Reduced production of the major allergen Bla g 1 and Bla g 2 in *Blattella germanica* after antibiotic treatment

Running title: Antibiotics lower Bla g 2 levels in *B. germanica*

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ABSTRACT

Purpose: Allergens present in the feces or frass of cockroaches can cause allergic sensitization in humans. The use of fecal and frass extracts for immunotherapy has been previously investigated but has not yet been fully standardized. Here, we treated cockroaches with ampicillin to produce extracts with reduced amounts of total bacteria.

Methods: We performed targeted high-throughput sequencing of 16S rDNA to compare the microbiomes of ampicillin-treated and untreated (control) cockroaches. RNA-seq was performed to identify differentially expressed genes (DEGs) in ampicillin-treated cockroaches.

Results: Analysis of the microbiome revealed that alpha diversity was lower in the ampicillin-treated group than in the control group. Beta diversity analysis indicated that ampicillin treatment altered bacterial composition in the microbiome of cockroaches. Quantitative polymerase chain reaction revealed that almost all bacteria were removed from ampicillin-treated cockroaches. RNA-seq analysis revealed 1,236 DEGs in ampicillin-treated cockroaches (compared to untreated cockroaches). Unlike bacterial composition, the DEGs varied between the two groups. Among major allergens, the expression of *Bla g 2* decreased significantly in ampicillin-treated cockroaches (compared to untreated group).

Conclusions: In this study, the reduced level of allergens observed in cockroaches may be related to lower amounts of total bacteria caused by treatment with antibiotics. It is possible to make a protein extract with few bacteria for use in immunotherapy.

Key words: cockroaches, antibiotics, microbiome, allergens

INTRODUCTION

Cockroaches have existed for some 300-350 million years, with more than 4,000 species described globally. They spread pathogenic bacteria through their feces or frass while traveling between locations, such as homes, shops, and hospitals [1]. Their omnivorous nature enables them to survive under a wide variety of conditions. The German cockroach, *Blattella germanica,* and its microbiome have been extensively investigated [2–4, 6]. Different gut microbiomes of *B. germanica* were identified at various locations inside and outside laboratory settings, likely due to differences in the diets available at these locations [2]. A laboratory-based study investigating the effect of diet on *B. germanica* confirmed that their microbiome changed dynamically according to food intake [3].

Several studies have reported that antibiotics directly affect the bacterial composition in the cockroach microbiota. Rosas *et al*. showed that rifampicin altered the *B. germanica* microbiota and that the second generation of insects following antibiotic treatment underwent microbiota recovery through fecal intake. Antibiotic-treated cockroaches showed changes in bacterial diversity and composition, including the removal of the endosymbiont *Blattabacterium* [4]. Another study reported difficulties in cockroach reproduction and growth following antibiotic treatment [5]. In *Riptortus pedestris*, the absence of an endosymbiont led to a decrease in hexamerin and vitellogenin, which affected egg production and insect development [6].

Cockroaches can spread pathogenic bacteria present in their gut or body to places with high human traffic, such as restaurants or hospitals [7], and the allergens in their feces or frass can cause allergen sensitization in humans [8]. Production of the major cockroach allergen Bla g 1 in female cockroaches is related to their reproductive cycle and is also affected by their food intake [9]. Bla g 1 can bind various lipids, suggesting that it has a digestive function related to the nonspecific transport of lipid molecules [10]. Similar to Bla g 1, Bla g 2 is present at high concentrations in the

77 digestive organs of cockroaches (esophagus, gut, and proventriculus), suggesting that
78 Bla g 2 functions as a digestive enzyme [11]. Bla g 2 is regarded the most important *B.*
79 *germanica* allergen, with the rate of sensitization being the highest among common
80 cockroach allergens at 54–71% generally [12].

81 Because the potency of the cockroach protein extract was different for each
82 cohort depending on the allergen content of the extract—as recently demonstrated in
83 several studies on allergen immunotherapy [13, 14]—it is important to select a suitable
84 protein extract for each patient [13].

85 Despite these variables, no studies have been conducted to determine the effect
86 of bacteria in the cockroach before extracting the protein for immunotherapy. In the
87 present study, we aimed to obtain a protein extract of *B. germanica* with reduced levels
88 of bacteria using ampicillin, a broad-spectrum antibiotic.

89

MATERIALS AND METHODS

Rearing conditions

Cockroaches (*B. germanica*) were reared for several generations under the same laboratory conditions to minimize the potential influence of environmental factors and diet on their performance. All cockroaches were reared in plastic boxes (27 cm × 34 cm × 19 cm) and incubated at 25°C and 50% relative humidity. *B. germanica* were fed sterilized fish food and provided with sterilized untreated or ampicillin-containing (autoclaved before the addition of 0.025% ampicillin) tap water *ad libitum*. The concentration of ampicillin was set based on data from a preliminary study, which showed that concentration did not significantly affect the survival of *B. germanica* (data not shown).

Experimental design

Newly hatched cockroaches (G1) were divided into two groups. Group A was offered ampicillin-treated water, while group C (control) was offered untreated water. Twenty-one days after becoming adults, ampicillin-treated (A) and untreated (C) female cockroaches were sampled for further analyses. *B. germanica* were sacrificed with CO_2 then surface-sterilized using alcohol and flash-frozen in liquid nitrogen. They were then individually crushed using a mortar and pestle and stored at −80 °C until further analysis.

DNA extraction

Cockroaches (n = 5 from each group) were frozen in liquid nitrogen and individually crushed using a mortar and pestle. Total DNA was extracted using the NucleoSpin DNA Insect Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The DNA extracted from each sample was eluted in 20 µL of elution buffer. Procedures were all conducted at a clean bench, under a sterilized hood,

and in a DNA-free room. DNA concentrations were quantified using an ND-1000 Nanodrop system (Thermo-Fisher Scientific, Waltham, MA, USA).

Next-generation sequencing

The 16S rDNA V3–V4 region was amplified through polymerase chain reaction (PCR) using forward and reverse primers (Table. 1) [15, 16], in an Illumina MiSeq V3 cartridge (San Diego, CA, USA) with a 600-cycle format. A limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters to the samples. The libraries were normalized, pooled, and sequenced on the Illumina MiSeq V3 cartridge platform according to the manufacturer's instructions.

Primer Name	Primer Sequence (5' → 3')
16S rDNA V3–V4_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S rDNA V3–V4_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
ActinF	CACATACAACTCCATTATGAAGTGCGA
ActinR	TGTCGGCAATTCCGGTACATG
BACT1369	CGGTGAATACGTTCYCGG
PROK1492R	GGWTACCTTGTTACGACTT
Blag1F	CTATATGACGCCATCCGTTCTC
Blag1R	CACATCAACTCCCTTGTCCTT
Blag2F	TGATGGGAATGTACAGGTGAA A

Blag2R	TGTTGAGATGTCGTGAGGTTAG
Blag5F	GATTGATGGGAAGCAAACACAC
Blag5R	CGATCTCCAAGTTCTCCCAATC

Table. 1. Primers used in this study

Bioinformatics and statistical analyses

Bioinformatics analyses were performed according to the methods described in our previous study [16]. Taxonomic assignment was performed using the EzBioCloud database (https://www.ezbiocloud.net/) [15] and BLAST (v. 2.2.22), and pairwise alignments were generated to measure sequence similarity [16, 17]. All analyses were performed using BIOiPLUG, a commercially available ChunLab bioinformatics cloud platform for microbiome research (https://www.bioiplug.com/) [16]. The reads were normalized to 11,000 to perform the analyses. Phylogenetic analysis was performed, and Shannon indexes, unweighted pair group method with arithmetic mean (UPGMA) clustering, principal coordinates analysis (PCoA), permutational multivariate analysis of variance, linear discriminant analysis (LDA), and linear discriminant analysis effect size (LEfSe) were determined according to our previous study [16].

Protein extraction

Total protein was extracted by first adding 2 mL of PBS to each sample. The samples were then sonicated (QSonica Q500, Fullerton, CA, USA) and centrifuged at 10,000 ×*g* for 30 min at 4°C. The resulting supernatants were filtered using a 0.22-μm membrane filter (Millex®, Tullagreen, Carrigtwohill, Co. Cork, Ireland).

Enzyme-linked immunosorbent assay (ELISA)

The Bla g 1, Bla g 2, and Bla g 5 allergens of *B. germanica* were quantified using corresponding ELISA kits (Indoor Biotechnologies, Charlottesville, VA, USA).

RNA extraction and cDNA synthesis

Total RNA was extracted by adding 1 mL of TRIZOL Reagent (GeneAll, Seoul, Korea) to each sample. The RNA extracted from each sample was eluted in 20 µL of the elution buffer. A master mix comprised 5× cDNA synthesis mix and 20× RTase was added to mRNA samples in PCR tubes for cDNA synthesis.

Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) was performed to quantify *Bla g 1, Bla g 2, Bla g 5*, and bacterial 16S rRNA in whole cockroaches. Actin 5C (accession number AJ861721.1) was used as the internal control, and primers specific to this gene were designed for this experiment: ActinF and ActinR (Table. 1) [4]. All bacterial 16S rRNA were amplified using the forward primer BACT1369 and the reverse primer PROK1492R (Table. 1) from XenoTech with AMPIGENE qPCR Mixes (ENZO, USA) [19]. *Bla g 1* (accession number EF202179.1), *Bla g 2* (accession number EF203068.1), and *Bla g 5* (accession number EF202178.1) gene expression was used as a measurement of major allergen content. We designed the following primers for this experiment: Blag1F and Blag1R, Blag2F and Blag2R, and Blag5F and Blag5R (Table. 1). qPCR analyses were performed using the 2× SensiFAST™ SYBR® Hi-ROX kit (Bioline Meridian Bioscience, Humber Rd, London) with SYBR Green as the fluorescent reporter, H_2O, corresponding primers, and either genomic or complementary DNA. At the end of each reaction, a melting curve was generated to check the specificity of amplification and to confirm the absence of primer dimers. All reactions, including negative controls (containing water instead of DNA), were run in duplicate in 96-well plates.

RNA-seq analysis

We used total RNA and the TruSeq Stranded mRNA LT Sample Prep Kit (San Diego, California, USA) to construct cDNA libraries. The protocol consisted of polyA-selected RNA extraction, RNA fragmentation, random hexamer primed reverse transcription, and 100 nt paired-end sequencing by Illumina NovaSeq 6000 (San Diego, California, USA). The libraries were quantified using qPCR according to the qPCR Quantification Protocol Guide and qualified using an Agilent Technologies 2100 Bioanalyzer.

Raw reads from the sequencer were preprocessed to remove low-quality and adapter sequences. The processed reads were aligned to the *B. germanica* genome using HISAT v2.1.0 [20]. HISAT utilizes two types of indexes for alignment (a global, whole-genome index, and tens of thousands of small local indexes). These index types are constructed using the same Burrows–Wheeler transform, and graph Ferragina Mangini index as Bowtie2. HISAT generates spliced alignments several times faster than the Burrows–Wheeler Aligner (BWA) and Bowtie because of how efficiently it utilizes these data structures and algorithms. The reference genome sequence of *B. germanica* and annotation data were downloaded from NCBI. Known transcripts were assembled using StringTie v1.3.4d [21, 22], and the results were used to calculate the expression abundance of transcripts and genes as read count or FPKM (fragments per kilobase of exon per million fragments mapped) value per sample. Expression profiles were used to further analyze differentially expressed genes (DEGs). DEGs or transcripts from groups with different conditions can be filtered through statistical hypothesis testing.

Statistical analysis of gene expression

The relative abundances of gene expression were measured in the read count using StringTie. We performed statistical analyses to detect DEGs using the estimates of abundance for each gene in individual samples. Genes with more than one “zero” read

count value were excluded. Filtered data were log2-transformed and subjected to trimmed mean of M-values (TMM) normalization. The statistical significance of the fold change in expression (i.e., differential expression data) was determined using the exact test from edgeR [23], wherein the null hypothesis was that no difference exists among groups. The false discovery rate (FDR) was controlled by adjusting the p-value using the Benjamini-Hochberg algorithm. For DEGs, hierarchical clustering analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Gene-enrichment and KEGG pathway analyses for DEGs were also performed based on the Gene Ontology (http://geneontology.org/) and KEGG pathway (https://www.genome.jp/kegg/) databases, respectively. We used the multidimensional scaling (MDS) method to visualize the similarities among samples and applied the Euclidean distance as a measure of dissimilarity. Hierarchical clustering analysis was also performed using complete linkage and Euclidean distance as a measure of similarity to display the expression patterns of differentially expressed transcripts that satisfied a |fold change| ≥ 2 and a raw P-value <0.05.

RESULTS

First-generation cockroaches reached the adult stage and were kept for an additional 21 days before being sacrificed for further analysis (Fig. 1). qPCR analysis showed that the number of total bacteria in the cockroaches was 2,000 times higher in the control group than that in the ampicillin-treated group (Fig. 2).

Fig. 1. Experimental design depicting ampicillin treatment of *B. germanica*. The cockroaches were divided into two groups (A and C) and individuals were either treated with ampicillin (A) or left untreated as control specimens (C). Ampicillin was administered to cockroaches from the G1 (i.e., offspring from G0) generation, 21 days after they had reached the adult stage. Whole bodies were then collected and analyzed.

Fig. 2. Relative quantification of the total bacterial population in the ampicillin-treated (A) and untreated (C) cockroach groups.

Comparison of the microbial composition data showed, in the control group, the endosymbiont *Blattabacterium* CP001487_s was the most abundant (27.43%), followed by *Fusobacterium varium*. By contrast, *Desulfovibrio*_g2 was the most abundant (64.39%) in the ampicillin-treated group (Fig. 3A). LEfSe analysis of sampled cockroaches showed that, in the control group, *Blattabacterium* CP001487_s showed the greatest difference in composition, followed by *F. varium*, *Rhodopila*_uc, and *Dysgonomonas*_uc (Fig. 3B). In the ampicillin-treated group, Desulfovibrio_g2 and Planctomycetes were the bacteria that showed the greatest differences in abundance (Fig. 3B). Analysis of alpha diversity revealed a significantly lower number of operational taxonomic units (OTUs) ($P = 0.009$) in the ampicillin-treated group (Fig. 4A). Although not statistically significant, phylogenetic diversity tended to be low in the ampicillin-treated group ($P = 0.076$) (Fig. 4B), indicating low overall abundance. A significant

248 difference was noted in the Shannon diversity index, reflecting richness and equity
249 simultaneously (P = 0.009) (Fig. 4C). Analysis of diversity using UPGMA clustering
250 showed that the samples from the control and ampicillin-treated groups were clustered
251 separately (Fig. 5A). Similarly, PCoA showed that both groups were clustered separately,
252 with samples from the ampicillin-treated and control groups located on the left and right
253 sides of the plot, respectively (Fig. 5B).
254
255 **Fig. 3.** Bacterial composition at the species level in the control and ampicillin-treated
256 groups. (A) Microbiome composition of each group (n = 5). (B) Linear discriminant
257 analysis effect size (LEfSe) analysis of differentially abundant bacterial taxa among the
258 two groups. Only taxa meeting a significant (>4) LDA threshold are shown.
259
260 **Fig. 4.** Box plots showing alpha diversity in the control and ampicillin-treated groups. (A)
261 The number of operational taxonomic units (OTUs). (B) Phylogenetic diversity
262 (abundance). (C) Shannon diversity index (measuring richness and equity in the
263 distribution of the species). * indicates a P-value < 0.05 from the Wilcoxon rank-sum test.
264
265 **Fig. 5.** Beta diversity in the control and ampicillin-treated groups. (A) UPGMA
266 (unweighted pair group method with arithmetic mean) clustering. (B) Principal
267 coordinates analysis (PCA) depicting differences in the taxonomic compositions of the
268 bacterial communities among the two groups.
269
270 RNA-sequencing was performed to explore the effect of ampicillin on gene
271 expression in cockroaches. UPGMA clustering results showed that the control group was
272 grouped together, but that one ampicillin-treated sample was clustered separately (Fig.
273 6A). Principal components analysis (PCA) confirmed that separation was achieved
274 between the control and ampicillin-treated groups (Fig. 6B). Hierarchical clustering

analysis between the control and ampicillin-treated groups generated a heat map of 1,236 DEGs for both groups (Fig. 6C). Gene Ontology (GO) functional classification analysis revealed that these 1,236 DEGs were divided among three main categories (biological process, cellular component, and molecular function), where differential expression more than doubled in 28, 16, and 13 items, respectively, between the control and ampicillin-treated groups (Fig. 7).

Fig. 6. Transcriptome analysis in ampicillin-treated and control cockroaches. (A) UPGMA (unweighted pair group method with arithmetic mean) clustering. (B) Principal components analysis (PCA) depicting the differences in the differentially expressed genes (DEGs) between the control and ampicillin-treated groups. (C) Heat map of transcriptional expression patterns of the two groups, displaying the expression profile of the top 1,236 DEGs for each sample in the RNA-seq dataset.

Fig. 7. Gene Ontology (GO) functional classification analysis of differentially expressed genes (DEGs) between the control and ampicillin-treated groups. Based on sequence homology, 1,236 DEGs were categorized into three main categories, biological process, cellular component, and molecular function, with 28, 16, and 13 functional groups, respectively.

RNA-sequencing showed that the expression of *Bla g 2* RNA more than doubled in the ampicillin-treated group (data not shown). Subsequently, RNA levels of the genes encoding the three major allergens Bla g 1 (Fig. 8A), Bla g 2 (Fig. 8B), and Bla g 5 (Fig. 8C) were measured using qPCR. Our findings confirmed that the expression levels of *Bla g 1* ($P = 0.000594$) and *Bla g 2* ($P < 0.00001$), but not *Bla g 5* ($P = 0.05067$), were significantly decreased in the ampicillin-treated group compared to those in the control group. Additionally, we noted a larger decrease in the level of *Bla g 2* than that of *Bla g*

1 (Fig. 8).

Fig. 8. Quantitative PCR (qPCR) analysis showing gene expression levels in cockroaches. (A) *Bla g 1*, (B) *Bla g 2*, and (C) *Bla g 5*.

At the protein level, we measured the amounts of Bla g 1 (Fig. 9A), Bla g 2 (Fig. 9B), and Bla g 5 (Fig. 9C). The results were similar to those obtained from transcriptomic analyses. No significant difference was detected in Bla g 5 ($P = 0.296897$), whereas a significant decrease in the expression of Bla g 1 ($P = 0.000463$) and Bla g 2 ($P = 0.00001$) was observed in the ampicillin-treated group compared to that in the control group. Here, Bla g 2 sustained yet again a more notable decrease than Bla g 1 (Fig. 9).

Fig. 9. Allergen levels in the extracts from the two cockroach groups. Concentrations of (A) Bla g 1, (B) Bla g 2, and (C) Bla g 5 in the extracts were measured using enzyme-linked immunosorbent assays (ELISAs).

DISCUSSION

We treated cockroaches (*B. germanica*) with ampicillin to obtain a protein extract containing a minimal number of bacteria for immunotherapy. Analysis of *B. germanica* following treatment revealed several changes.

First, the total bacterial population was notably affected. Compared with the control group, total bacteria in cockroaches from the ampicillin-treated group disappeared almost completely, perhaps because ampicillin eliminated both gram-positive and gram-negative bacteria.

The microbiome study revealed marked differences at the species level. A ‘super-resistant’ taxon was previously identified in *B. germanica* treated with rifampicin

329 instead of ampicillin [4]. The *Desulfovibrio* and *Plantomycetes* genera occurred in lower
330 numbers in control cockroaches but comprised most of the microbiota of ampicillin-
331 treated cockroaches. This finding is supported by the fact that all *Plantomycetes* are
332 resistant to β-lactam antibiotics [24], including ampicillin, which belongs to the penicillin
333 group of antibiotics. Similarly, *Desulfovibrio* and *Adiutrix* are resistant to ampicillin, which
334 eliminated other bacterial species. Among the several bacterial species found in the
335 control group, *Blattabacterium* is the most important. In a previous study, treatment with
336 rifampicin failed to eliminate *Blattabacterium* from the first generation of cockroaches;
337 however, with continued treatment, the bacteria were eliminated from second-generation
338 specimens [4]. Our data showed that treatment with ampicillin immediately eliminated
339 *Blattabacterium* from first-generation adults. *Blattabacterium* is an endosymbiont of *B.*
340 *germanica*, in which it is involved in the synthesis of essential amino acids and various
341 vitamins, as well as in nitrogen recycling [25]. A previous study showed that tetracycline
342 removed the endosymbiont of *Riptortus pedestris*, and that the expression of genes
343 encoding hexamerin and vitellogenin was reduced. Consequently, these findings
344 confirmed the factors that affected egg production and development [6]. Here, we
345 expected that the absence of an endosymbiont would lead to several changes.
346 Essentially, the reported decrease in bacterial composition produced differences in alpha
347 and beta diversity. Because ampicillin reduced the bacterial load, OTUs were
348 significantly lower in the ampicillin-treated group than in the control group. Phylogenetic
349 diversity (indicating abundance) was not significantly different, but tended to be lower in
350 the ampicillin-treated group. The Shannon diversity index significantly decreased,
351 reducing both richness and equity. We noted a decrease in both the species number and
352 the ratio of bacteria in ampicillin-treated cockroaches (compared to the total bacteria
353 found in control specimens). Analysis of beta diversity using UPGMA and PCoA showed
354 a clear difference in clustering between the two groups.

355 RNA-seq was performed to identify changes in gene expression at the RNA level

caused by ampicillin. Results similar to those from microbiome clustering analysis were confirmed at the RNA level. Hierarchical clustering and heat map analysis showed that one of the ampicillin-treated samples was clustered separately, but that clustering occurred first among the control group. PCA confirmed that each group was well clustered. Most of the ampicillin-treated *B. germanica* showed decreased levels of DNA; however, gene levels were either substantially increased or decreased at the RNA level. DEGs were enriched in biological, metabolic, and cellular processes. Differential expression of various genes from the two groups was also noted in developmental process and growth, as well as in cellular component, with several differences noted between the cells and the organelles. A significant difference was observed in the molecular functions related to catalytic activity and binding. Therefore, at the RNA level, gene expression may increase compared to the control group, instead of decrease with antibiotic treatment, with differential expression occurring in various functions.

Allergen data were obtained at the transcriptomic and proteomic levels, following analyses to confirm changes in the allergens according to these changes. Similar patterns were observed at both levels. There was no significant difference in Bla g 5, but its abundance tended to be lower in the ampicillin-treated group than in the control group. By contrast, Bla g 1 and Bla g 2 showed a significant decrease in abundance in the ampicillin-treated group compared to the control group.

The exact mechanism of allergen production in German cockroaches is unknown. However, clear differences in Bla g 1 production were observed based on the insect's level of starvation or its stage in the gonadotropic cycle [9]. Therefore, we expected to observe changes caused by several factors in the present study as well. Bacteria were removed by ampicillin treatment and likely included species that promoted the growth of cockroaches, accounting for the difference in total bacteria. The inhibition of bacterial growth may have affected allergen production, with *Blattabacterium* being probably the most influential member of the cockroach microbiota. This endosymbiont is

383 responsible for the nitrogen cycle and the production of essential amino acids and
384 vitamins in the German cockroach [25]. Moreover, in other insects, *Blattabacterium*
385 reduces the expression of genes involved in reproduction and growth inhibition. Similar
386 growth rates were observed in *R. pedestris* originally without the endosymbiont and in
387 those treated with antibiotics to remove the bacterium [6]. German cockroaches may
388 also experience changes in reproduction and growth due to the removal of
389 *Blattabacterium*. RNA-seq highlighted numerous changes in gene expression. Therefore,
390 we suggest that ampicillin may have influenced the production of allergens. Although
391 antibiotics primarily affect bacteria, they may also indirectly affect allergens through their
392 effects on bacteria.

393 A limitation of this experiment was that it was not possible to culture
394 *Blattabacterium* alone. If *Blattabacterium* in German cockroaches could be specifically
395 targeted, it would be possible to study only the effects of *Blattabacterium* while excluding
396 those of other bacteria.

397 In the present study, protein extraction from ampicillin-treated *B. germanica* was
398 optimized to obtain an extract containing a small amount of Bla g 2 compared to Bla g 1
399 and Bla g 5, with very few bacteria. Ampicillin treatment reduced total numbers of
400 bacteria associated with cockroaches. As a result, we suggest that reduced numbers of
401 bacteria may have influenced the production of allergens. Future studies should
402 investigate the effect of bacteria on patients undergoing immunotherapy using protein
403 extracts obtained from the German cockroach. In addition, methods to prepare protein
404 extracts with varying amounts of allergens should be optimized.

405

406 **Acknowledgments**:

407 This study was supported by a National Research Foundation of Korea (NRF) Grant
408 funded by the Korean Government (MEST; Numbers NRF-2019R1A2B5B01069843
409 and NRF-2020R1I1A2074562).

410

411 **Disclosure:** The authors declare no conflicts of interest.

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References

1. Guzman J, Vilcinskas A. Bacteria associated with cockroaches: health risk or biotechnological opportunity? Appl Microbiol Biotechnol 2020;104:10369-87. https://doi.org/10.1007/s00253-020-10973-6 PMID: 33128616

2. Kakumanu ML, Maritz JM, Carlton JM, Schal C. Overlapping Community Compositions of Gut and Fecal Microbiomes in Lab-Reared and Field-Collected German Cockroaches. Appl Environ Microbiol 2018;84:e01037-18. https://doi.org/10.1128/AEM.01037-18 PMID: 29959246

3. Pérez-Cobas AE, Maiques E, Angelova A, Carrasco P, Moya A, Latorre A. Diet shapes the gut microbiota of the omnivorous cockroach Blattella germanica. FEMS Microbiol Ecol 2015;91:fiv022. https://doi.org/10.1093/femsec/fiv022 PMID: 25764470

4 - Rosas T, García-Ferris C, Domínguez-Santos R, Llop P, Latorre A, Moya A. Rifampicin treatment of Blattella germanica evidences a fecal transmission route of their gut microbiota. FEMS Microbiol Ecol 2018;94:fiy002. https://doi.org/10.1093/femsec/fiy002 PMID: 29325007

5- Pietri JE,Tiffany C, Liang D. Disruption of the microbiota affects physiological and evolutionary aspects of insecticide resistance in the German cockroach, an important urban pest. PLoS One 2018;13:e0207985. https://doi.org/10.1371/journal.pone.0207985 PMID: 30540788

6- Lee JB, Park KE, Lee SA, Jang SH, Eo HJ, Jang HA et al. Gut symbiotic bacteria stimulate insect growth and egg production by modulating hexamerin and vitellogenin gene expression. Dev Comp Immunol 2017;69:12-22. https://doi.org/10.1016/j.dci.2016.11.019 PMID: 27932027

7. Guzman J, Vilcinskas A. Bacteria associated with cockroaches: health risk or biotechnological opportunity? Appl Microbiol Biotechnol 2020;104:10369-87. https://doi.org/10.1007/s00253-020-10973-6 PMID: 33128616

8. Do DC, Zhao Y, Gao P. Cockroach Allergen Exposure and Risk of Asthma. Allergy 2016;71:436-74. https://doi.org/10.1111/all.12827 PMID: 26706467

9. Gore JC, Schal C. Expression, production and excretion of Bla g 1, a major human allergen, in relation to food intake in the German cockroach, Blattella germanica. Med Vet Entomol 2005;19:127-34. https://doi.org/10.1111/j.0269-283X.2005.00550.x PMID: 15958021

10. Mueller GA, Pedersen LC, Lih FB, Glesner J, Moon AF, Chapman MD et al. The novel structure of the cockroach allergen Bla g 1 has implications for allergenicity and exposure assessment. J Allergy Clin Immunol 2013;132:1420-6. https://doi.org/10.1016/j.jaci.2013.06.014 PMID: 23915714

11. Arruda LK, Vailes LD, Mann BJ, Shannon J, Fox JW, Vedvick TS et al. Molecular Cloning of a Major Cockroach (Blattella germanica) Allergen, Bla g 2. Sequence homology to the aspartic proteases. J Biol Chem 1995;270:19563-8. https://doi.org/10.1074/jbc.270.33.19563 PMID: 7642642

12. Sohn MH, Kim KE. The Cockroach and Allergic Diseases. Allergy Asthma Immunol Res 2012;4:264-9. https://doi.org/10.4168/aair.2012.4.5.264 PMID: 22950031

13. Birrueta G, Frazier A, Pomés A, Glesner J, Filep S, Schal C et al. Variability in German Cockroach Extract Composition Greatly Impacts T Cell Potency in Cockroach-Allergic Donors. Front Immunol 2019;10:313. https://doi.org/10.3389/fimmu.2019.00313 PMID: 30891032

14. Glesner J, Filep S, Vailes LD, Wünschmann S, Chapman MD, Birrueta G et al. Allergen content in German cockroach extracts and sensitization profiles to a new expanded set of cockroach allergens determine in vitro extract potency for IgE reactivity. J Allergy Clin Immunol 2019;143:1474-81. https://doi.org/10.1016/j.jaci.2018.07.036 PMID: 30170124

15. Claudia SLV, Sota O, Koichi H. Composition of the Cockroach Gut Microbiome in the Presence of Parasitic Nematodes. Microbes Environ 2016;31:314-20. https://doi.org/10.1264/jsme2.ME16088 PMID: 27524304

16. Lee S, Kim JY, Yi MH, Lee IY, Fyumagwa R, Yong TS. Comparative microbiomes of ticks collected from a black rhino and its surrounding environment. Int J Parasitol Parasites Wildl2019;28:243-6. https://doi.org/10.1016/j.ijppaw.2019.05.008 PMID: 31198683

17. Myers EW, Miller W. Optimal alignments in linear space. Comput Appl Biosci 1988;4:11-7. https://doi.org/10.1093/bioinformatics/4.1.11 PMID: 3382986

18. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;11:403-10. https://doi.org/10.1016/S0022-2836(05)80360-2 PMID: 2231712

19. Lee J, Kim JY, Yi MH, Hwang Y, Lee IY, Nam SH et al. Comparative microbiome analysis of Dermatophagoides farinae, Dermatophagoides pteronyssinus, and Tyrophagus putrescentiae. J Allergy Clin Immunol 2019;143:1620-3. https://doi.org/10.1016/j.jaci.2018.10.062 PMID: 30528614

20. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods 2015;12:357-60. https://doi.org/10.1038/nmeth.3317 PMID: 25751142

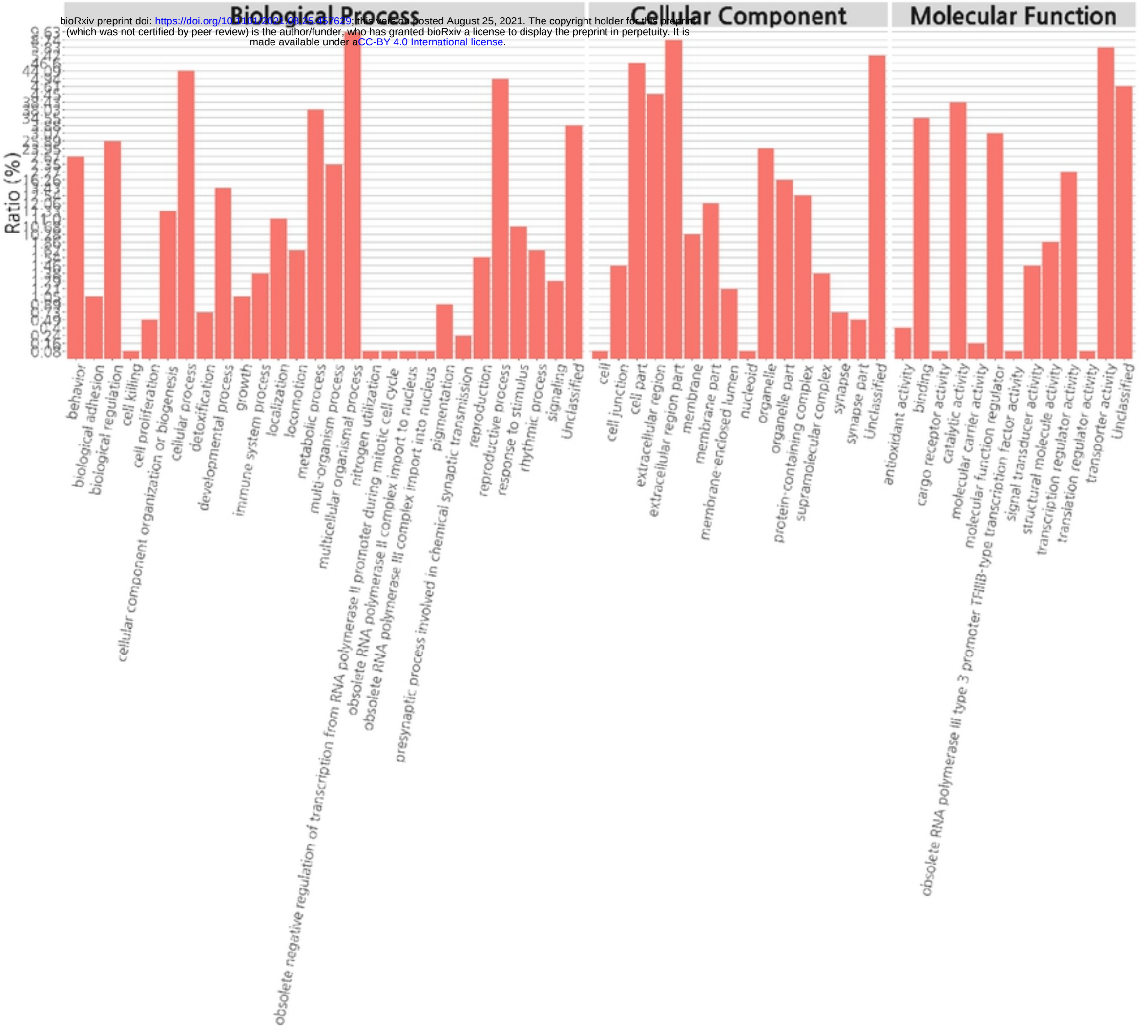
21. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol 2015;33:290-5. https://doi.org/10.1038/nbt.3122 PMID: 25690850

22. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc 2016;11:1650-67. https://doi.org/10.1038/nprot.2016.095 PMID: 27560171

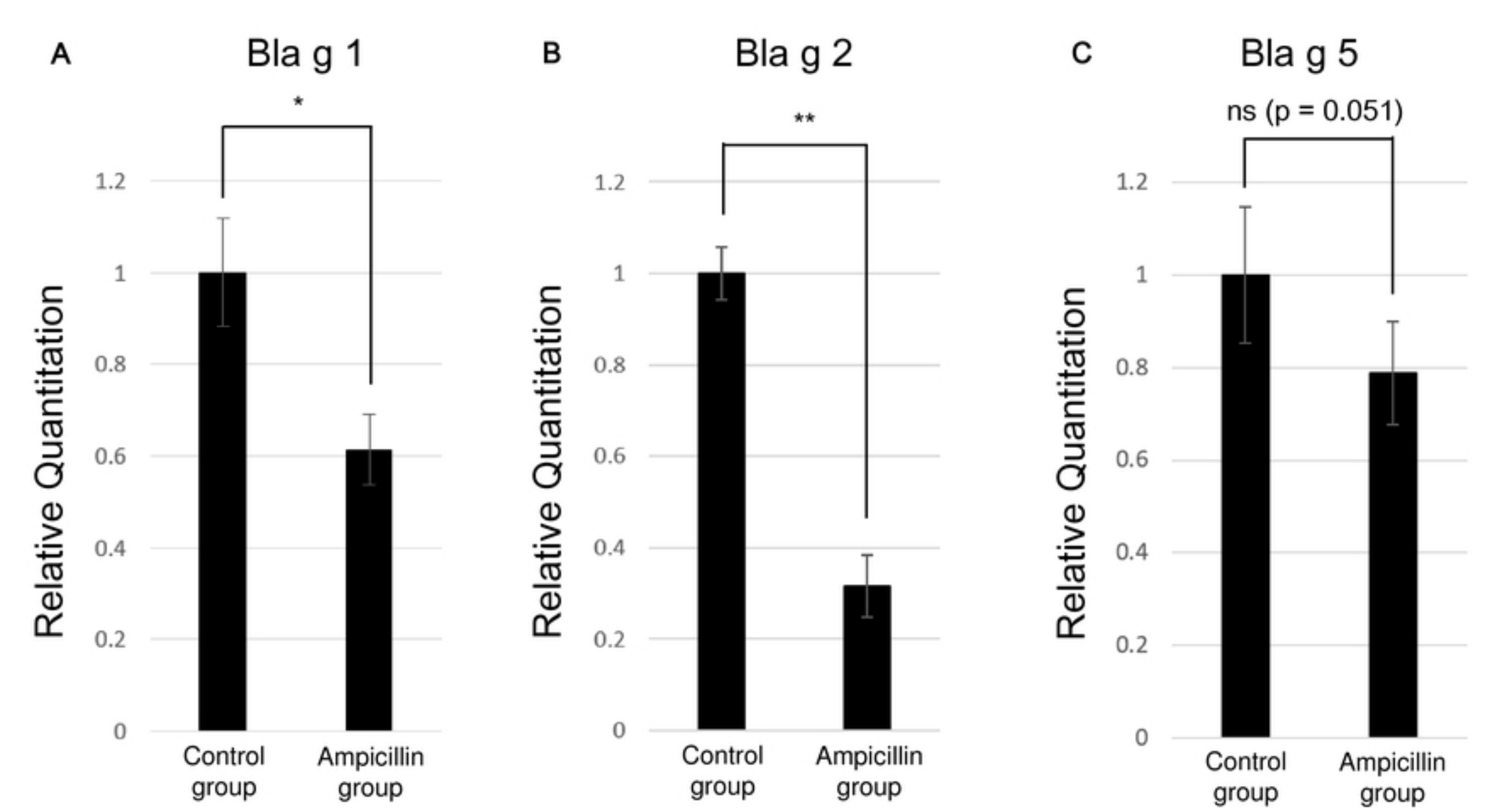
23. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010;26:139-40. https://doi.org/10.1093/bioinformatics/btp616 PMID: 19910308

24. Cayrou C, Raoult D, Drancourt M. Broad-spectrum antibiotic resistance of Planctomycetes organisms determined by Etest. J Antimicrob Chemother 2010;65:2119-22. https://doi.org/10.1093/jac/dkq290 PMID: 20699245

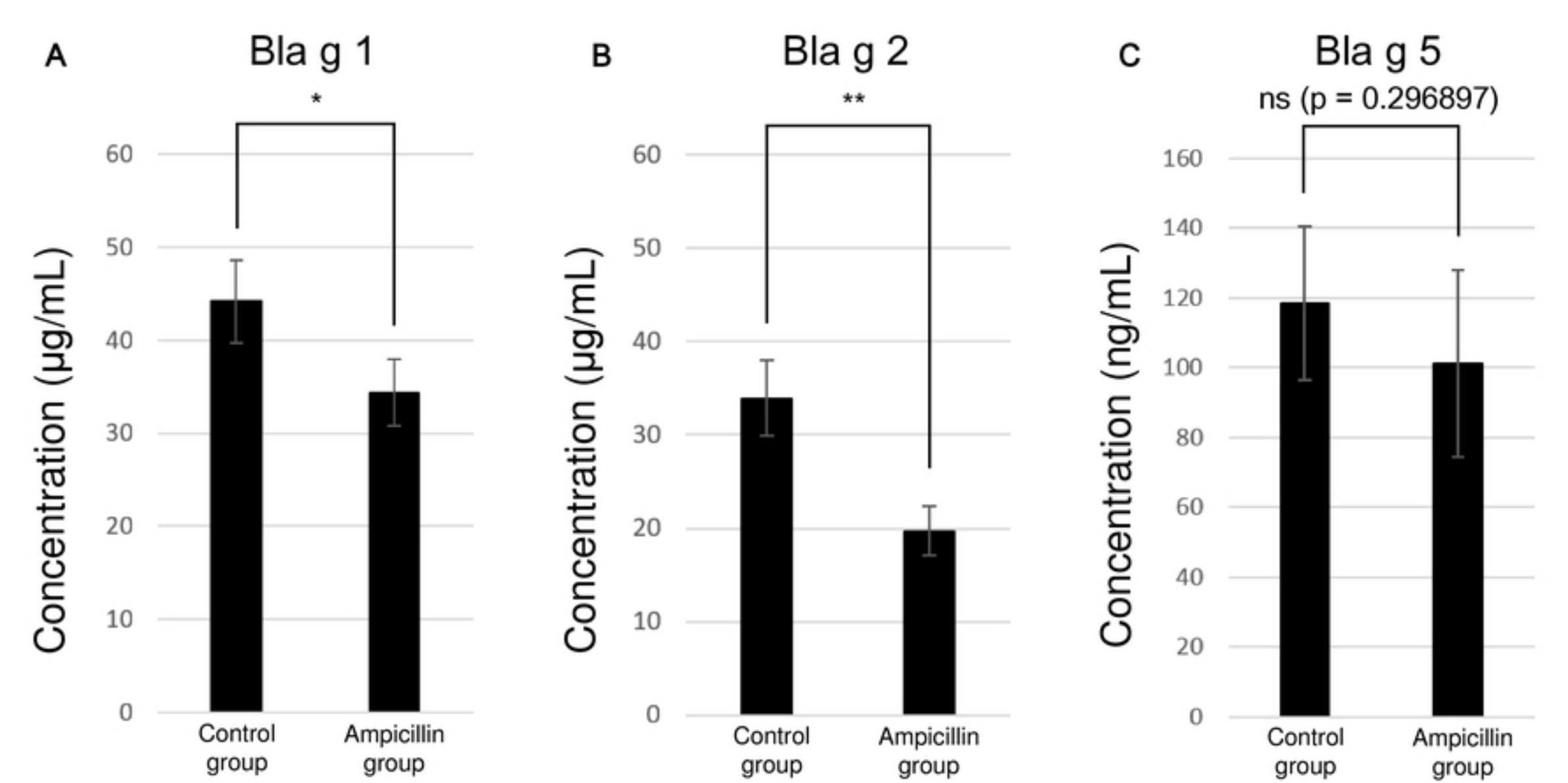
25. Sabree ZL, Kambhampati S, Moran NA. Nitrogen recycling and nutritional provisioning by Blattabacterium, the cockroach endosymbiont. Proc Natl Acad Sci U S A 2009;106:19521-6. https://doi.org/10.1073/pnas.0907504106 PMID: 19880743



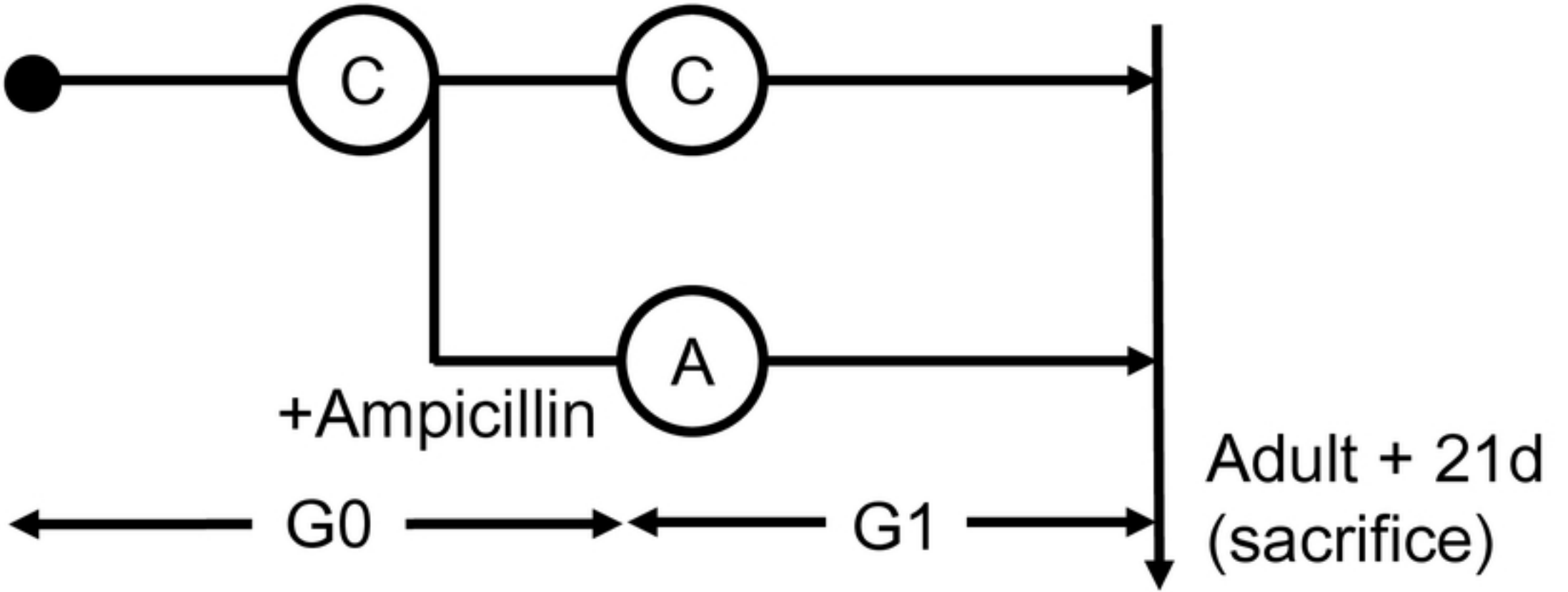
Figure


A
Bla g 1
*
Relative Quantitation
1.2
1
0.8
0.6
0.4
0.2
0
Control group
Ampicillin group
B
Bla g 2
**
Relative Quantitation
1.2
1
0.8
0.6
0.4
0.2
0
Control group
Ampicillin group
C
Bla g 5
ns (p = 0.051)
Relative Quantitation
1.2
1
0.8
0.6
0.4
0.2
0
Control group
Ampicillin group

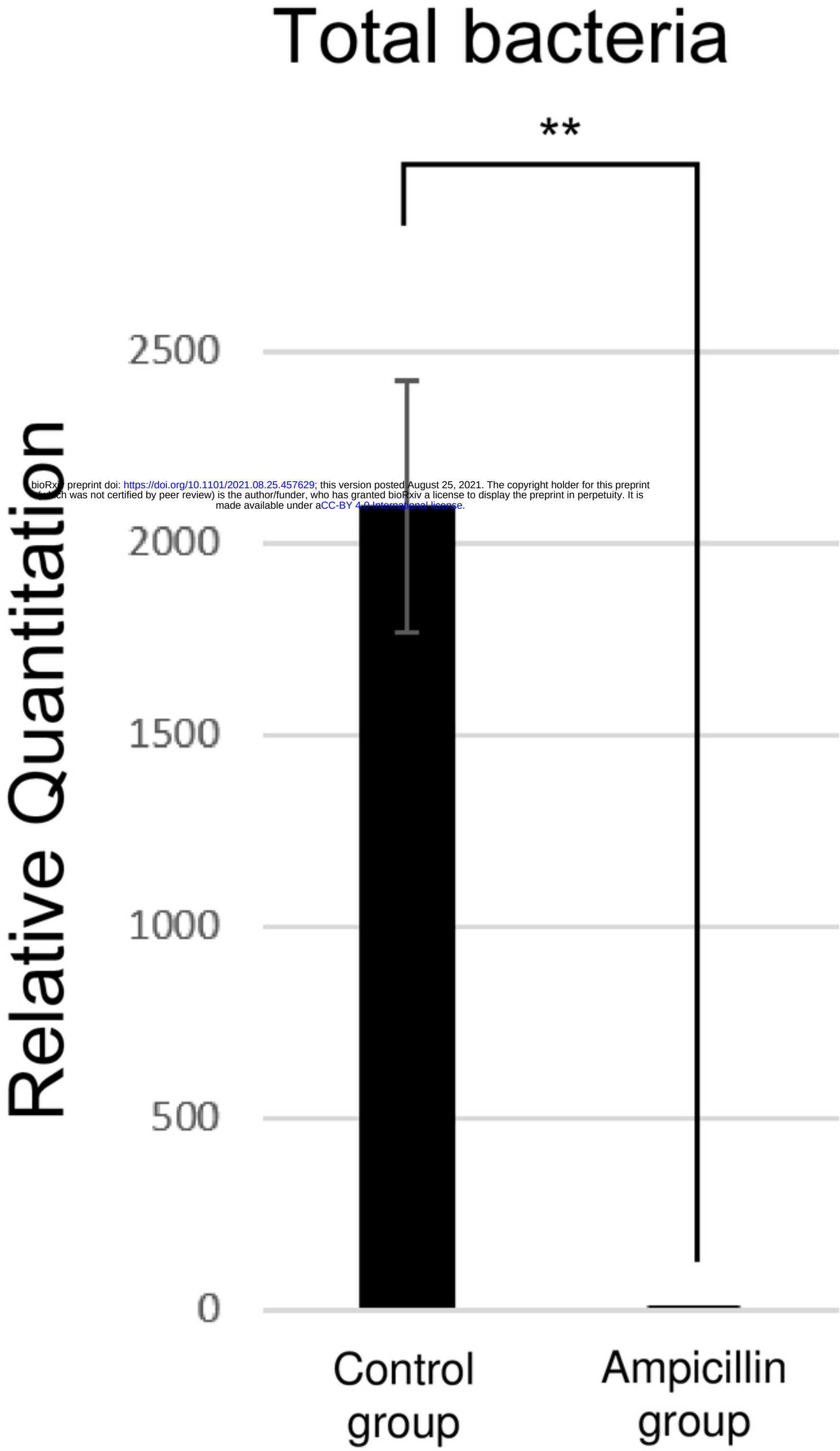
Figure


A
Bla g 1
*
Concentration (µg/mL)
60
50
40
30
20
10
0
Control group
Ampicillin group
B
Bla g 2
**
Concentration (µg/mL)
60
50
40
30
20
10
0
Control group
Ampicillin group
C
Bla g 5
ns (p = 0.296897)
Concentration (ng/mL)
160
140
120
100
80
60
40
20
0
Control group
Ampicillin group

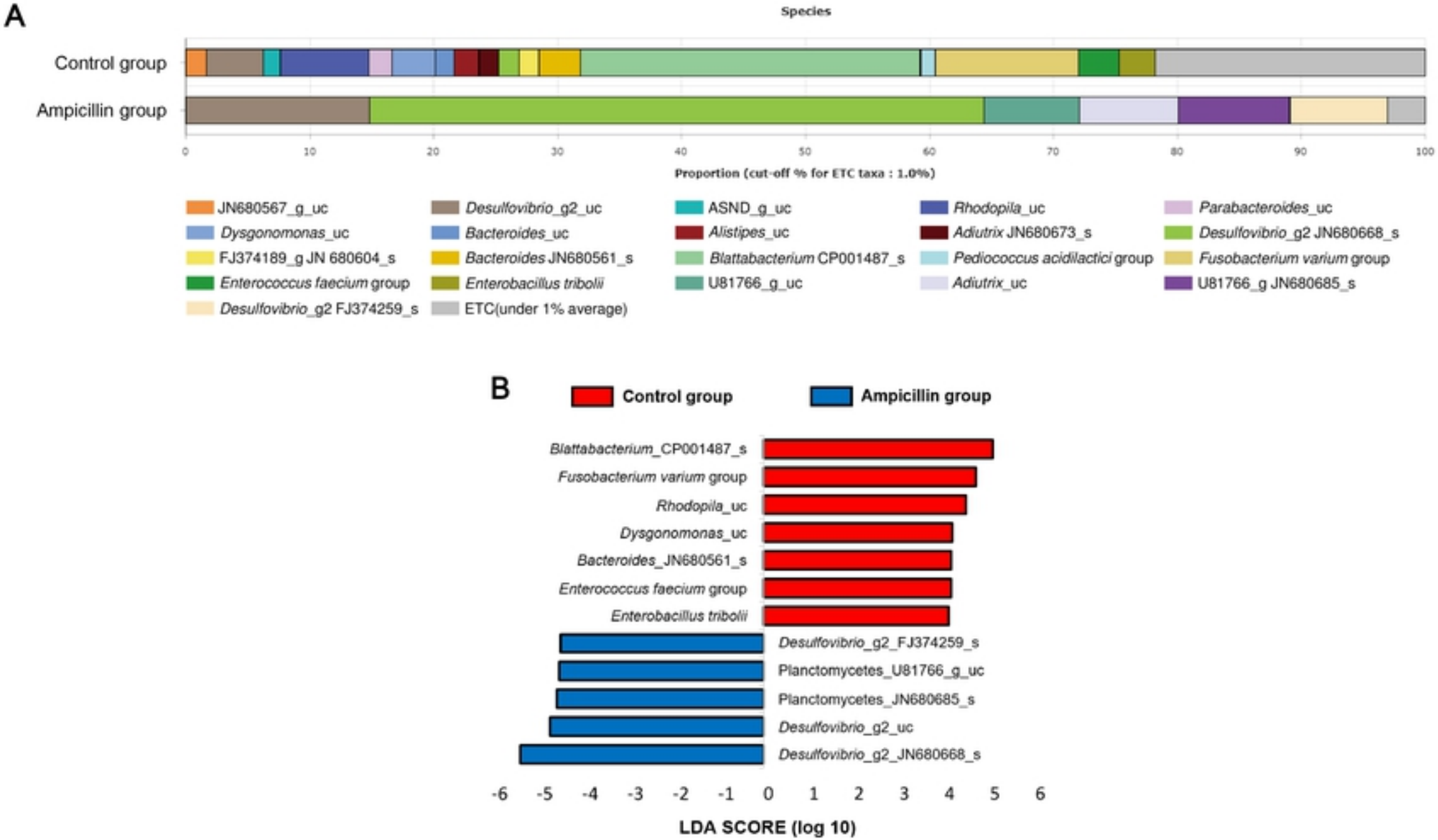
Figure



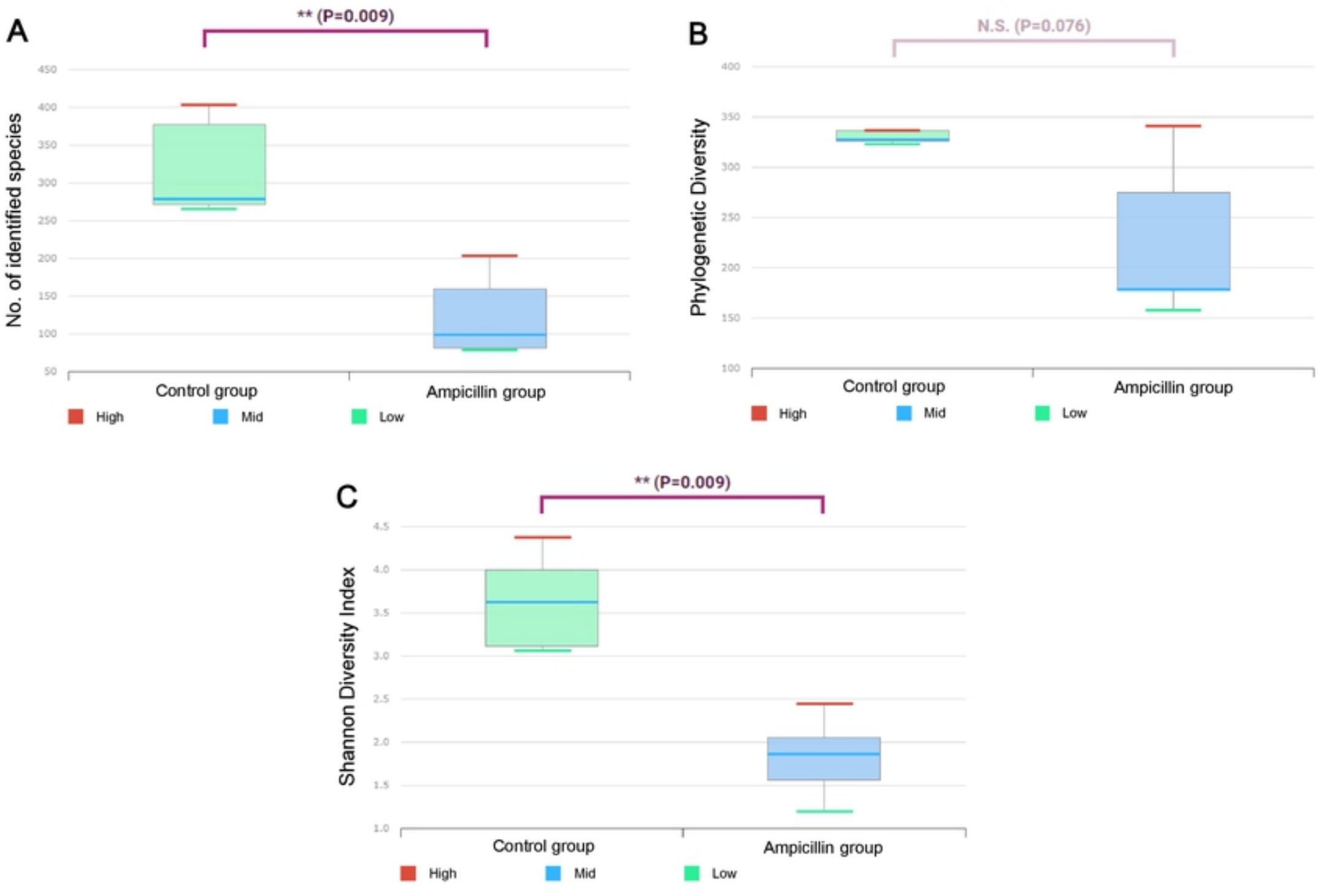
Figure



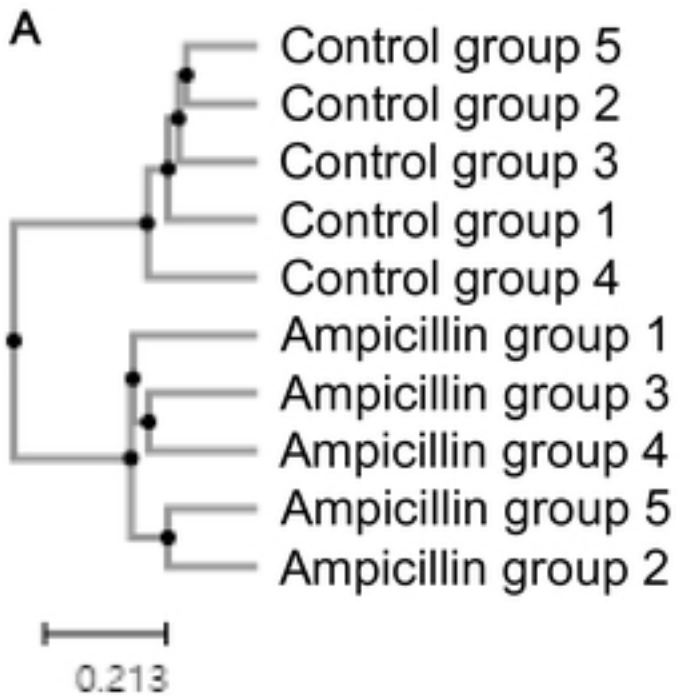
Figure

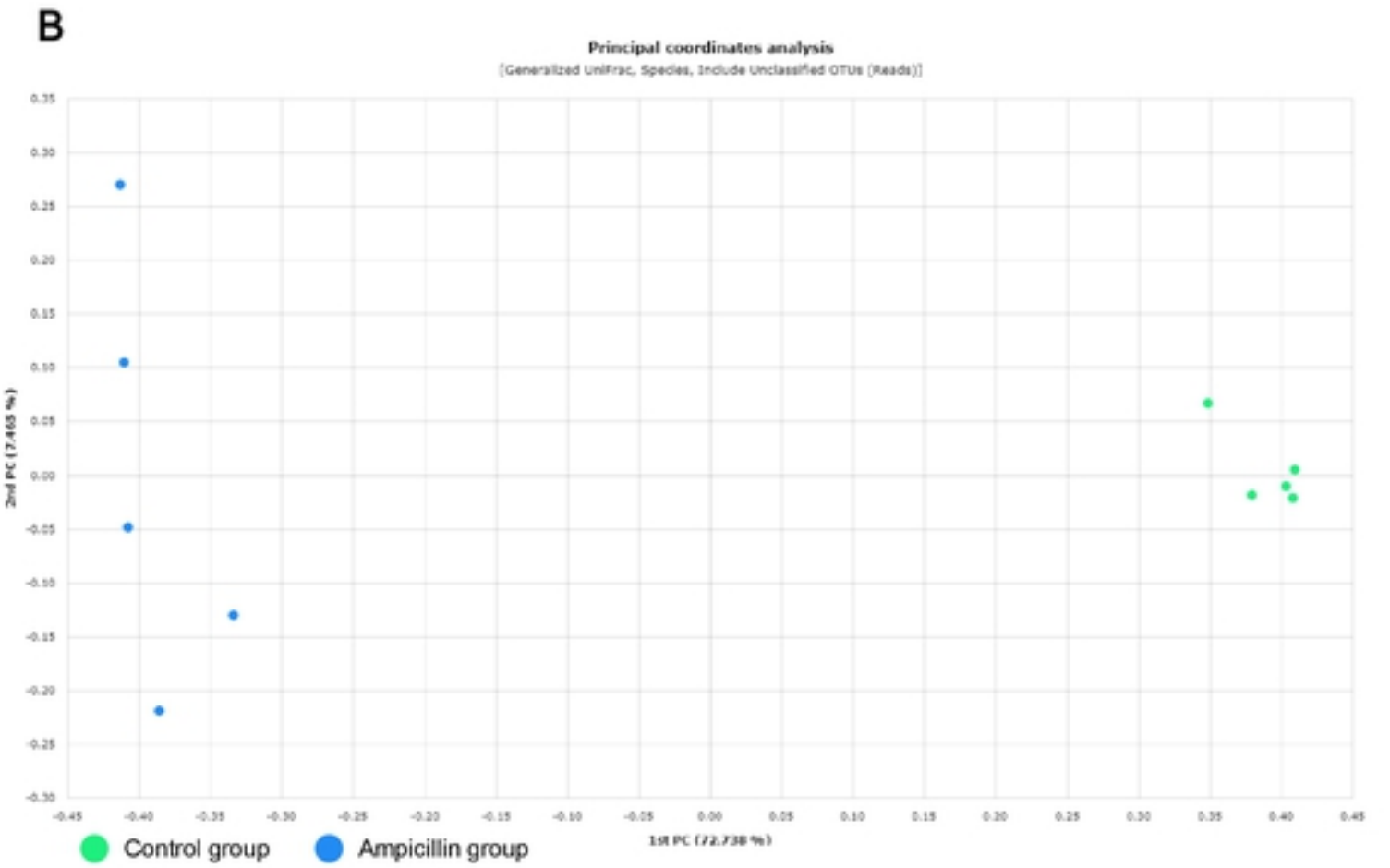

A
Species
Control group
Ampicillin group
0
10
20
30
40
50
60
70
80
90
100
Proportion (cut-off % for ETC taxa : 1.0%)
JN680567_g_uc
Desulfovibrio_g2_uc
ASND_g_uc
Rhodopila_uc
Parabacteroides_uc
Dysgonomonas_uc
Bacteroides_uc
Alistipes_uc
Adiutrix JN680673_s
Desulfovibrio_g2 JN680668_s
FJ374189_g JN 680604_s
Bacteroides JN680561_s
Blattabacterium CP001487_s
Pediococcus acidilactici group
Fusobacterium varium group
Enterococcus faecium group
Enterobacillus tribolii
U81766_g_uc
Adiutrix_uc
U81766_g JN680685_s
Desulfovibrio_g2 FJ374259_s
ETC(under 1% average)
B
Control group
Ampicillin group
Blattabacterium_CP001487_s
Fusobacterium varium group
Rhodopila_uc
Dysgonomonas_uc
Bacteroides_JN680561_s
Enterococcus faecium group
Enterobacillus tribolii
Desulfovibrio_g2_FJ374259_s
Planctomycetes_U81766_g_uc
Planctomycetes_JN680685_s
Desulfovibrio_g2_uc
Desulfovibrio_g2_JN680668_s
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
LDA SCORE (log 10)

Figure

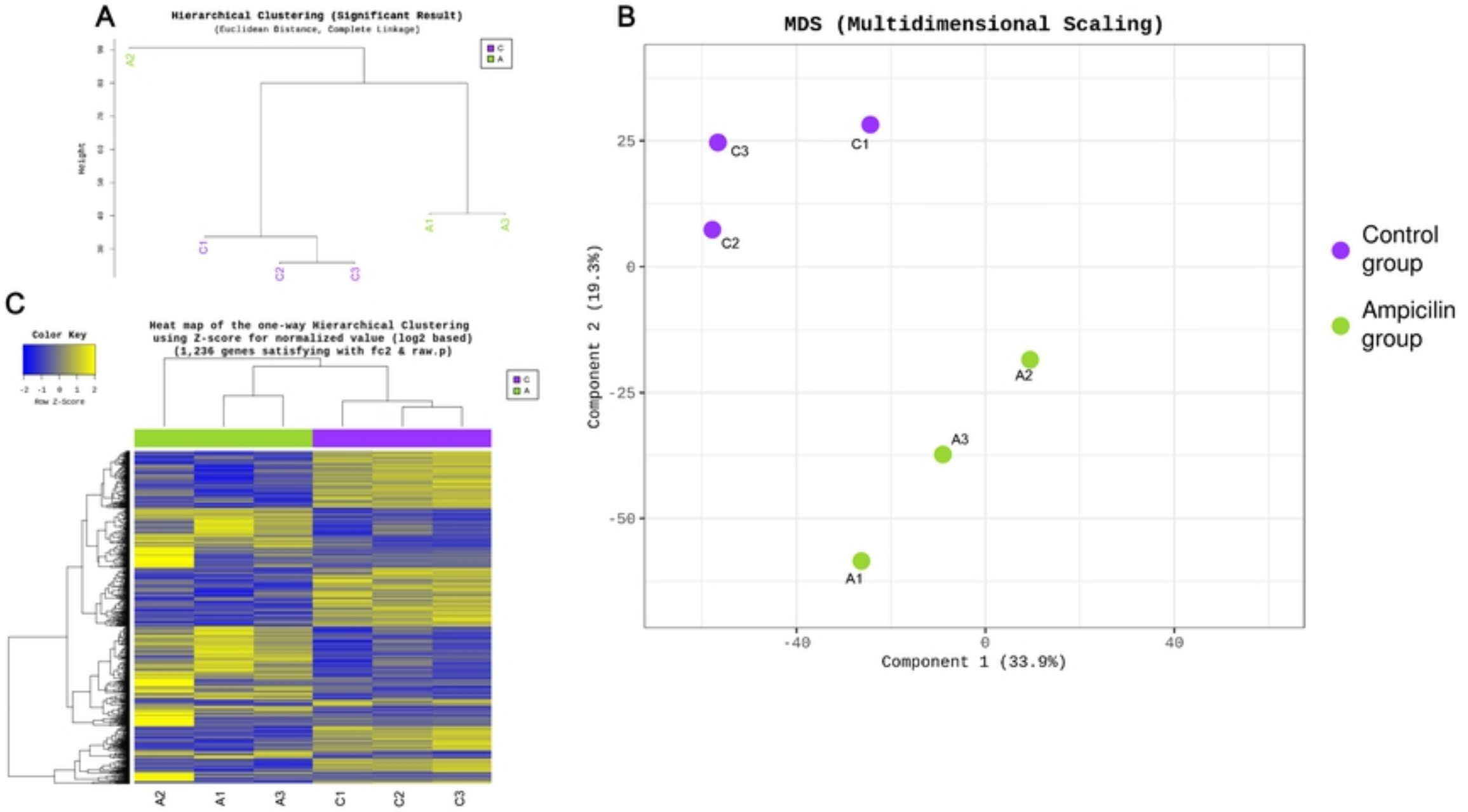

A
** (P=0.009)
No. of identified species
450
400
350
300
250
200
150
100
50
Control group
Ampicillin group
High
Mid
Low
B
N.S. (P=0.076)
Phylogenetic Diversity
400
350
300
250
200
150
100
Control group
Ampicillin group
High
Mid
Low
C
** (P=0.009)
Shannon Diversity Index
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Control group
Ampicillin group
High
Mid
Low

Figure


A
Control group 5
Control group 2
Control group 3
Control group 1
Control group 4
Ampicillin group 1
Ampicillin group 3
Ampicillin group 4
Ampicillin group 5
Ampicillin group 2
0.213


B
Principal coordinates analysis
[Generalized UniFrac, Species, Include Unclassified OTUs (Reads)]
2nd PC (7.465 %)
1st PC (72.738 %)
Control group
Ampicillin group

Figure


A
Hierarchical Clustering (Significant Result)
(Euclidean Distance, Complete Linkage)
Height
A2
C1
C2
C3
A1
A3
C
A
B
MDS (Multidimensional Scaling)
Component 2 (19.3%)
Component 1 (33.9%)
25
0
-25
-50
-40
0
40
C3
C1
C2
A2
A3
A1
Control group
Ampicilin group
C
Color Key
-2 -1 0 1 2
Row Z-Score
Heat map of the one-way Hierarchical Clustering
using Z-score for normalized value (log2 based)
(1,236 genes satisfying with fc2 & raw.p)
A2
A1
A3
C1
C2
C3

Figure