Investigation of Systemic Granulomatosis in cultured meagre, *Argyrosomus regius*, using clinical metagenomics

Christina Pavloudi[1,2], Maria Ioanna Tsertou[1,3], Efthimia Antonopoulou[3], Pantelis Katharios[1]

[1] Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Heraklion, 71500 Crete, Greece

[2] Department of Biological Sciences, The George Washington University, 2029 G St NW, Bell Hall 302, Washington DC, 20052, USA

[3] Laboratory of Animal Physiology, Department of Zoology, School of Biology, Faculty of Science, Aristotle University of Thessaloniki, Thessaloniki, Greece

Corresponding author: Pantelis Katharios

e-mail: katharios@hcmr.gr

phone number: +30 2810 337760

ABSTRACT

Systemic granulomatosis is a peculiar disease of unknown etiology affecting meagre, *Argyrosomus regius*, an important aquaculture fish species. Several pathogenic microorganisms have been suggested as causative agents. In this study, we have applied amplicon metagenomics of the 16S rRNA gene in kidneys of fish exhibiting different health states, ranging from apparently healthy to kidneys with calcification. Comparison of the kidney microbiomes of the different fish groups showed that all fish had similar bacterial communities. Linear discriminant analysis effect size revealed only three OTUs

significantly enriched in the sick individuals, with no known granuloma-causing species being present among them, reinforcing the hypothesis of a non-infectious cause of the disease in this fish species.

Keywords: 16S rRNA amplicon sequencing, metagenomics, granuloma, meagre, microbiome, lesion

Highlights

- No known granuloma-causing species identified in calcified meagre kidneys
- Non-infectious cause of Systemic Granulomatosis in *Argyrosomus regius*
- Potential for clinical metagenomics to identify pathogens in fish

1. INTRODUCTION

Meagre, *Argyrosomus regius*, is one of the most promising fish species for the expansion and diversification of the Mediterranean aquaculture since it is a fast grower especially in comparison to gilthead seabream and European seabass, the two species which currently dominate marine aquaculture production in the Mediterranean, and it has excellent flesh quality widely appreciated by the consumers (Duncan et al., 2013). Meagre is very well adapted in the rearing process and is very resilient to infectious diseases. However, this species is affected by a condition known as Systemic Granulomatosis (SG) that seems to affect almost all farmed populations throughout the Mediterranean (Ghittino et al., 2004; Katharios et al., 2011; Manuali et al., 2005). It is a disease of unknown etiology manifested by the presence of multiple granulomas in all soft tissues. The most affected organ is the kidney, however in severe cases granulomas can be found literally everywhere including the brain and the eyes. As the disease progresses, granulomas may become necrotic and calcified rendering the fish unacceptable for the consumer. The disease is not directly linked to mortalities, however affected

organs eventually contribute to reduced rearing performance of the fish. The severity of granulomatosis varies between years and rearing locations (Katharios, personal observations).

A granuloma is an organized collection of inflammatory cells. Typically, the granuloma consists of macrophages which are tightly arranged to wall-off the causative agent from the healthy tissue. These macrophages are also called histiocytes, or epithelioid cells due to their morphological resemblance to epithelial cells. Granulomas can have both infectious and non-infectious etiology (Ramakrishnan, 2012; Shah et al., 2017). Relevant to human pathology, the most extensively studied are the infectious granulomas especially those caused by Mycobacteria, while non-infectious granulomas are present in diseases which are considered metabolic, autoimmune or of unknown mechanism like Crohn's disease, sarcoidosis, and Granulomatosis with Polyangiitis (formerly Wegener's granulomatosis) and because of the absence of an adequate animal model, are difficult to study. One of the criteria that often leads pathologists to assume an infectious origin of a granuloma is the presence of necrosis (Aubry, 2012). This is because necrosis is induced by the toxicity of the invoking agent (usually pathogenic bacteria) leading to macrophage death (Ramakrishnan, 2012), whereas in foreign-body granulomas, sarcoidosis and Crohn's disease there is no necrotic area inside the granuloma. An exception to this rule is Granulomatosis with Polyangiitis, a rare non-infectious systemic disorder characterized by vascular inflammation leading to necrotizing granulomas (Lutalo and D'Cruz, 2014). However, even in the case of granulomatous diseases once considered to be of non-infectious etiology like Whipple's disease and cat scratch disease, recent developments have revealed an infectious causative agent, the bacteria *Tropheryma whipplei* for the former and *Bartonella henselae* for the latter (Ramakrishnan, 2012).

Metagenomic Next Generation Sequencing (mNGS) has revolutionized microbial ecology over the past two decades since being a culture-independent technique it can be used to reveal the true microbial diversity otherwise missed when using conventional cultivation methods (Handelsman, 2004). Metagenomics have been extensively used in environmental microbiology, biotechnology and have

become the gold standard for gut microbiome research (Wang et al., 2015). The use of metagenomics in clinical practice, especially for identifying pathogens within patient's samples, is an emerging and highly promising application (Brown et al., 2018; Chiu and Miller, 2019; Huang et al., 2020). It is particularly relevant in cases where the pathogen is not cultivable, "protected" in lesions surrounded by fibrous tissue, and not readily visible in histopathology. It has also been applied in several studies assessing the gut microbiome of cultured and wild fish (e.g. Burtseva et al., 2021; Yukgehnaish et al., 2020).

Both infectious and non-infectious etiologies have been hypothesized regarding the systemic granulomatosis affecting meagre. Initially it was linked to fungal infection (Manuali et al., 2005) and later to infection caused by *Nocardia* sp. (Elkesh et al., 2013). The latest research points rather to a nutritional imbalance (Katharios et al., 2011; Kotzamanis et al., 2018; Ruiz García et al., 2019; Ruiz et al., 2019; Tsertou et al., 2020). We have recently challenged the "infectious" etiology of the disease (Tsertou et al., 2018), which is fueled by incidental findings of granuloma-inducing bacterial pathogens like *Nocardia* sp. (Elkesh et al., 2013; Tsertou et al., 2018) and *Mycobacterium marinum* (Avsever et al., 2014; Timur et al., 2015) in fish cultured in the open sea. Nevertheless, the histopathological picture of the disease with the necrosis centrally located in meagre's granuloma cannot exclude entirely the possibility of a "cryptic" pathogen which is both invisible in the histological sections and not cultivable. Therefore, the aim of this study was to use metagenomic Next Generation Sequencing directly in DNA extracted from kidney samples of meagre at three health states (a) healthy with no visible granulomas, (b) SG-affected and (c) kidneys with calcification, in order to investigate the presence of pathogens known to cause granulomas and to compare the microbiome in these three states. To our knowledge, this is the second clinical application of mNGS in fish lesions as a tool for disease diagnosis in aquaculture.

2. MATERIALS AND METHODS

2.1 Fish samples

All experimental procedures and handling that include the use of animals were conducted at the Hellenic Center of Marine Research (HCMR) licensed facility (EL91-BIOexp-04) under the protocol 255.325 approved by the regional veterinary authority, which is the competent agency according to the Directive 2010/63/EU. The fish used in this study were obtained by the meagre broodstock maintained at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC), HCMR, Crete, Greece and they were exclusively reared in tanks in inland facilities using borehole seawater (salinity: 35‰, pH: 7.5, T: 19.0 ± 1.0°C). In total, 27 fish with average body weight 28.90 ± 9.17 g and average total length 13.72 ± 1.40 cm were euthanized with an overdose of anesthetic (Benzoak® Vet). Following dissection, internal organs were visually examined. Samples from kidneys were removed, snap-frozen in liquid nitrogen and stored at -80°C until analysis. In addition, fresh squash preparations of each sample were examined and evaluated under a microscope for the presence of granulomas. For the visual evaluation of granulomas, a modification of a semi-quantitative ordinal-scale scoring system was used, which is described in detail in Tsertou et al. (2020). The evaluation of the kidney samples revealed 7 samples that did not have granulomas (health state: healthy/healthy with no visible granulomas, samples with prefix “O”), 10 samples with granulomas visible macroscopically or microscopically (health state: sick/SG-affected, samples with prefix “KOK”) and 10 samples with tissue calcification (health state: sick/kidneys with calcification, samples with prefix “ASB”).

2.2 DNA extraction, PCR amplification and 16S rRNA sequencing

DNA was extracted from the kidney samples using the NucleoSpin Tissue kit (MACHEREY‑NAGEL), as recommended by the manufacturer. The quality of the extracted DNA was evaluated by gel electrophoresis.

PCR amplification was performed targeting the V3–V4 region of the 16S rRNA gene using the bacterial primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′) (Herlemann et al., 2011; Klindworth et al., 2013) and the revised 805RB (5′-GACTACNVGGGTATCTAATCC-3′) (Apprill et al., 2015; Pavloudi et al., 2017). The Two-Step PCR Approach was used for this study, as described in Pavloudi et al. (2017). Briefly, the first-step PCR was performed with the aforementioned primers containing a universal 5′ tail as specified in the Nextera library protocol from Illumina. The resulting PCR amplicons (~531 bp) were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA), quantified and used as templates for the second-step PCR in order to include the indexes (barcodes), as well as the Illumina adaptors. Amplifications were carried out using T100 Thermal Cycler (BIORAD, Hercules, CA, USA). Again, the resulting PCR amplicons (~600 bp) were purified and quantified, mixed in equimolar amounts and sequenced using a MiSeq Reagent Kit v3 (2 × 300-cycles) at the IMBBC (HCMR). The PCR negative control sample (blank) was also sequenced, so that possible contamination during the library preparation could be assessed.

All the raw sequence files of this study were submitted to the European Nucleotide Archive (ENA) (Cummins et al., 2022) with the study accession number PRJEB43864 (available at http://www.ebi.ac.uk/ena/data/view/PRJEB43864).

2.3 Bioinformatics and Statistical analysis

Raw sequence reads retrieved from all the samples were processed using PEMA (version 2.1.4) (Zafeiropoulos et al., 2020) using VSEARCH for the assignation of OTUs (97% cut off) (Rognes et al., 2016). The detailed parameters of the PEMA processing are given in Table S1. Taxonomic assignment was performed with the SILVA database (version 132) (Quast et al., 2013). PEMA analyses were supported by the IMBBC High Performance Computing system (Zafeiropoulos et al., 2021).

Similarity matrices between samples were generated using the Bray–Curtis coefficient, calculated based on the relative abundances of the OTUs, and the Jaccard coefficient, calculated based on the presence and absence of OTUs at each sample. Afterwards, non-metric multidimensional scaling (nMDS) was used to visualize the relationship between the samples. An upset plot was created, as an alternative to the Venn Diagram, to illustrate all the possible logical relations between the three health states. LEfSe was used to estimate which microbiome attributes differ significantly by health state.

The phyloseq (version 1.36) (McMurdie and Holmes, 2013), microbiomeMarker (version 1.0.2) (Cao, 2022), ggplot2 (version 3.3.5) (Wickham, 2016) and ComplexUpset (version 1.3.3) (Krassowski, 2020; Lex et al., 2014) packages were used in R (version 4.1.1) (R Core Team, 2021) for the creation of bar charts, for the nMDS and PERMANOVA, simper, upset plot and for the linear discriminant analysis (LDA) effect size (LEfSe).

In addition, analysis was carried out using Geneious software (v9.1, Biomatters, Auckland, Australia) following the metagenomic analysis workflow for each state of granulomas (healthy, granulomas, calcification). Briefly, reads were paired, trimmed with the BBDuk plugin to remove the Illumina adapters, any base below an average quality score of 30 from the ends and reads that are less 100bp after end-trimming Paired end reads were then merged to produce a consensus sequence for each pair with chimeric reads being removed. A high stringency de-novo assembly was then carried out with a minimum overlap identity of 98% in order to cluster the contigs into OTU's and then BLAST searched against a local copy of the NCBI 16S microbial database. These were then used to set up a local database for each group which was analyzed using the Geneious Sequence Classifier plugin. The minimum overlap identity for the lowest taxonomic level (species level) was 97%. Data outputs from these tools were then used for comparative analysis.

3. RESULTS

3.1 Microbial community composition as retrieved by PEMA

The results of the processing of the sequences using PEMA are shown in Table S2. Due to a low number of reads, sample ASB9_OO10_3 was removed from the further analysis of the PEMA results. The final number of OTUs, after removal of the OTUs that were also found on the blank sample, was 462. Overall, the most abundant phyla were Proteobacteria (~40% on the average of all the samples), followed by Firmicutes (~29%) and Bacteroidetes (~22%) (Figure 1).

Although there seem to be differences in the microbial communities between the different health states, the differences are much less pronounced when the presence/absence of the OTUs is considered (Figure 2).

The nMDS of the microbial OTUs showed that there is no difference between the sick ("SG_affected" and "kidneys with calcification") and the healthy individuals, both when the relative abundances were used (Figure 3) as well as when they were constructed based on presence/absence of the OTUs (Figure 4); this was also confirmed by the PERMANOVA results (F.Model = 1.3567, $p = 0.203$; F.Model = 1.0661, $p = 0.302$, respectively).

However, there was a statistically significant grouping based on the condition of the individuals, i.e., healthy, SG-affected and samples with tissue calcification (relative abundances: F.Model = 2.0155, $p < 0.05$; Presence/Absence: F.Model = 1.4098, $p < 0.05$). The OTUs that contribute most to this grouping, as identified by the simper analysis, their taxonomy, as well as the significance of any given OTU's contribution, are shown in Table S3. Interestingly, there was no difference between the healthy and the SG affected OTUs, based on the relative abundance of the OTUs.

As shown in the upset plot (Figure 5), the three conditions share the majority of OTUs (178 OTUs). There are 59 OTUs that are shared only between the SG affected and the samples with kidney calcification and 16 OTUs that are shared only between the healthy samples and those with kidney calcification (Table 1).

3.2 LEfSe analysis and LDA

The LefSe analysis identified 12 OTUs with significant abundance differences between the three groups (Figure 6, Figure S1, Table 2); out of those OTUs, only 2 were significantly enriched in the calcified samples, namely Otu124 and Otu239. When the LefSe analysis was performed using two groups, i.e., healthy and sick, there were only 2 OTUs enriched in the sick samples: Otu130, Otu185 and Otu229 (Figures S2-S3, Table 1).

As shown in Figure 7, the healthy samples and the SG affected samples were very similar, while certain OTUs from the list of the 12, were lost in the calcified kidneys. This loss of OTUs is even more pronounced when the healthy and sick samples are compared (Figure S4).

3.3 Microbial community composition as retrieved by Geneious

The metagenomic analysis from the Geneious software showed that the phyla with the most reads were Bacteroidetes (50.1% for the healthy kidneys, 39.4% for the kidneys with granulomas and 63.3% for the calcified kidneys), Proteobacteria (30.4% for the healthy, 30.9% for the kidneys with the granulomas and 31.9% for the kidneys with calcification) and Firmicutes (13.4% for the healthy, 26.2% for the kidneys with granulomas and 1.7% for the calcified kidneys). The total reads per phylum and their corresponding prevalence for each of the health state of the kidneys are presented in Table 3.

In the phylum Bacteroidetes, the classes with the most reads for all the health states of kidneys were Flavobacteriia (76.3% for healthy samples, 75.2% for samples with granulomas and 78% for calcified samples) with most abundant species *Myroides ceti*, followed by Sphingobacteriia (22%, 22.2% and 20.1% for healthy kidneys, kidneys with granulomas and calcified kidneys, respectively) with *Pedobacter nutrimenti* as the most abundant species.

Accordingly, in the phylum of Proteobacteria, the class of Gammaproteobacteria had the most reads in all three groups (49% in healthy kidneys, 42.3% in kidneys with granulomas and 45% in calcified kidneys) followed by the Alphaproteobacteria (38.9% in healthy, 42.2% in kidneys with granulomas and 43.0% in kidneys with calcification). The main representatives of Gammaproteobacteria in the healthy samples were the species *Pseudomonas fildesensis* and *P. miguale*, in the samples with granulomas were the species *P. fildesensis* and *P. lini* and in the samples with calcification the species *P. veronii* and *P. migulae*. Regarding Alphaproteobacteria the main representative in all groups was *Brevudimonas albigilva.*

Finally in the phylum Firmicutes the class with the most reads in all groups was Bacilli (99.1% in healthy kidneys, 99.6% in kidneys with granulomas, 95.7% in calcified kidneys) with the main representative the species *Anoxybacillus flavithermus* subsp. *yunnanensis.* The taxa with the most reads per health state of the kidneys are presented in Table 4. It should be stated that the identification of the taxa to species level is based on an approximately 600bp fragment of the 16S and therefore the resolution for certain genera might not be ideal.

The results obtained using Geneious are in accordance with the results of PEMA processing, since there is no pathogen nor any unknown bacterium lacking from the healthy tissues while being highly abundant in the SG-affected and calcified tissues.

4. DISCUSSION

Due to the similarities between the healthy and the SG affected samples, it can be suggested that there is no apparent bacterial species that drives the onset of the systemic granulomatosis. The principal bacterial species that have been identified as agents causing chronic and necrotizing granulomas in fish and shellfish belong to the genera *Francisella* (Birkbeck et al., 2011), *Mycobacterium* (Jacobs et al., 2009), *Nocardia* (Martínez-Lara et al., 2021), which were not found in our samples.

Pathogens of the last two genera like *Mycobacterium marinum* and *Nocardia seriolae* have been associated with granulomatous diseases in meagre from Greece and Turkey (Avsever et al., 2014; Elkesh et al., 2013; Timur et al., 2015; Tsertou et al., 2018). It should be noted however, that chronic infections by these two pathogens are not uncommon in fish cultured in sea cages especially in fish that remain in the cages for long (more than one year). The fish examined in the current study were young, continuously grown in inland facilities provided with borehole water and never exposed to natural seawater. Furthermore, previous extensive investigation in several stocks of meagre grown in our facility using histology with special stains like Ziehl-Neelsen were always negative for these two pathogens which are common suspects of granulomatous lesions verifying the results of this study. In addition, apart from the aforementioned pathogens, *Edwarsiella tarda* (Miyazaki and Kaige, 1985), *Lactococcus garvieae* (Castro et al., 2019) and *Streptococcus iniae* (Ortega et al., 2018) have also been identified as etiological agents of granulomatosis, which were also not found in our samples. There was one OTU classified as *Lactococcus* (Otu111) and three OTUs classified as *Streptococcus* (Otu36, Otu56, Otu287). However, out of those only Otu56 was identified by the LefSe as a discriminating OTU, it was enriched in the healthy samples and lost in the samples with granulomatosis; therefore, it cannot be the agent causing the disease.

Several species of the genus *Pseudomonas* have been identified as pathological agents causing granulomas in fish such as *P. plecoglossicida* (Sun et al., 2020), *P. putida* (Urku, 2021), *P. stutzeri* and *P. oleovorans/pseudoalcaligenes* (Emam et al., 2022). There were 4 OTUs in our study that belonged to the genus *Pseudomonas* (Otu3, Otu7, Otu294, Otu397) and there were found in all the three conditions. In addition, none of them was included in the results of the LefSe analysis and none of them was identified as one of the aforementioned species when blasted against the NCBI database, further corroborating the fact that they were not responsible for the granulomatosis. On the other hand, further analysis with Geneious showed that *Pseudomonas fildesensis* was present in both healthy and SG-affected fish but

almost absent in fish with calcification, while *Pseudomonas lini* was present in SG-affected fish with very few reads obtained from the fish of the other groups and *Pseudomonas veronii* present only in fish with calcification and completely absent from the other two groups. The genus of *Pseudomonas* contains 259 valid species and continues to expand with the addition of novel species every year (Girard et al., 2021). The distinction of *Pseudomonas* species using 16S rRNA is rather challenging, especially for species which are phylogenetically close like, *P. fildesensis* and *P. veronii* (Pavlov et al., 2020). Nevertheless, it should be noted that none of the presumptively identified species have been previously reported as pathogens nor are phylogenetically close to known pathogenic species of the genus capable of causing granulomas like *P. plecoglocissida*. Until now, no pathological agents have been found to be responsible for non-infectious systemic granulomatosis in meagre (Iaria et al., 2019). This is also suggested from the results of our study, which further support the hypothesis of a non-infectious cause of this peculiar fish disease (Tsertou et al., 2018).

Several studies have employed amplicon metagenomics of the 16S rRNA gene, as well as shotgun metagenomics, for the assessment of microbial communities in skin mucus, stomach (Nurul et al., 2019) and gut (Tyagi et al., 2019; Xing et al., 2013) of fish. The application of metagenomic analyses has been increasingly applied in clinical microbiology the last years as a diagnostic tool for various infectious diseases (Chiu and Miller, 2019; Forbes et al., 2018). Traditional microbiology techniques applied to date are based on culturing the pathogen in the laboratory and identifying it through PCR analyses using specific primers. Although these techniques are time-consuming, they are useful in case the culture conditions, the sensitivity of the assays and the primers used are compatible and suitable for the target microbe (Takhampunya et al., 2019). The fact that a large number of pathogens cannot be isolated and cultured in the laboratory leads in many cases to the lack of identification of the causative agent which is responsible for disease outbreaks, thus the development and use of techniques that are

283 independent of the culture of microorganisms is proving valuable, particularly for emerging pathogens
284 (Afshinnekoo et al., 2017; Miller et al., 2013; Mulcahy-O'Grady and Workentine, 2016).
285 To our knowledge, there has been only one other study so far where 16S rRNA high throughput
286 sequencing was used to suggest that the main causative pathogen responsible for the haemorrhagic
287 disease in turbot (*Scophthalmus maximus*) was *Edwardsiella* (Si et al., 2021). Although in this study only
288 diseased tissues were selected and there was no comparison with tissues from healthy individuals, it
289 was clear that the genus *Edwardsiella* was the most abundant in all the examined tissues. With the
290 present study, and due to the comparison between healthy and diseased individuals, we were able to
291 conclude that there was no bacterial pathogen responsible for the onset and development of systemic
292 granulomatosis in cultured meagre (*Argyrosomus regius*). This indicates the potential for further
293 applications of clinical metagenomics in fish, in an attempt to decipher whether known, or unknown,
294 pathogens are indeed the aeatiological agents responsible for the onset and the progression of disease
295 or there are other causes of non-infectious nature involved.
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6. CRediT author statement

Christina Pavloudi: Methodology, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing

Maria Ioanna Tsertou: Formal analysis, Conceptualization, Writing - Original Draft, Writing - Review & Editing

Efthimia Antonopoulou: Writing - Review & Editing

Pantelis Katharios: Conceptualization, Resources, Supervision, Funding acquisition, Writing - Original Draft, Writing - Review & Editing

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496

FIGURE LEGENDS

Figure 1: Bar chart showing the relative abundances of the main microbial taxa, at the phylum level, at each sample.

Figure 2: Bar chart based on the presence/absence of the main microbial taxa, at the phylum level, at each sample.

Figure 3: nMDS of the similarity matrix of the samples based on the relative abundances of the microbial OTUs.

Figure 4: nMDS of the similarity matrix of the samples based on the presence/absence of the microbial OTUs.

Figure 5: Upset plot showing the number of shared and unique OTUs in the three groups.

Figure 6: The LDA scores of the enriched OTUs in each of the three health conditions.

Figure 7: Heatmap of the abundance of the LefSe identified OTUs in the three groups.

Supplementary Figure 1: The abundance of the OTUs that were identified by the LefSe, in the three health groups.

Supplementary Figure 2: The abundance of the OTUs that were identified by the LefSe, in the two health groups (healthy, sick).

Supplementary Figure 3: The LDA scores of the enriched OTUs in each of the two health conditions (healthy, sick).

Supplementary Figure 4: Heatmap of the abundance of the LefSe identified OTUs in the two groups (healthy, sick).

Figure 1

Click here to access/download;Figure;Figure 1.png

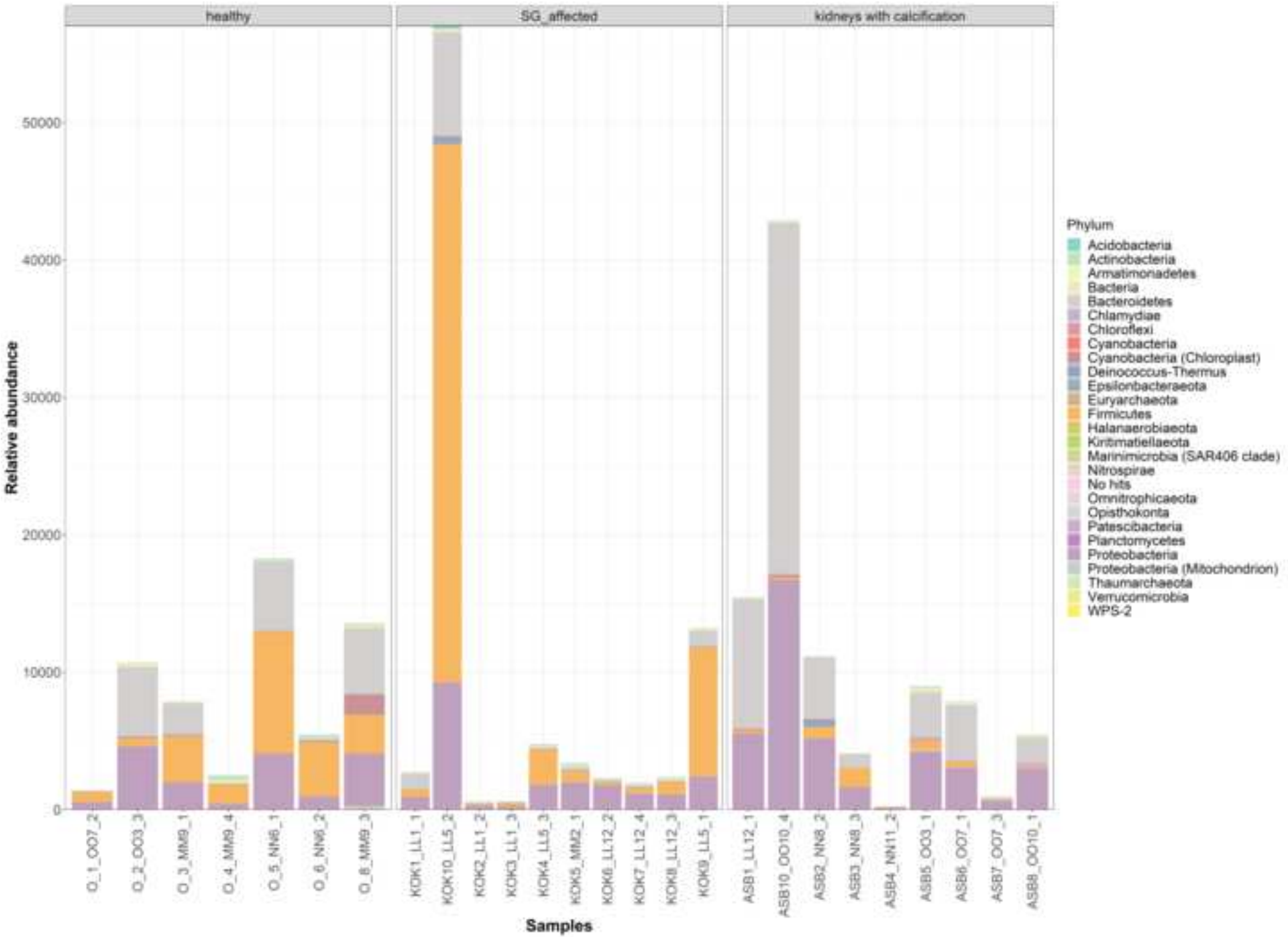

Figure 2

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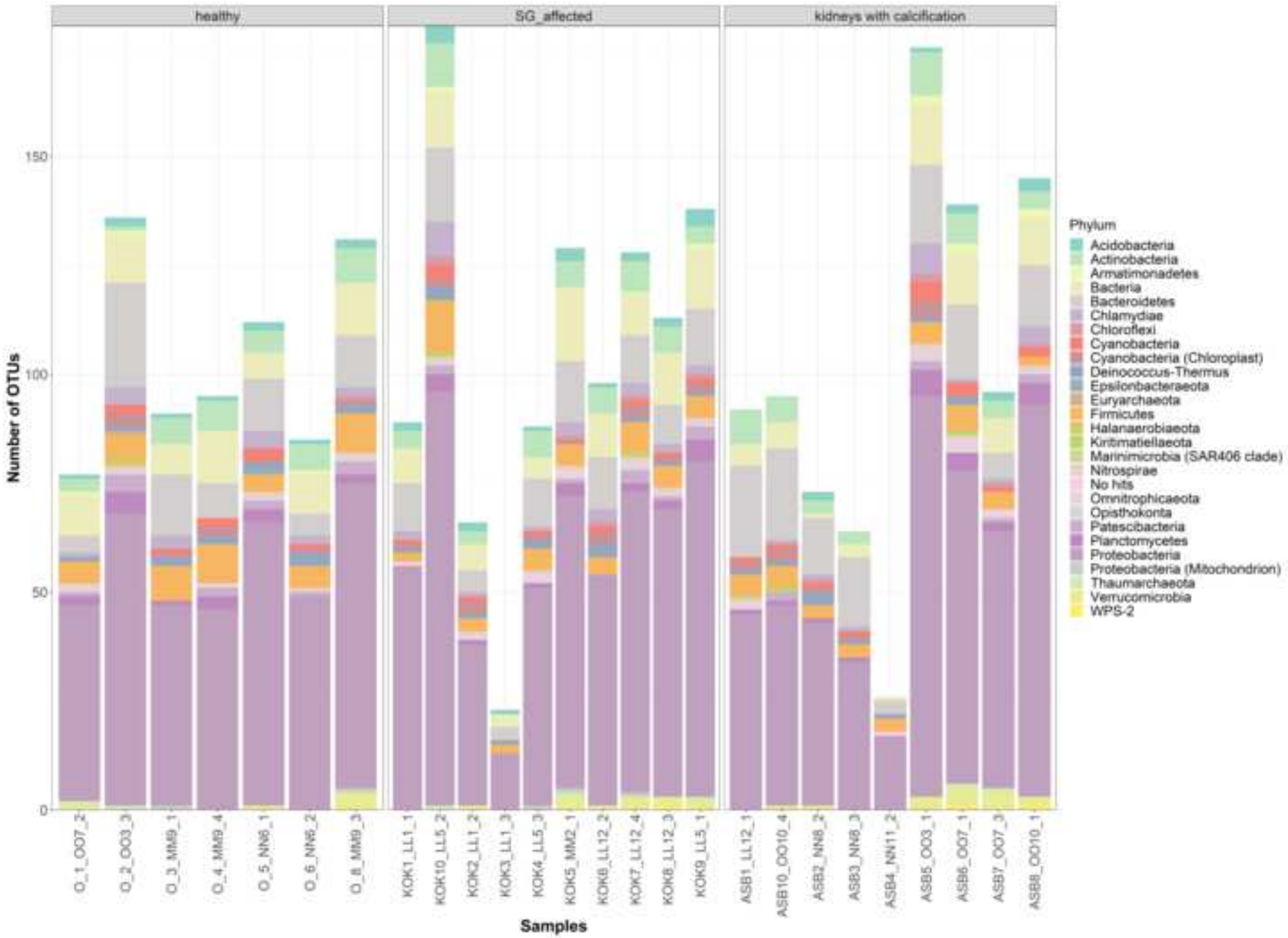

Figure 3

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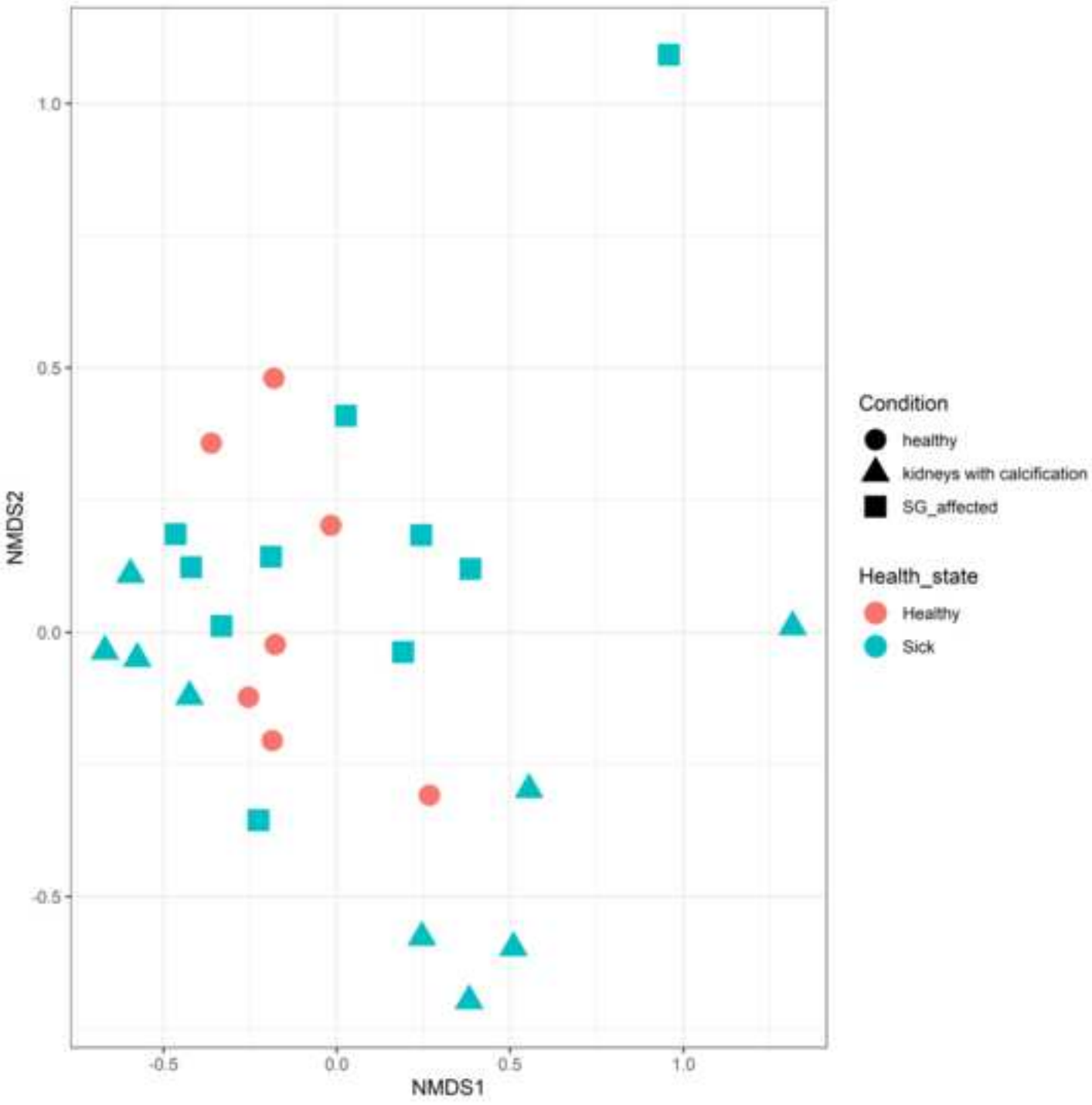

Figure 4

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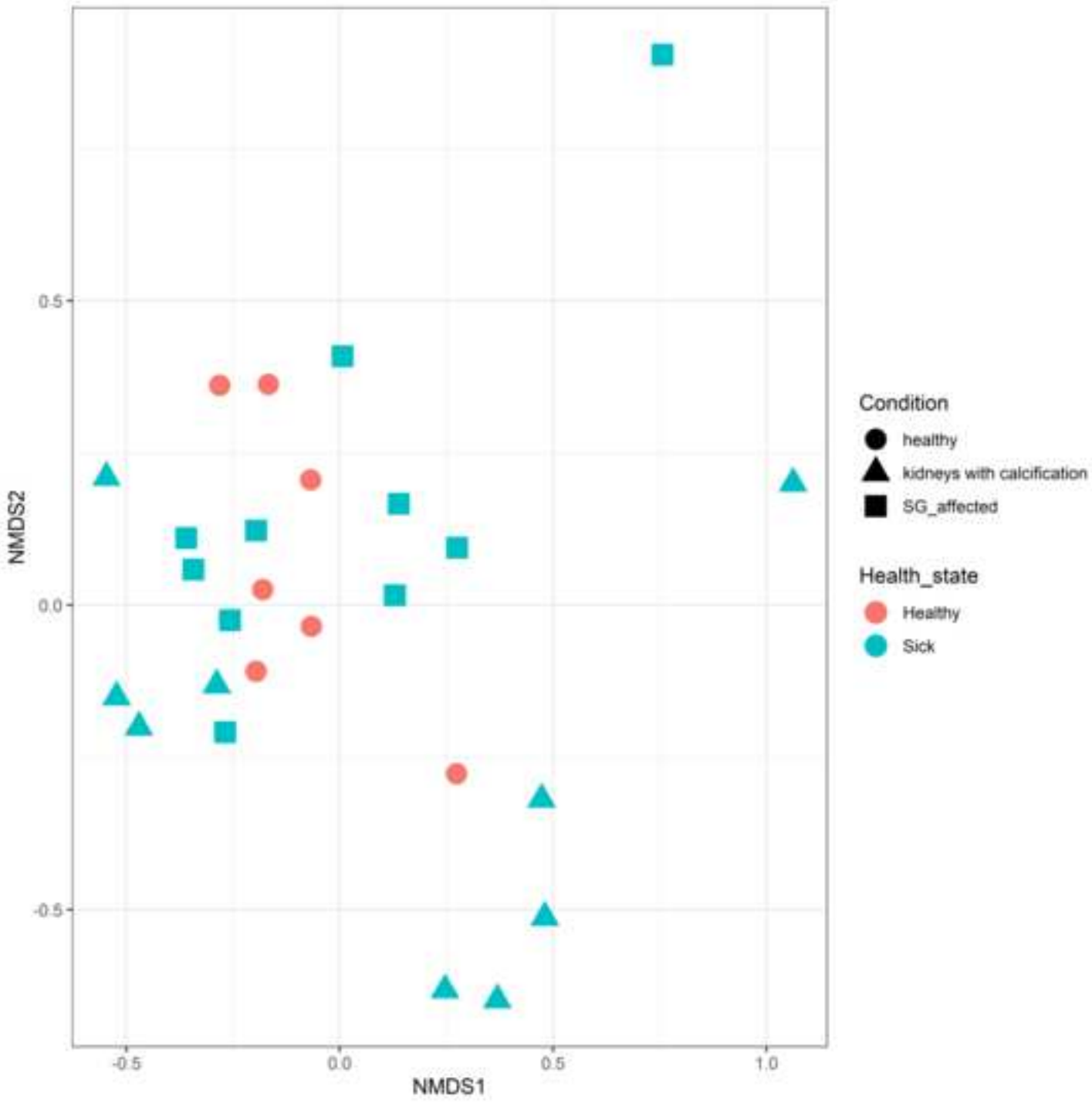

Figure 5

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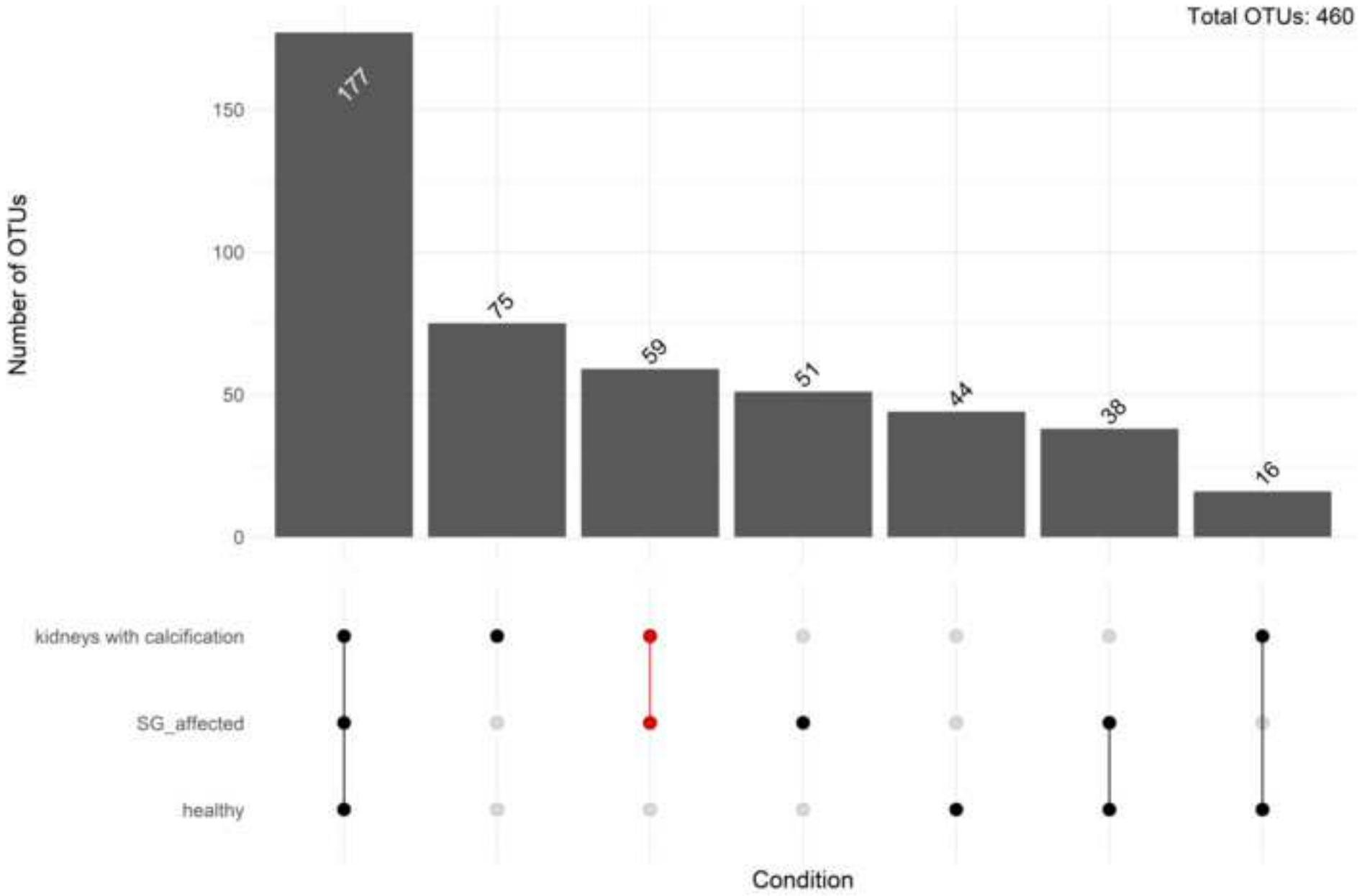

Figure 6

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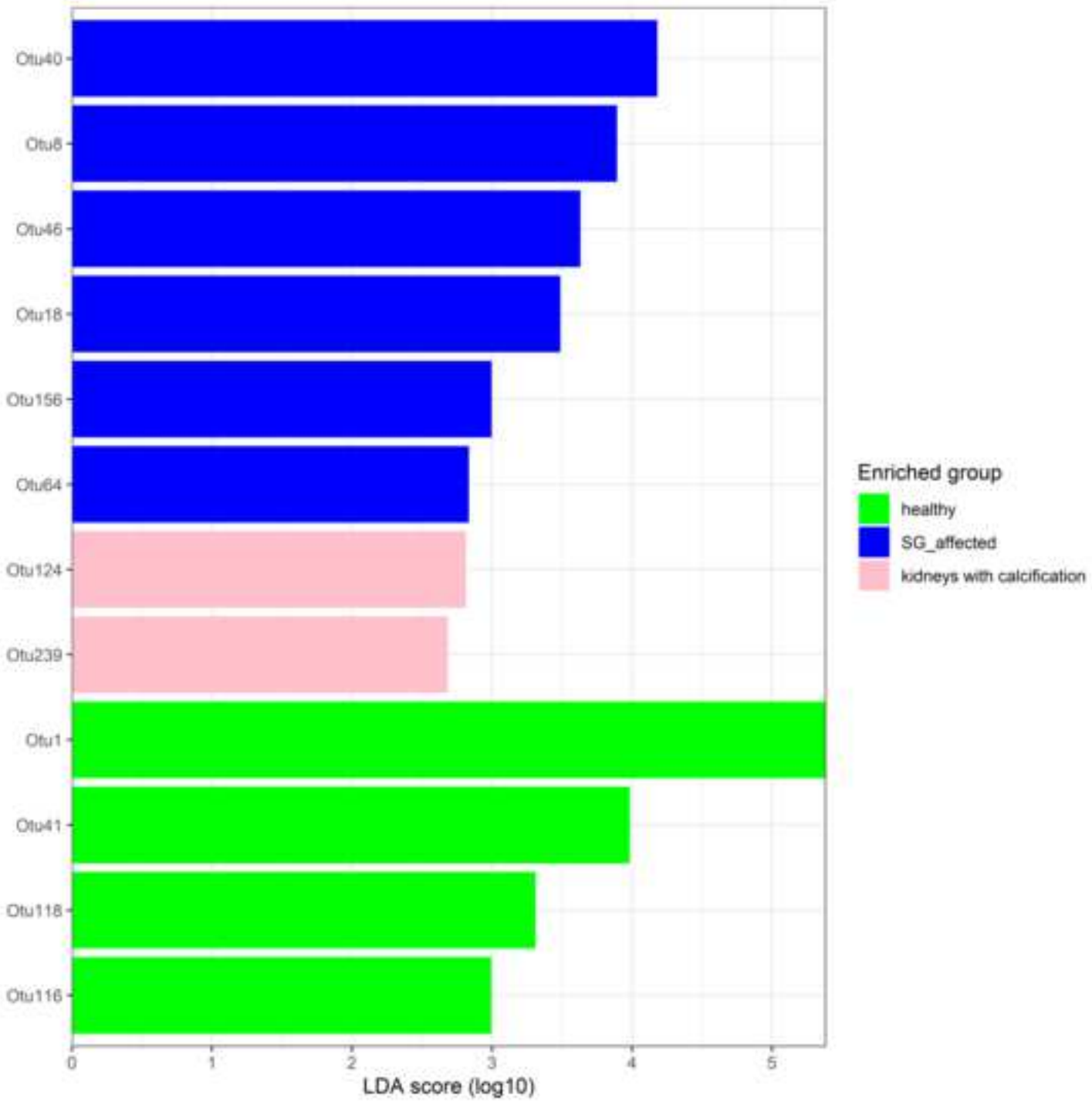

Figure 7

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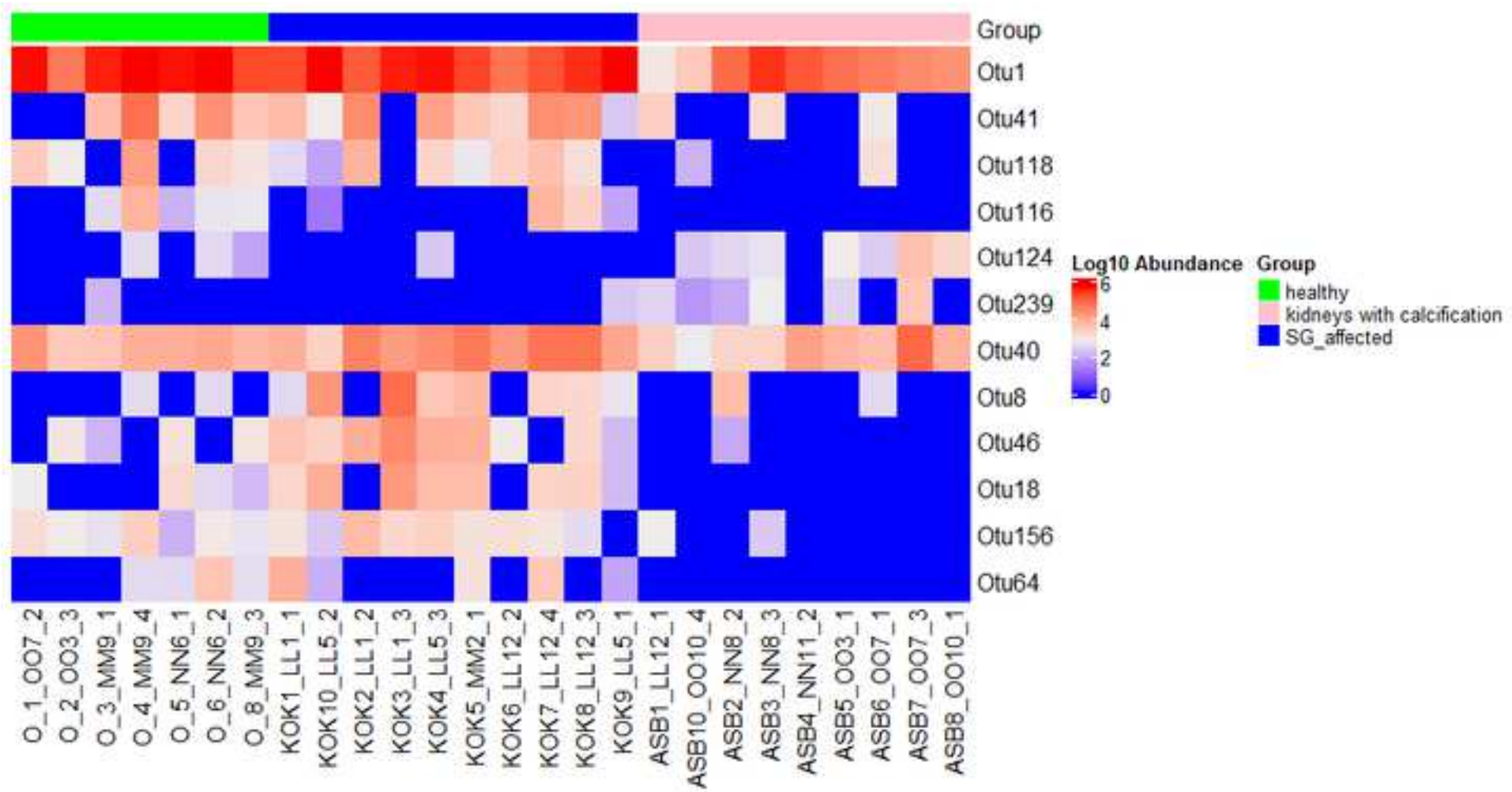

Table 1

Table 1: Common OTUs between different health states.			
kidneys with calcification & SG_affected		**healthy & kidneys with calcification**	
Otu 77	Main genome;Bacteria;Kiritimatiellaeota;Kiritimatiellae;Kiritimatiellales;Kiritimatiellaceae;R76-B128	Otu 56	Main genome;Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus
Otu 84	Main genome;Bacteria;Chlamydiae;Chlamydiae (kingdom);Chlamydiales;Simkaniaceae;Ga0074140	Otu 138	Main genome;Bacteria;Omnitrophicaeota
Otu 88	Main genome;Bacteria;Bacteroidetes;Bacteroidia;Flavobacteriales;Weeksellaceae	Otu 238	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Pasteurellales;Pasteurellaceae;Actinobacillus
Otu 98	Main genome;Bacteria;Verrucomicrobia;Verrucomicrobiae;Methylacidiphilales;Methylacidiphilaceae	Otu 266	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Litoricolaceae;Litoricola
Otu 99	Main genome;Bacteria;Verrucomicrobia;Verrucomicrobiae;Pedosphaerales;Pedosphaeraceae	Otu 288	Chloroplast;Bacteria (Chloroplast);Cyanobacteria (Chloroplast);Oxyphotobacteria (Chloroplast)
Otu 105	Main genome;Bacteria;Bacteroidetes;Bacteroidia	Otu 291	Main genome;Bacteria;Firmicutes;Clostridia;Clostridiales;Family XI;Finegoldia
Otu 132	Main genome;Bacteria;Chloroflexi;OLB14	Otu 320	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Diplorickettsiales;Diplorickettsiaceae
Otu 135	Main genome;Bacteria;Acidobacteria;Blastocatellia (Subgroup 4);Blastocatellales;Blastocatellaceae	Otu 322	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Devosiaceae;Devosia
Otu 141	Main genome;Bacteria;Chlamydiae;Chlamydiae (kingdom);Chlamydiales;Simkaniaceae	Otu 335	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;Sphingomonas
Otu 147	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria	Otu 344	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Puniceispirillales;SAR116 clade
Otu 149	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;SAR11 clade	Otu 363	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae;Hyphomicrobium

Otu 180	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Rhodocyclaceae	Otu 369	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;Legionellaceae;Legionella
Otu 193	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae	Otu 454	Main genome;Bacteria;Bacteroidetes;Bacteroidia
Otu 198	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Gallionellaceae	Otu 458	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Caulobacterales;Hyphomonadaceae;SWB02
Otu 199	Main genome;Bacteria;Actinobacteria;Actinobacteria (kingdom);Bifidobacteriales;Bifidobacteriaceae;Gardnerella	Otu 460	Main genome;Bacteria;Actinobacteria;Actinobacteria (kingdom);PeM15
Otu 203	No hits	Otu 461	Main genome;Bacteria;Planctomycetes;Planctomycetacia;Planctomycetales
Otu 206	Main genome;Bacteria		
Otu 218	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;TRA3-20		
Otu 224	Main genome;Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Porphyromonas		
Otu 229	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Gallionellaceae		
Otu 232	Main genome;Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Alloprevotella		
Otu 233	Main genome;Bacteria;WPS-2		
Otu 235	Main genome;Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus		

Otu 253	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae		
Otu 255	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Reyranellales;Reyranellaceae;Reyranella		
Otu 347	Main genome;Bacteria;Chlamydiae;Chlamydiae (kingdom);Chlamydiales;Simkaniaceae;Ga0074140		
Otu 348	Main genome;Bacteria		
Otu 352	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae		
Otu 354	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Rhodocyclaceae		
Otu 359	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Rhodocyclaceae;Sulfuritalea		
Otu 360	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Beijerinckiaceae		
Otu 362	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Gallionellaceae		
Otu 366	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Pasteurellales;Pasteurellaceae;Haemophilus		
Otu 378	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae		
Otu 380	Main genome;Bacteria		

Otu 388	Main genome;Bacteria;Acidobacteria;Subgroup 6		
Otu 402	No hits		
Otu 405	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae;Alcaligenes		
Otu 406	Main genome;Bacteria;Proteobacteria;Deltaproteobacteria;Oligoflexales;0319-6G20		
Otu 407	Main genome;Bacteria		
Otu 410	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Methylophilaceae;OM43 clade		
Otu 411	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Glaciecola		
Otu 412	Main genome;Bacteria;Armatimonadetes;Fimbriimonadia;Fimbriimonadales;Fimbriimonadaceae		
Otu 413	Main genome;Bacteria;Chloroflexi;P2-11E		
Otu 417	Main genome;Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus		
Otu 422	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Gallionellaceae		
Otu 424	Chloroplast;Bacteria (Chloroplast);Cyanobacteria (Chloroplast);Oxyphotobacteria (Chloroplast)		
Otu 429	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Azospirillales;Inquilinaceae;Inquilinus		

Otu 435	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Diplorickettsiales;Diplorickettsiaceae;Aquicella		
Otu 437	Main genome;Bacteria;Bacteroidetes;Bacteroidia		
Otu 439	Main genome;Bacteria;Acidobacteria;Subgroup 17		
Otu 441	Main genome;Bacteria;Bacteroidetes;Rhodothermia;Balneolales;Balneolaceae;Balneola		
Otu 443	Main genome;Bacteria;Chlamydiae;Chlamydiae (kingdom);Chlamydiales;Simkaniaceae		
Otu 445	Main genome;Bacteria;Planctomycetes;Planctomycetacia;Gemmatales;Gemmataceae;Fimbriiglobus		
Otu 447	Main genome;Bacteria;Omnitrophicaeota;Omnitrophia;Omnitrophales;Omnitrophaceae;Candidatus Omnitrophus		
Otu 448	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Salinisphaerales;Solimonadaceae;Nevskia		
Otu 449	Main genome;Bacteria;Acidobacteria;Subgroup 6		
Otu 450	Main genome;Bacteria;Verrucomicrobia;Verrucomicrobiae;Pedosphaerales;Pedosphaeraceae		
Otu 451	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae		

Table 2

Table 2: The OTUs that were enriched in each of the groups as identified by the LefSe. *:<0.05, **:<0.01, ***:<0.001

OTU	Taxonomy	Enriched Group	LDA	adjusted p value
Otu1	Main genome;Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Anoxybacillus	healthy	5.38	**
Otu41	Main genome;Bacteria;Actinobacteria;Actinobacteria (kingdom);Micrococcales;Micrococcaceae;Micrococcus	healthy	3.98	*
Otu118	Main genome;Bacteria;Actinobacteria;Actinobacteria (kingdom);Micrococcales;Dermacoccaceae;Dermacoccus	healthy	3.31	*
Otu116	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Paracoccus	healthy	3.00	*
Otu40	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae	SG_affected	4.18	*
Otu8	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Beijerinckiaceae	SG_affected	3.89	**
Otu46	Main genome;Bacteria;Acidobacteria;Blastocatellia (Subgroup 4);Pyrinomonadales;Pyrinomonadaceae;Pyrinomonas	SG_affected	3.63	***
Otu18	Main genome;Bacteria;Bacteroidetes;Bacteroidia;Sphingobacteriales;env.OPS 17	SG_affected	3.49	**
Otu156	Main genome;Bacteria	SG_affected	3.00	**
Otu64	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Paracoccus	SG_affected	2.84	*
Otu124	Main genome;Bacteria;Actinobacteria;Acidimicrobiia;Actinomarinales;Actinomarinaceae;Candidatus Actinomarina	kidneys with calcification	2.81	**
Otu239	Main genome;Bacteria;Bacteroidetes;Bacteroidia;Flavobacteriales;Flavobacteriaceae;NS5 marine group	kidneys with calcification	2.68	*
Otu16	Main genome;Bacteria;Deinococcus-Thermus;Deinococci;Thermales;Thermaceae;Meiothermus	Healthy	3.43	*
Otu75	Main genome;Bacteria;Firmicutes;Bacilli;Bacillales;Family XII	Healthy	2.93	*
Otu116	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Paracoccus	Healthy	2.90	*
Otu56	Main genome;Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus	Healthy	2.46	*
Otu457	Main genome;Bacteria;Verrucomicrobia;Verrucomicrobiae;Opitutales;Puniceicoccaceae;Coraliomargarita	Healthy	2.23	*
Otu316	Main genome;Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus	Healthy	2.10	*
Otu130	Main genome;Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae	Sick	3.22	*
Otu185	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae	Sick	2.93	*
Otu229	Main genome;Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Gallionellaceae	Sick	2.79	*

Table 3. Total reads and prevalence (%) of detected phyla per health state of the kidneys.

	Health state of kidneys					
	Healthy		With granulomas		With calcification	
	No of reads	%	No of reads	%	No of reads	%
Bacteroidetes	29,095	50.1	27,502	39.4	60,069	63.3
Proteobacteria	17,649	30.4	21,565	30.9	30,307	31.9
Firmicutes	7,768	13.4	18,300	26.2	1,612	1.7
Cyanobacteria	1,671	2.9	402	0.6	630	0.7
Acidobacteria	28	0.0	96	0.1	89	0.1
Actinobacteria	1,347	2.3	1,072	1.5	824	0.9
Armatimonadetes	5	0.0	8	0.0	30	0.0
Balneolaeota	2	0.0	6	0.0	15	0.0
Chlamydiae	178	0.3	292	0.4	507	0.5
Chloroflexi	11	0.0	12	0.0	32	0.0
Deinococcus-Thermus	174	0.3	415	0.6	444	0.5
Dictyoglomi	0	0.0	7	0.0	9	0.0
Elusimicrobia	0	0.0	0	0.0	2	0.0
Fibrobacteres	0	0.0	0	0.0	1	0.0
Fusobacteria	4	0.0	13	0.0	5	0.0
Gemmatimonadetes	0	0.0	2	0.0	1	0.0
Ignavibacteriae	2	0.0	0	0.0	0	0.0
Kiritimatiellaeota	0	0.0	8	0.0	18	0.0
Lentisphaerae	1	0.0	0	0.0	0	0.0
Nitrospirae	11	0.0	18	0.0	44	0.0
Planctomycetes	47	0.1	56	0.1	147	0.2
Rhodothermaeota	8	0.0	0	0.0	10	0.0
Spirochaetes	2	0.0	3	0.0	4	0.0
Tenericutes	0	0.0	0	0.0	5	0.0
Verrucomicrobia	24	0.0	42	0.1	66	0.1
Total reads	**58,027**	**100.0**	**69,819**	**100.0**	**94,871**	**100.0**

Table 4 (Editable version)

Table 4. Taxa with the most reads per health state of the kidneys

	Health state of kidneys		
	Healthy	With granulomas	With calcification
	No of reads	No of reads	No of reads
Myroides ceti	21,567	19,950	45,340
Anoxybacillus flavithermus subsp. yunnanensis	6,223	17,565	1,100
Pedobacter nutrimenti	5,943	5,023	11,391
Pseudomonas fildesensis	4,159	3,973	40
Brevundimonas albigilva	3,519	3,747	7,102
Pseudomonas migulae	2,125	14	4,200
Achromobacter kerstersii	875	789	1,266
Candidatus pelagibacter	813	1,089	1,721
Stenotrophomonas pavanii	576	502	1,243
Brucella abortus	306	375	673
Vulcaniibacterium thermophilum	282	846	487
Sphingomonas kyeonggiensis	206	158	169
Acinetobacter junii	143	118	422
Pseudomonas lini	2	2,081	2
Pseudomonas veronii	0	0	4,915

Cutoff value for species identification: 97%

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT author statement

Christina Pavloudi: Methodology, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing

Maria Ioanna Tsertou: Formal analysis, Conceptualization, Writing - Original Draft, Writing - Review & Editing

Efthimia Antonopoulou: Writing - Review & Editing

Pantelis Katharios: Conceptualization, Resources, Supervision, Funding acquisition, Writing - Original Draft, Writing - Review & Editing

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