15) were removed from this part of the analysis, since they did not contain matching weekday-weekend samples.

Fig 9: Beta diversity of urinary microbiota at weekdays compared to weekends. A. Variation in bacterial composition of samples collected during weekdays compared to weekends, using PCA of Hellinger transformed OTU abundances. Colors indicate donor while shape indicate day. B. Heat maps listing the 20 most common OTUs for each donor at weekdays compared to weekends. As for figure 9, three donors, one girl (donor 8) and two men (donors 13 and 15) were removed from this part of the analysis, since they did not contain matching weekday-weekend samples.

Supplementary information

S1_Metadata CP154. 16S rRNA gene sequencing metadata. Information describing the samples used for investigating storage.

S2_otus CP154. 16S rRNA gene sequences. FASTA file containing the DNA sequences generated using 16S rRNA gene sequencing of the samples used for investigating storage.

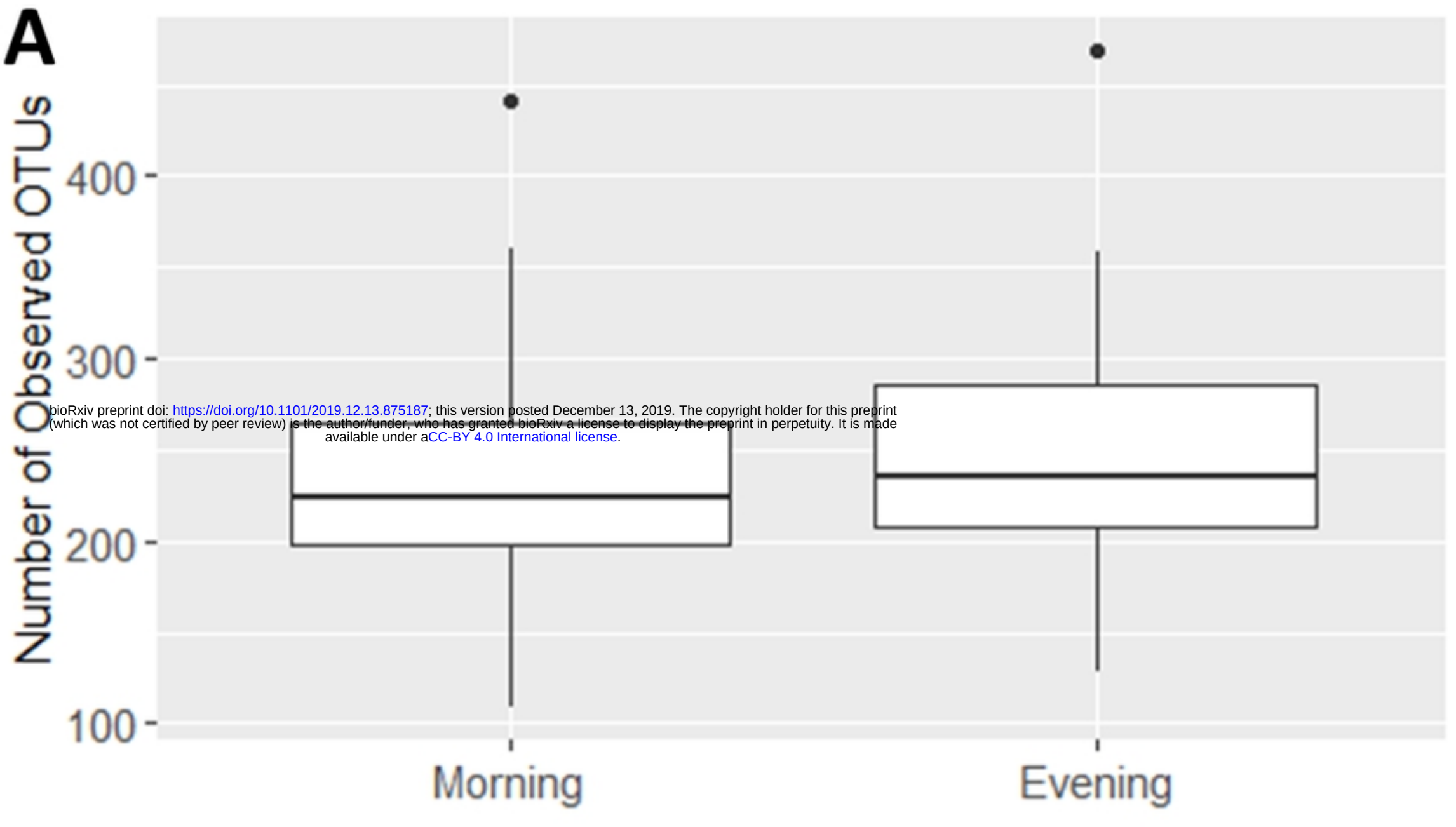
S3_otutable CP154. 16S rRNA gene sequencing OTUtable. OTU table documenting numbers of different OTUs generated during the 16S rRNA gene sequencing of the samples used for investigating storage.

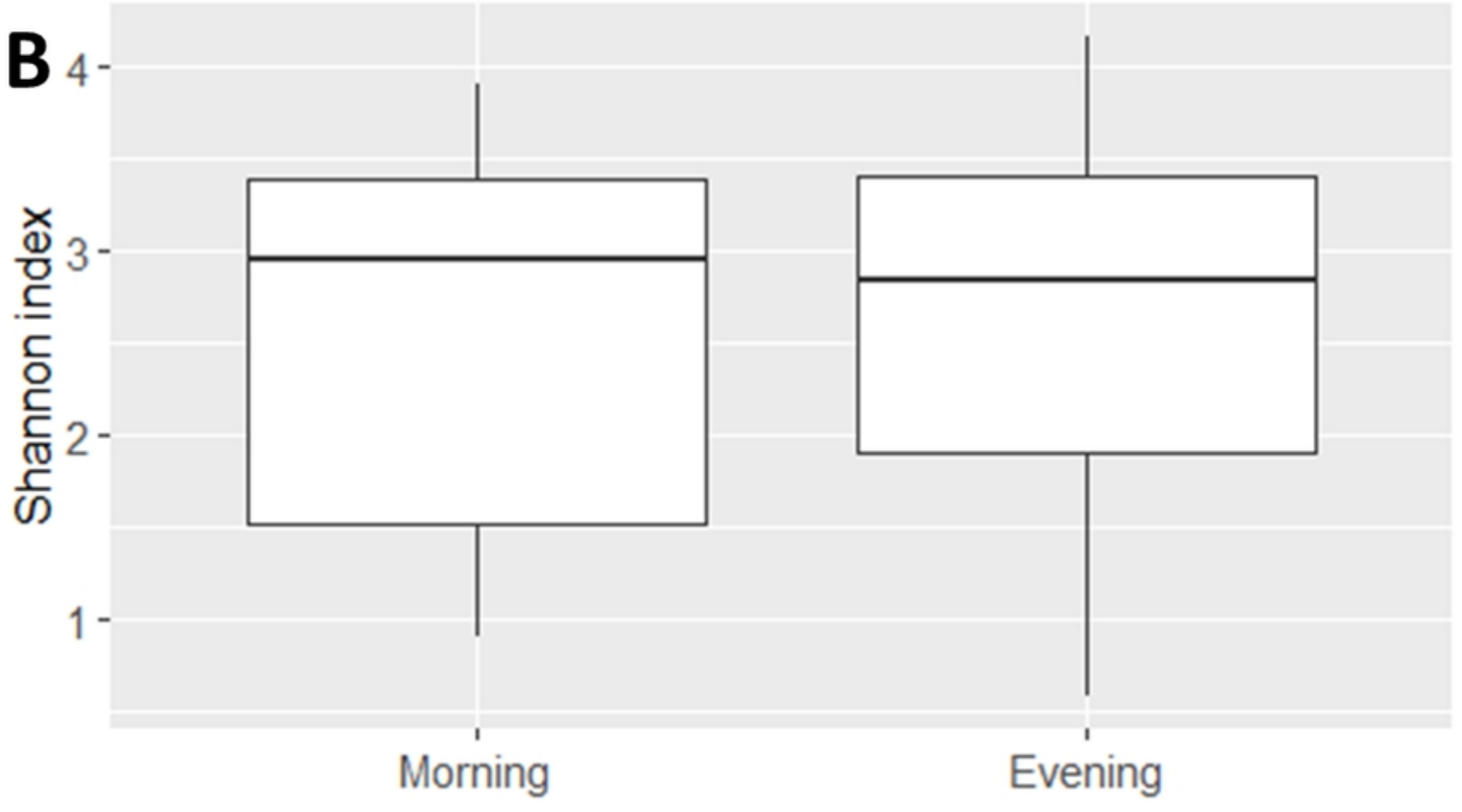
S4_metadata CP303. 16S rRNA gene sequencing metadata. Information describing the samples used for investigating bacterial variation across different time of day or between weekday or weekend.

S5_otus CP303. 16S rRNA gene sequences. FASTA file containing the DNA sequences generated using 16S rRNA

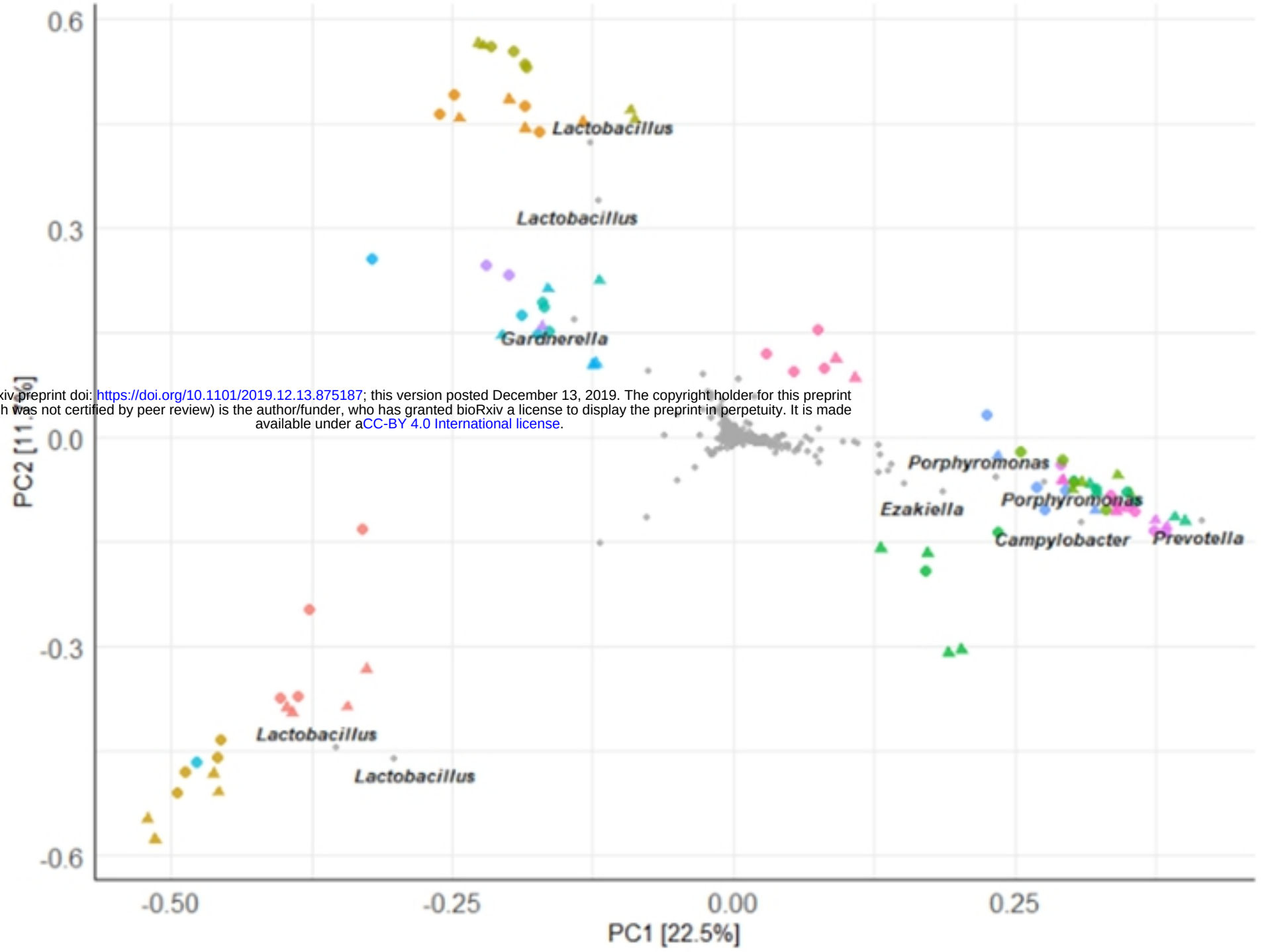
459 gene sequencing of the samples used for investigating bacterial variation across different time of day or between
460 weekday or weekend.

461 **S6_otutable CP303. 16S rRNA gene sequencing OTUtable.** OTU table documenting numbers of different OTUs
462 generated during the 16S rRNA gene sequencing of the samples used for investigating bacterial variation across
463 different time of day or between weekday or weekend.

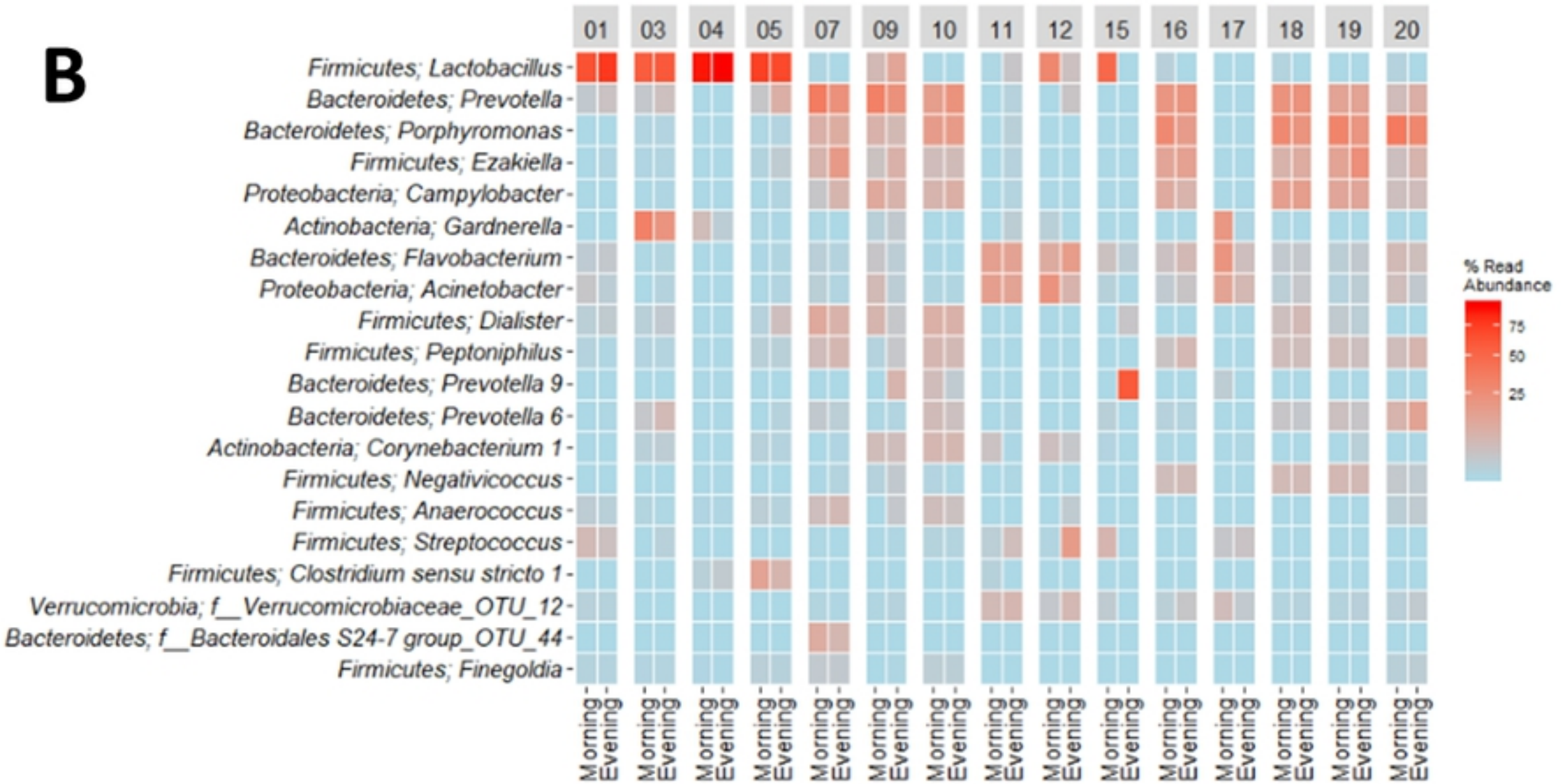


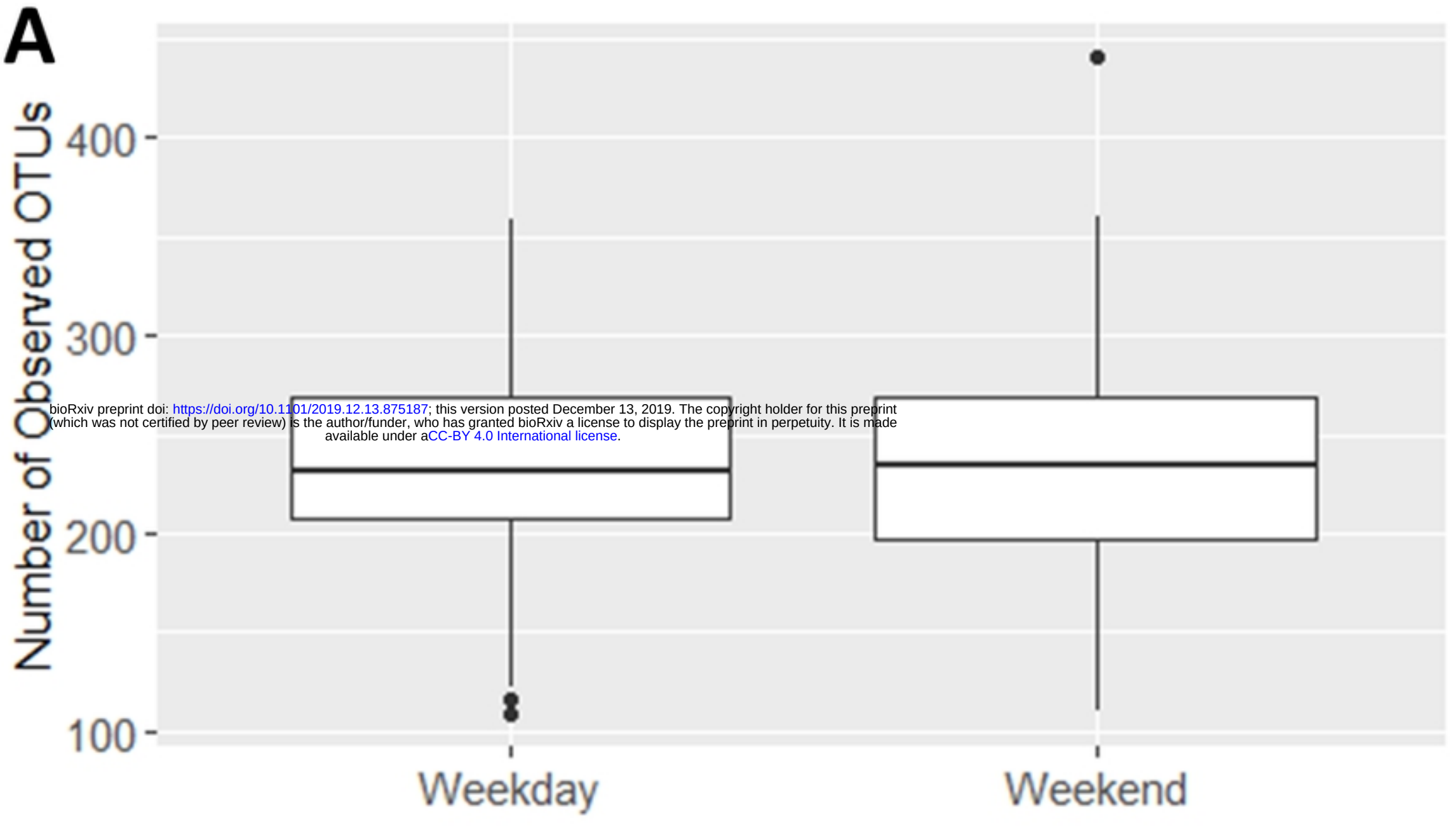


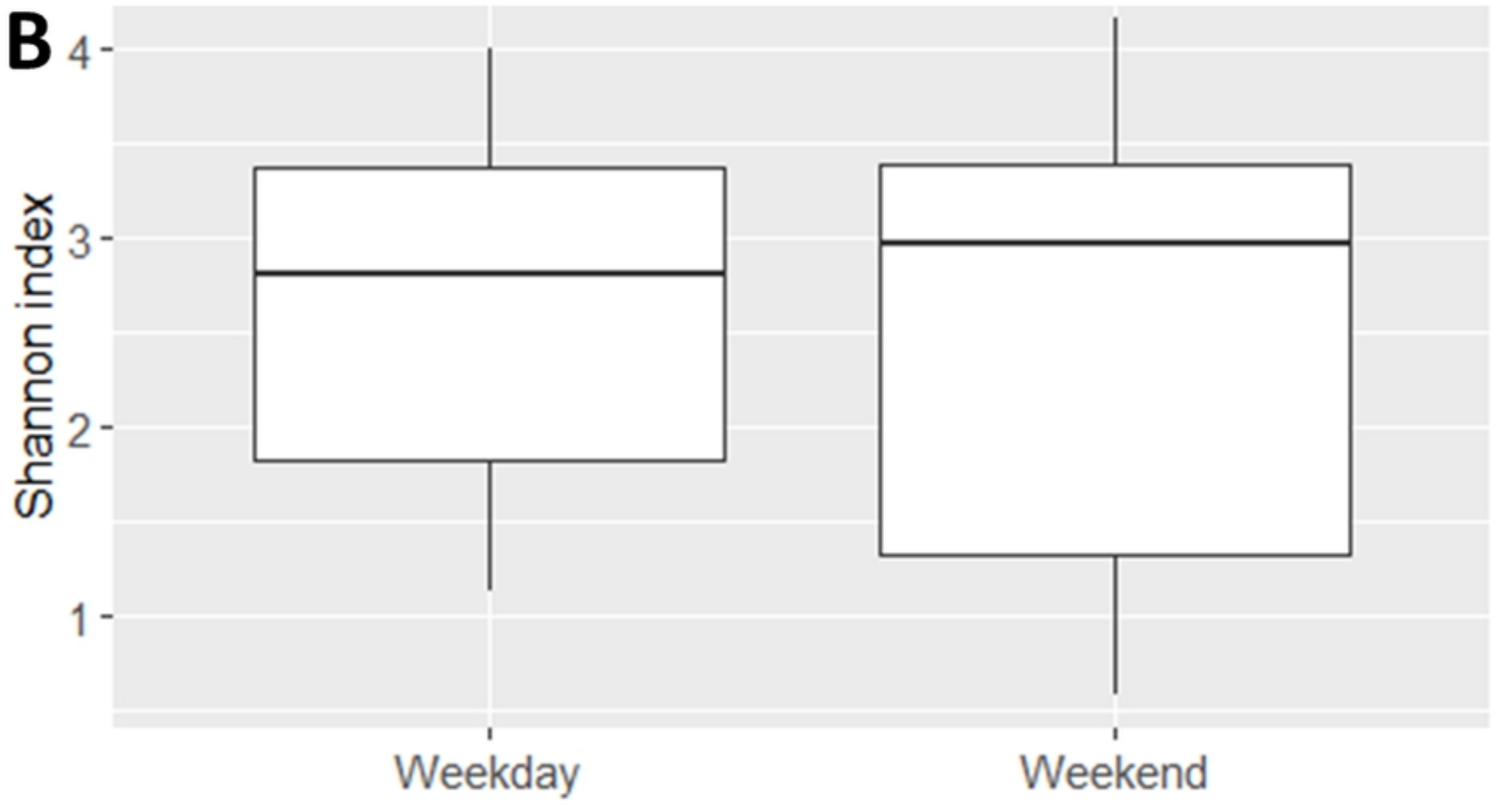
A



B

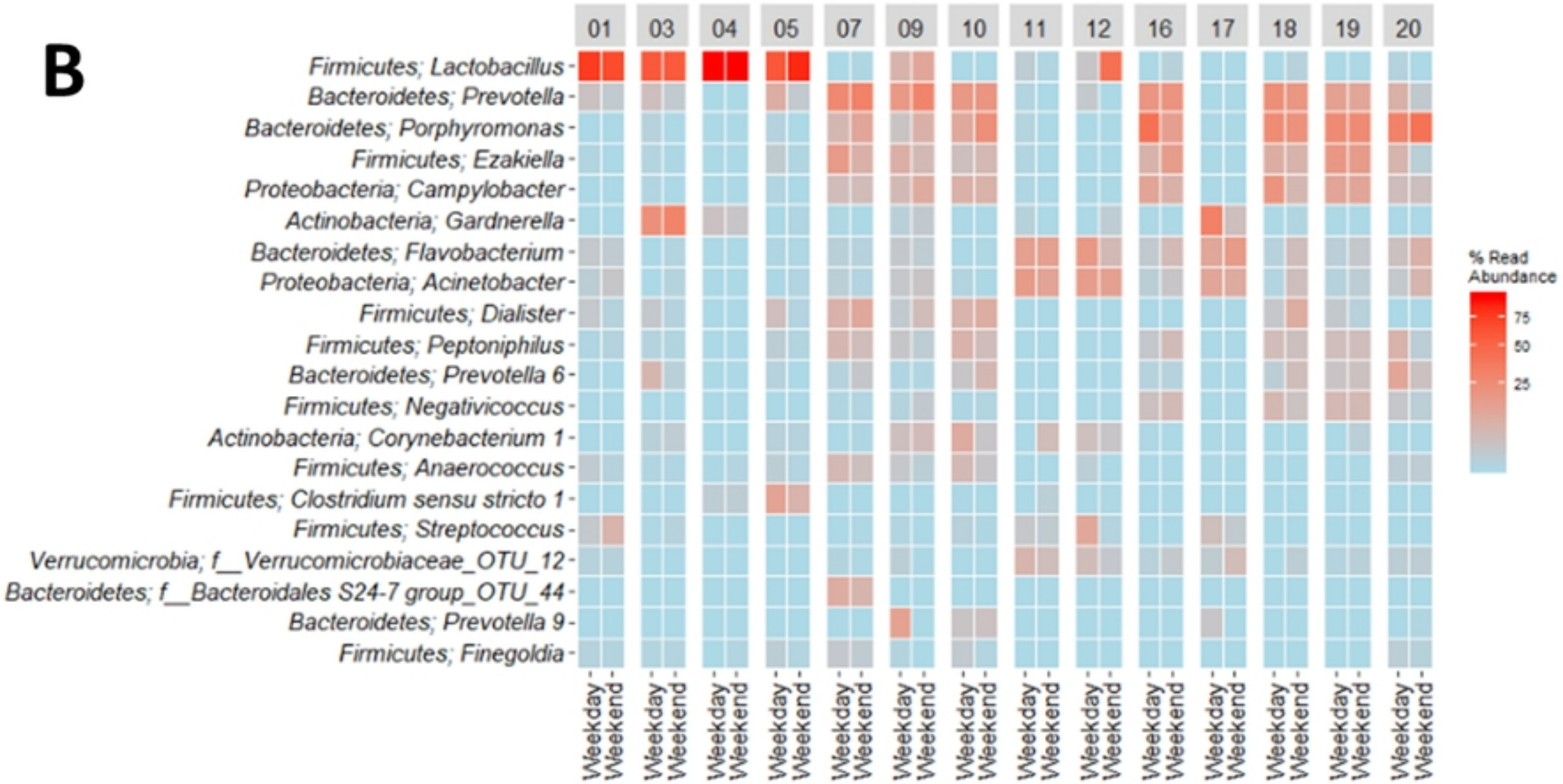


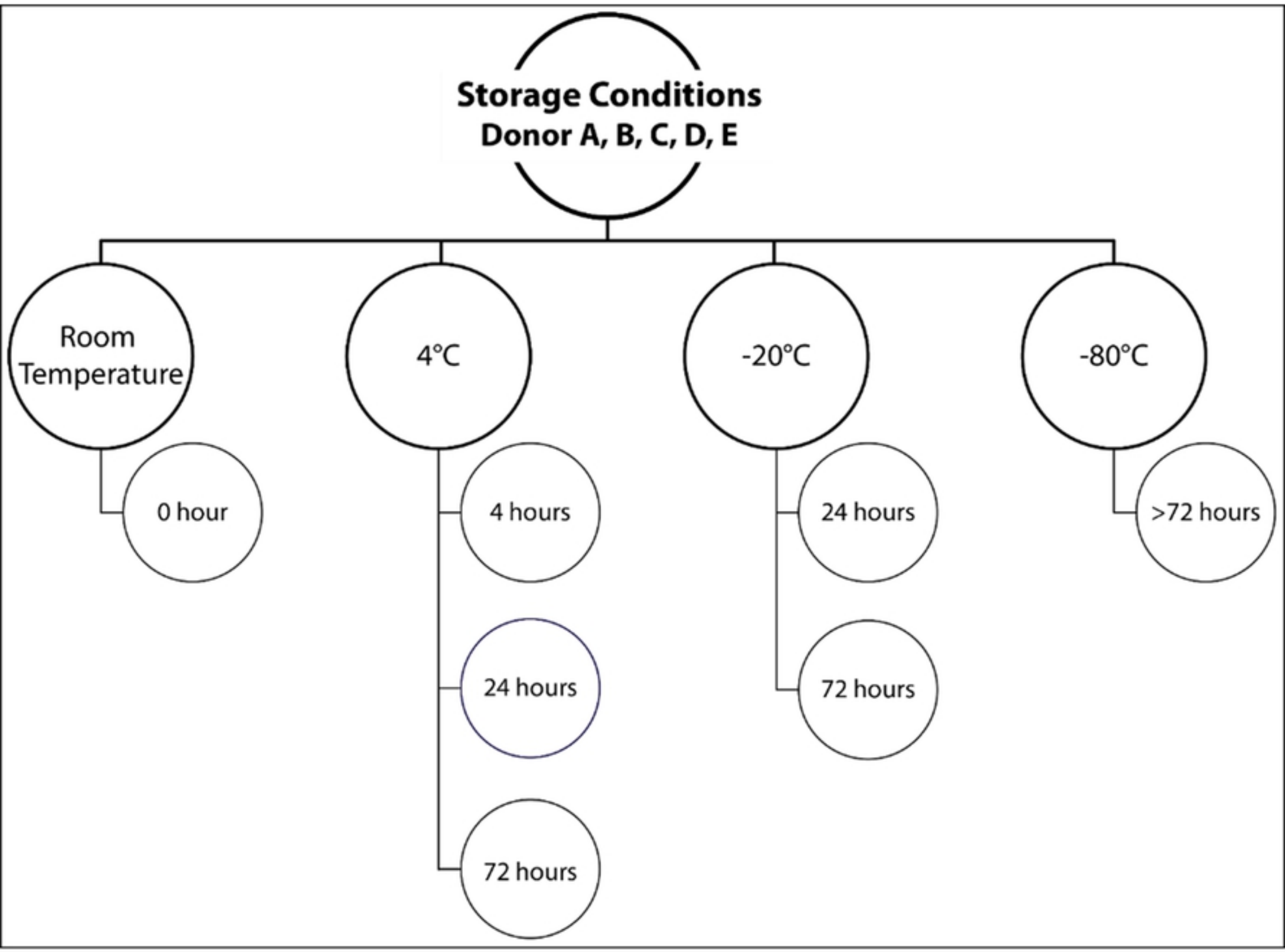


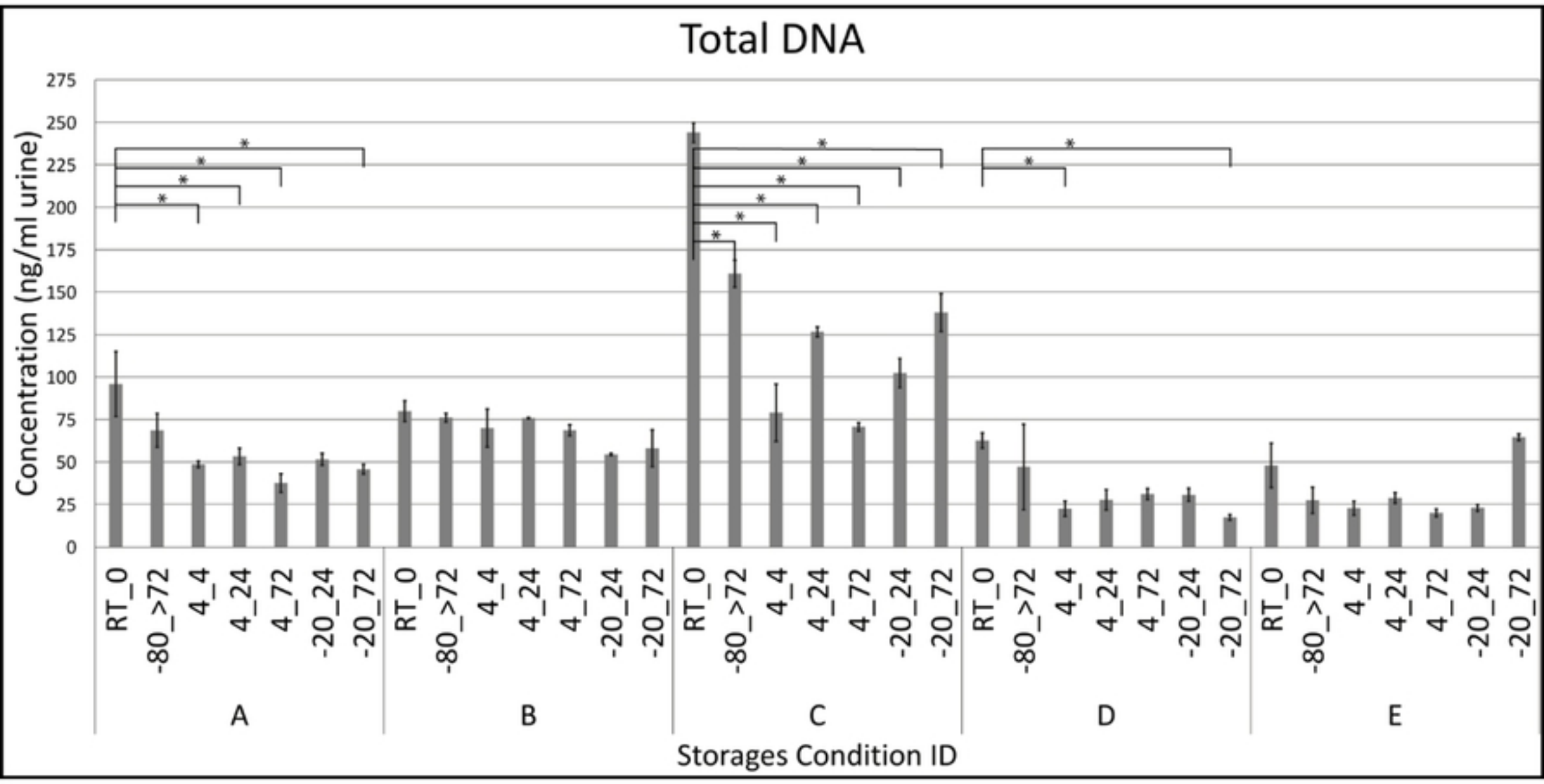


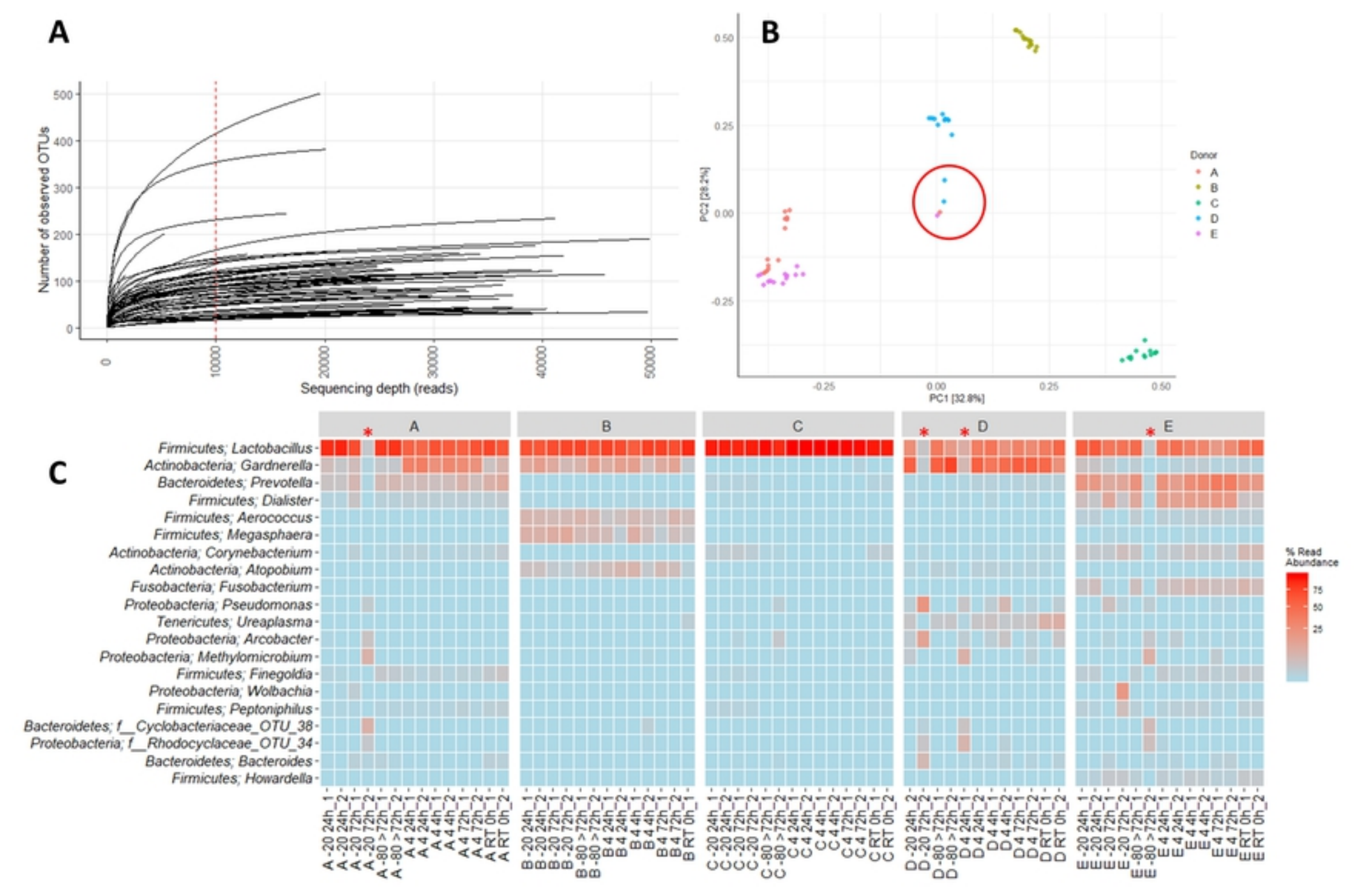
A

B

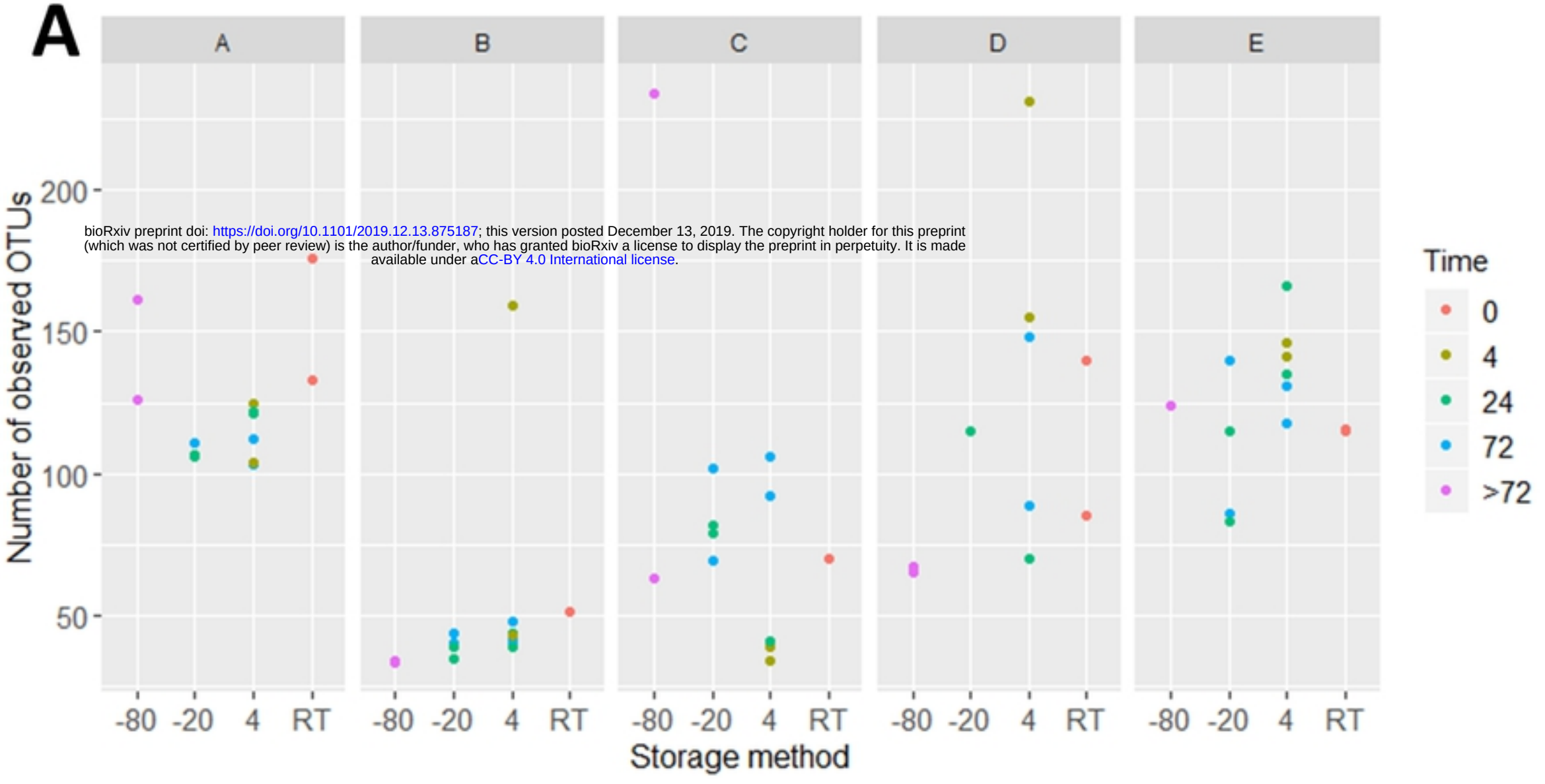



Storage Conditions
Donor A, B, C, D, E
Room Temperature
0 hour
4°C
4 hours
24 hours
72 hours
-20°C
24 hours
72 hours
-80°C
>72 hours

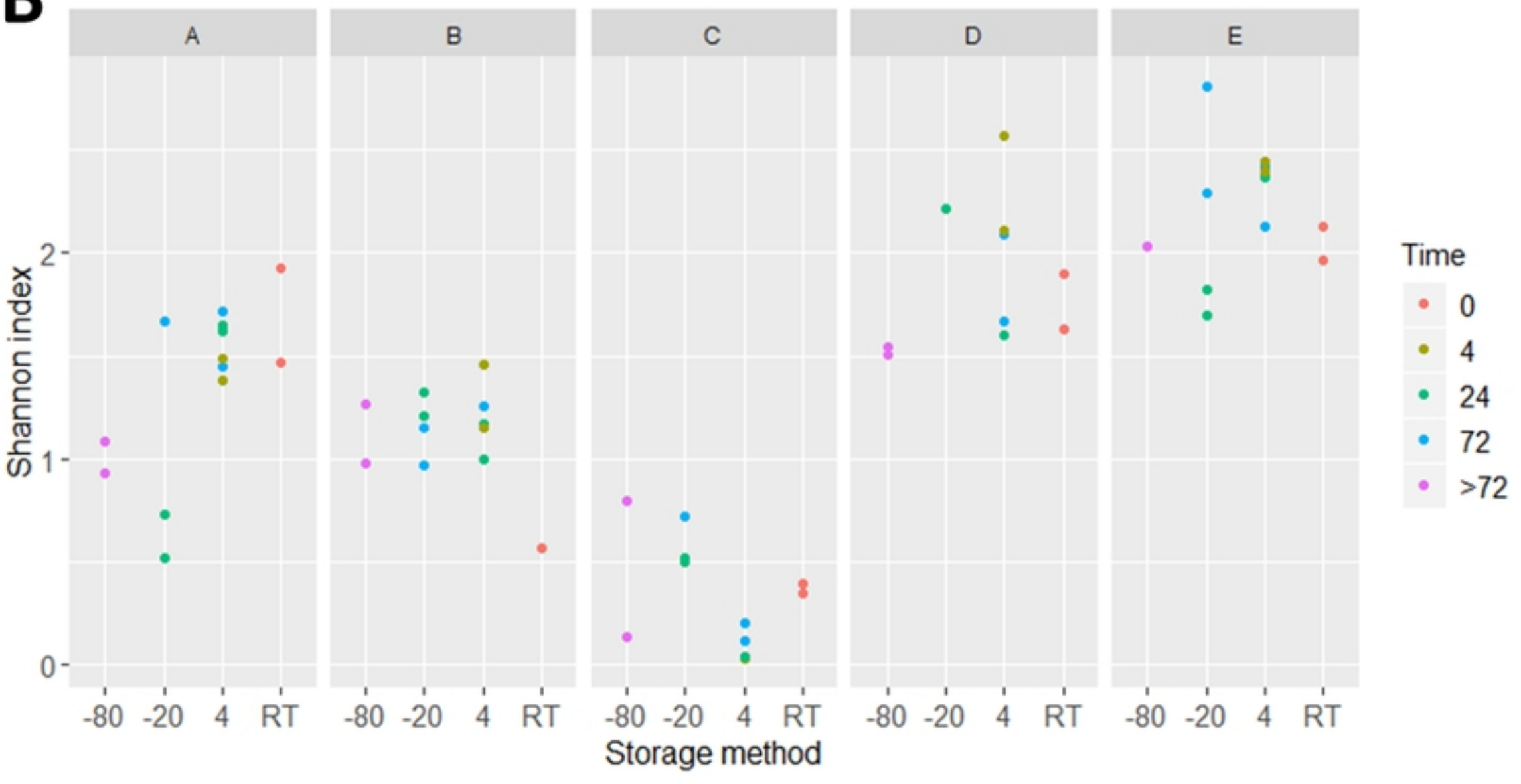

Total DNA
Concentration (ng/ml urine)
275
250
225
200
175
150
125
100
75
50
25
0
RT_0
-80_>72
4_4
4_24
4_72
-20_24
-20_72
A
B
C
D
E
Storages Condition ID


A
Number of observed OTUs
Sequencing depth (reads)
B
PC2 [28.2%]
PC1 [32.8%]
Donor
A
B
C
D
E
C
A
B
C
D
E
Firmicutes; Lactobacillus
Actinobacteria; Gardnerella
Bacteroidetes; Prevotella
Firmicutes; Dialister
Firmicutes; Aerococcus
Firmicutes; Megasphaera
Actinobacteria; Corynebacterium
Actinobacteria; Atopobium
Fusobacteria; Fusobacterium
Proteobacteria; Pseudomonas
Tenericutes; Ureaplasma
Proteobacteria; Arcobacter
Proteobacteria; Methylomicrobium
Firmicutes; Finegoldia
Proteobacteria; Wolbachia
Firmicutes; Peptoniphilus
Bacteroidetes; f__Cyclobacteriaceae_OTU_38
Proteobacteria; f__Rhodocyclaceae_OTU_34
Bacteroidetes; Bacteroides
Firmicutes; Howardella
% Read Abundance

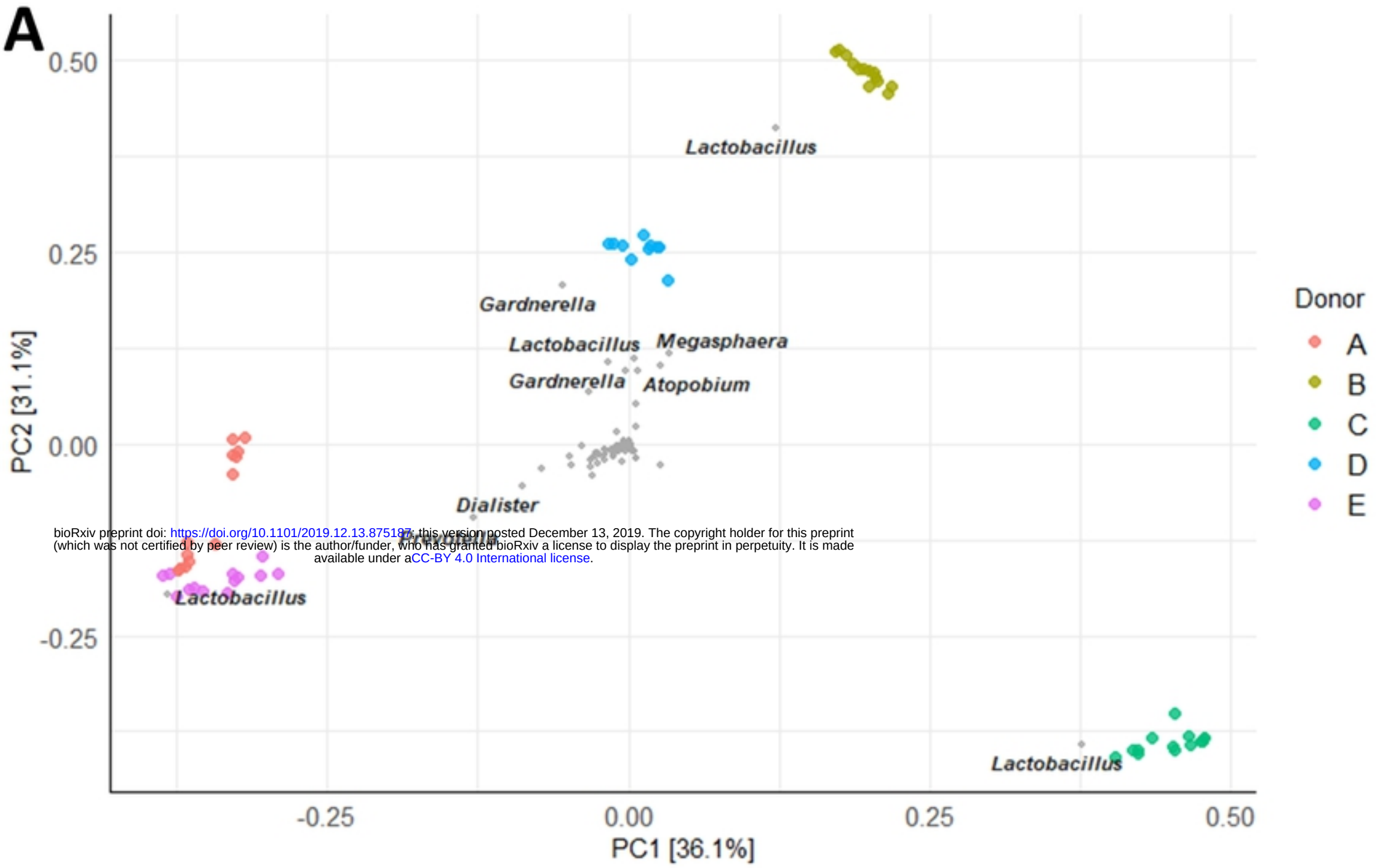
A



B



A



B