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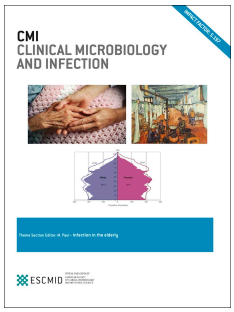

Metatranscriptomic assessment of burn wound infection clearance

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Title: Metatranscriptomic Assessment of Burn Wound Infection Clearance

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*To the Editor***,**

Skin and soft tissue infections are frequent infections in healthcare that vary in presentation and severity. The severe forms often require surgical intervention and antimicrobial therapy and their recovery should be assessed frequently to catch treatment failures [1]. Here, we used random sequencing of coding bacterial RNA (metatranscriptomics) and 16S rRNA-gene amplicon sequencing (16S-profiling) to characterise the post-treatment bacterial communities of a complicated burn wound and assess infection clearance.

A 2-year-old female sustained a ~5% total body surface area boiling water burn injury on the left side of her head and right arm. After initial conservative treatment by silver dressings, the healing was complicated by prolonged and persistent infections (**Fig. 1A**). During the course of the treatment, the patient underwent conventional microbiological testing, several antibiotic treatments, and multiple surgical debridements and skin graftings. The last adjustment of antibiotics occurred on day 96 (a switch to intravenous levofloxacin) and the last grafting operation three days later. Healing progressed and the wound was cured by day 147.

The post-treatment tissue samples (wound) for metatranscriptomics and 16S-profiling were extracted during the last grafting operation. Body site-matched, non-infected tissue was obtained from an independent patient and control samples with (spike-in) and without (normal) added *Staphylococcus epidermidis* ATCC 12228 were prepared. The metatranscriptomics workflow is outlined in **Figure 1B** and involved depletion of rRNAs and poly-adenylated-transcripts to enrich bacterial mRNAs. Illumina HiSeq (PE100) was used in sequencing. Data analysis followed our previous protocol [2] and employed an in-house built genome and gene sequence classification

databases (**Fig. 1C**). For 16S-profiling, DNA was extracted, V3-V4 region of 16S rRNA gene amplified, and amplicons sequenced using Illumina MiSeq (PE250). Reads were classified to species level. The detailed methodology is provided in the Supplementary text. The Operative Ethics Committee of the Hospital District for Helsinki and Uusimaa approved the study protocol (HUS 2076/2016). Written informed consent was obtained from all participants or their guardians.

Metatranscriptomics and 16S-profiling analyses both revealed samples to contain bacterial reads in varying portions (**Fig. 1D**). To account the variation, samples were sequenced to different total depths in both technologies.

A genus-level classification was assigned to 99.25% of metatranscriptomic (with an estimated precision of 0.94 and recall of 0.96; **Table S1**) and 99.99% of 16S-profiling bacterial reads. The identified major genera are listed in **Figures 1E** and **S1**. Correlation analysis revealed that genus-level abundancies correlated well across technologies in spike-in (r=0.95) and normal (r=0.87) but not in wound (r=-0.46; **Fig. S2**). A strong correlation was also identified in metatranscriptomics between wound and normal (r=0.86), while these were negatively associated in 16S-profiling (r=-0.89, **Fig. S2)**. Diversity analyses corroborated the trends observed in the correlation analysis and highlighted the highest diversities for wound samples (**Fig. 1F**, **Fig. S3-S4**).

Differential abundance analysis revealed eight genera enriched between wound and normal by either technology (**Fig. 1G, Fig. S5, Table S2**). The skin commensal *Cutibacterium* was underrepresented in wound compared to normal. Genera enriched in wound harboured clinically associated pathogens, established 16S-profiling contaminants, and environmental bacteria unlikely to exist in the sample. Clinically associated *Micrococcus*, *Dietzia*, and *Corynebacterium* were highly enriched especially in 16S-profiling. The species-level results agreed with genus-level findings and revealed enrichment of specific *Micrococcus*, *Corynebacterium*, and *Dietzia* species by 16S-profiling (**Fig. S6-S7, Table S3**).

Examination of microbial gene activity at the UniRef90-level (with an estimated precision of 0.95 and recall of 0.83; **Table S1**) revealed activity of 594 (normal), 960 (wound), and 5,325 (spike-in) bacterial protein groups with ≥2.5 counts per million reads. The genes expressed in wound were mostly involved in basic cellular activities. Pathway enrichment analysis revealed no functional differences (**Fig. 1H, Tables S4 and S5)**.

Metatranscriptomics is an interesting approach to assess treatment success. In this study, it revealed marked similarity between bacterial communities of post-treatment and normal samples and captured activity of normal cellular processes in wound; results which indicate infection clearance and agree with the clinical picture. The 16S-profiling in contrast supported the presence of an abnormal community and indicated marked enrichment of *Corynebacterium tuberculostearicum* associated to wound infections in patients with a long history of antibiotics [3] and an infection-associated and often misdiagnosed *Dietzia cinnamea* sensitive to levofloxacin [4]. Technical differences are unlikely to explain these discrepancies given the high cross-platform concordance among controls. Instead, the inability of DNA-amplicon-based approaches to differentiate the live and dead [5] might explain the differences, especially as microbiological cultures taken four days earlier listed coagulase-negative staphylococci and diphtheroids without detailing the species and were thereby partly in agreement with the 16S-profiling results unveiling unenriched levels of *Staphylococcus* and enriched levels of *Corynebacterium* in wound.

To conclude, this study demonstrated the value of metatranscriptomics in understanding rapid microbial alterations in complex host-microbe samples. Its use in infection clearance assessment warrants further investigations.

Transparency declaration

The authors declare no conflicts of interest related to the study. This work was supported by the Academy of Finland [grant numbers 292635, 292589, 292646, 292626, and 292605]; Business

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Appendix A. Supplementary data

Supplementary data to this article can be found online at xxx.

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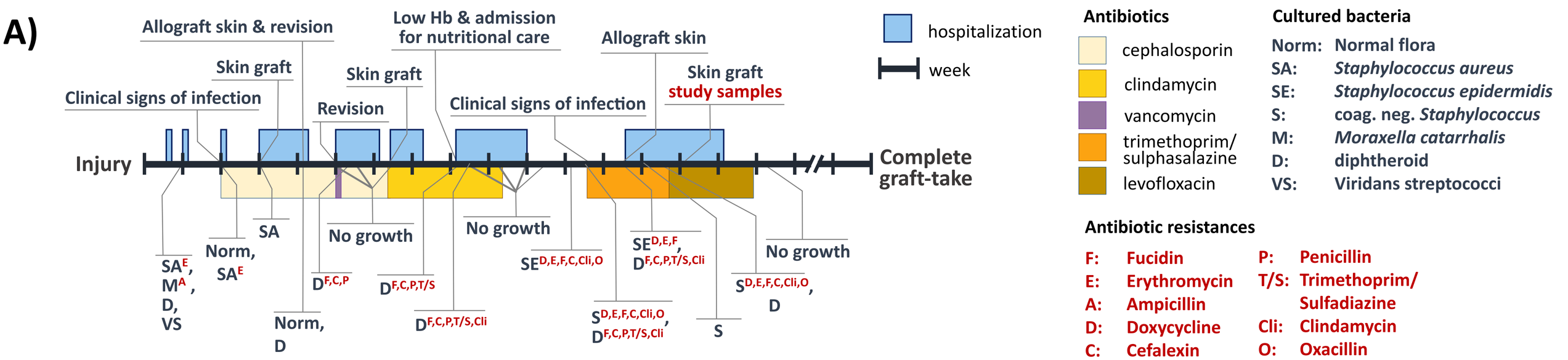
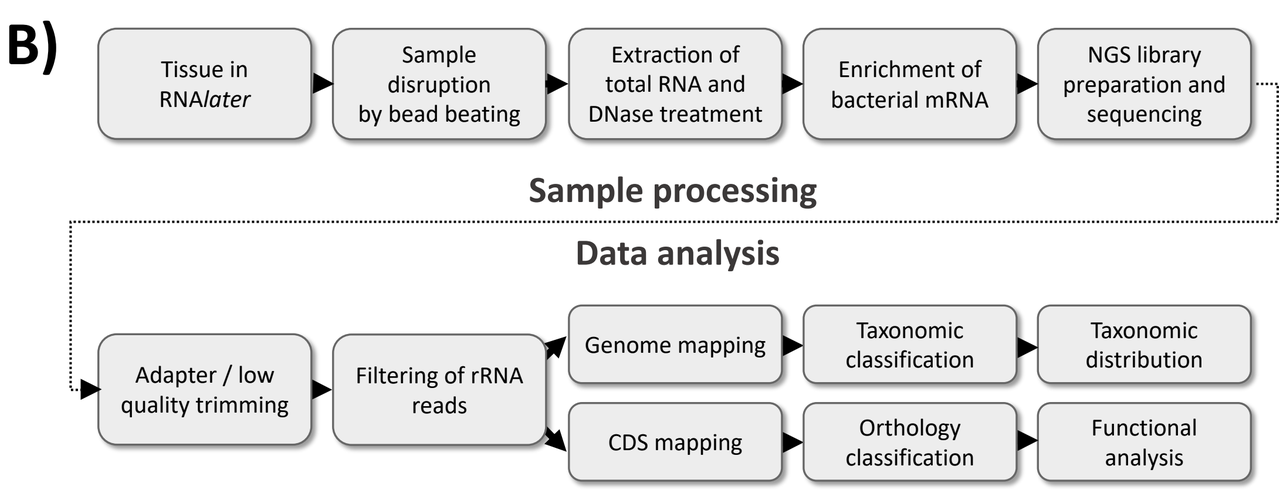
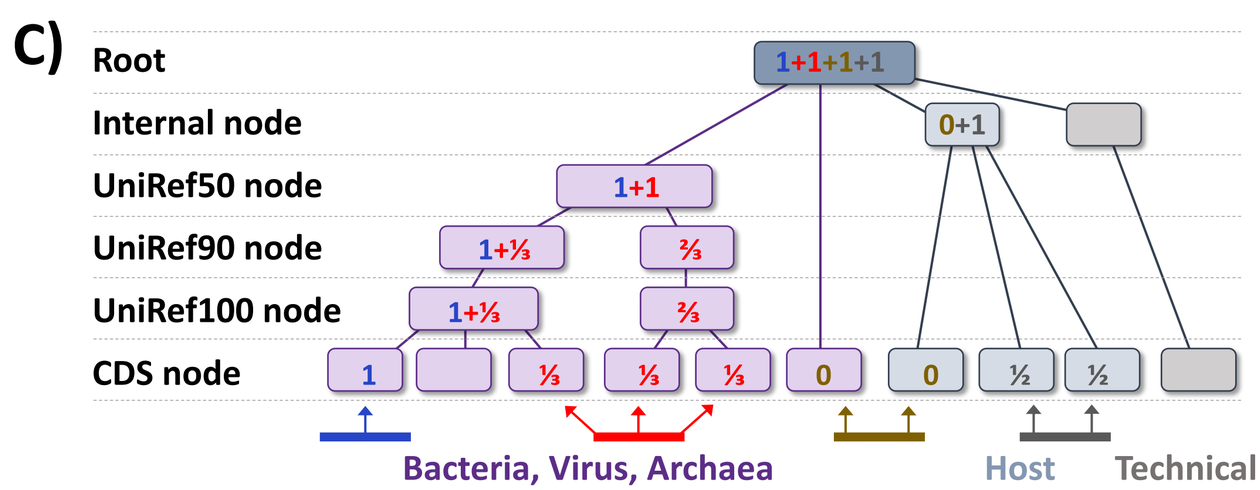
Figure legend:

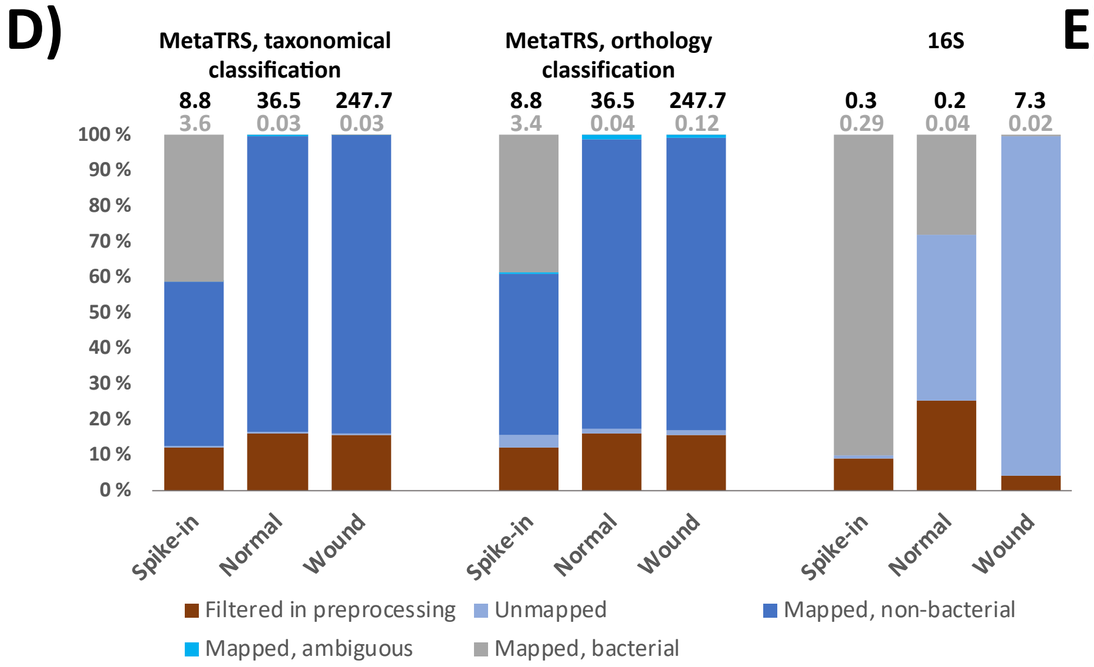
Fig 1. **A: Clinical course of the patient.** The timeline illustrates admissions, antimicrobial medications, and results of standard microbial diagnostics from injury to recovery. Study samples were taken 3-days after a switch from the oral trimethoprim and sulphasalazine to the intravenous levofloxacin at post-injury day 99 during a grafting operation. **B: Metatranscriptomics sample processing and data analysis workflow.** Samples were immersed in transcriptome preserving reagent immediately upon extraction, tissue structure and cells were disrupted, total RNA was extracted and DNase treated, and bacterial mRNA was enriched by depleting rRNAs and poly-adenylated-transcripts. **C: Gene classification scheme.** The reads were aligned against all known bacterial, archaeal, and viral coding sequences that were organized into sequence clusters in the presence of UniRef information as well as to human and technical sequences. Reads classified to human and technical references were ignored in analyses. **D: Read survival in metatranscriptomics and 16S-profiling.** For each sample, the stacked columns depict the portions of reads classified as bacterial (grey), classified ambiguously as bacterial and non-bacterial (cyan), classified solely as non-bacterial (blue), passing preprocessing but not classified (light blue), and failed in preprocessing (brown). The total number of reads (black) and the number of bacterial reads (grey) in millions are given above columns. **E: Relative distribution of bacterial genera.** Only genera accounting >5% of the bacterial reads in any samples by either metatranscriptomics (MetaTRS) or 16S-profiling (16S) are shown. Reads assigned to other genera are marked as ‘other

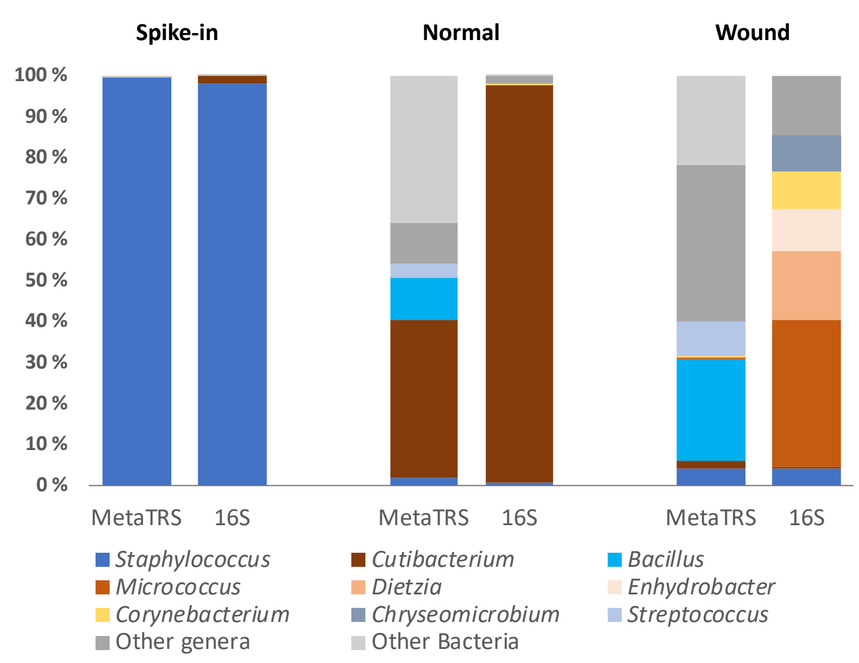
genera'. Bacterial reads without genus-level classifications are marked as 'other Bacteria'. Both technologies correctly identified the spiked-in *Staphylococcus* as the major genera in the spike-in sample and reported the normal skin commensal *Cutibacterium* in the normal sample. **F: Bacterial beta-diversity.** Bray-Curtis dissimilarity-based principal coordinates analysis at the genus level revealed a high similarity between control samples assessed with different platforms. Wound samples assessed with different platforms were more dissimilar. **G: Enriched genera in wound versus normal.** The bar graph depicts fold change (log_{10}) of the enriched genera in wound versus normal. The difference between fold changes determined by 16S-profiling and metatranscriptomics are given above columns. Small values mark taxa likely related to inter-sample variance. High values represent taxa likely associated with the treatment response. Asterisks highlight genera accounting ≥5% of the total bacterial read count and with ≥2-fold-change. Double asterisks highlight genera accounting ≥5% of the total bacterial read count and with ≥5-fold-change. Conservative cut-offs were used to avoid false findings. **H: Pathways in wound and normal.** Pathways with ≥10 UniRef90-level assignments in wound and normal are listed. For each pathway, shown are the number of assignments to the given pathway, number of assignments to any pathway, and the Bonferroni corrected *p*-value of Chi-squared test between expected and observed frequencies. Example of the glycolysis pathway is detailed in the figure. Blue, red, and purple colours indicate functions supported by ≥2.5 counts per million bacterial reads in wound, normal, or both, respectively.

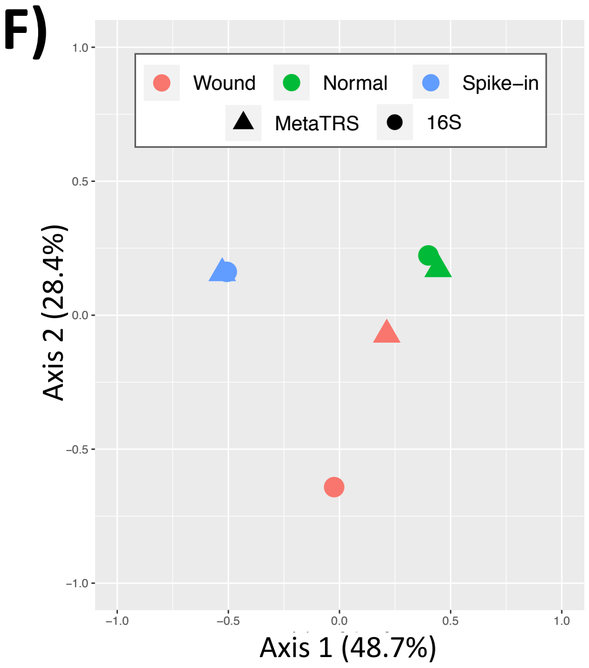


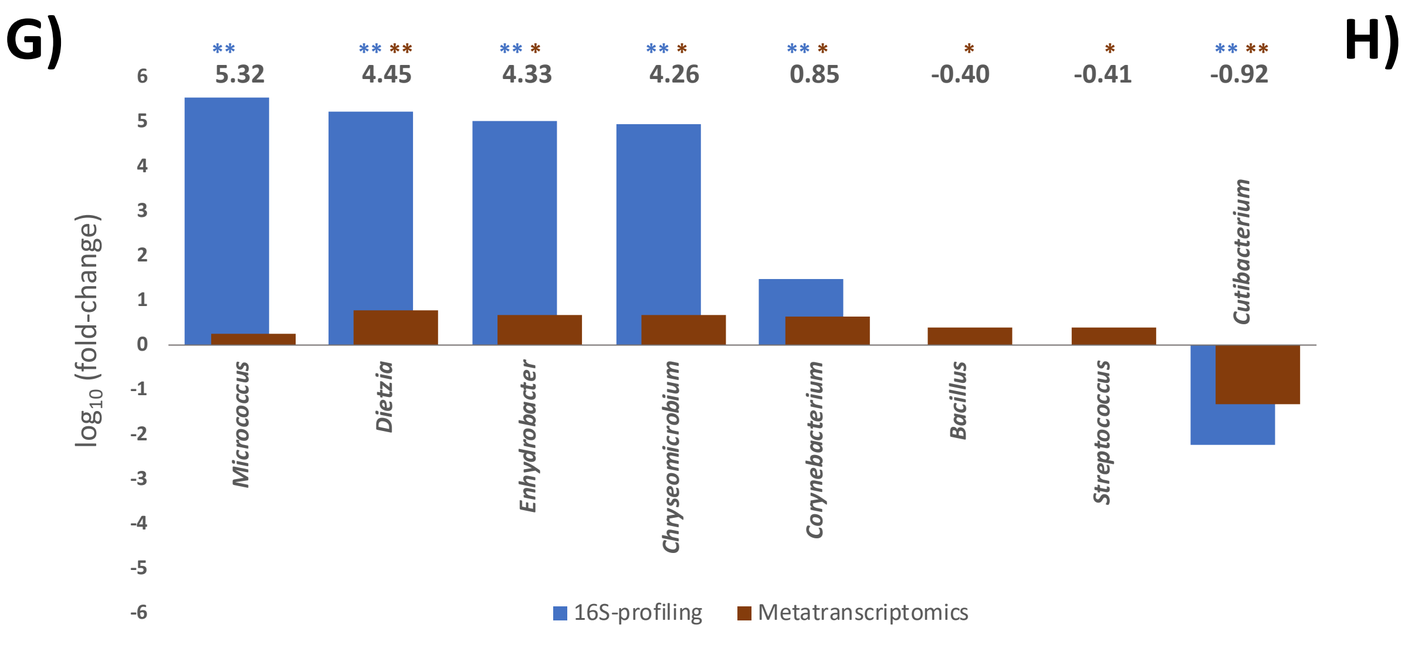












H)

Pathway	Name	Wound Pathway/Total	Normal Pathway/Total	*P* (bonf)
map01100	Metabolic pathways	110/294	63/182	1.00
map01110	Biosynthesis of secondary metabolites	54/294	30/182	1.00
map01130	Biosynthesis of antibiotics	40/294	22/182	1.00
map01120	Microbial metabolism in diverse environments	38/294	19/182	1.00
map01230	Biosynthesis of amino acids	25/294	14/182	1.00
map01200	Carbon metabolism	22/294	17/182	1.00
map02010	ABC transporters	18/294	5/182	1.00
map02020	Two-component system	11/294	5/182	1.00
map00620	Pyruvate metabolism	11/294	3/182	1.00
map00010	Glycolysis / Gluconeogenesis	11/294	7/182	1.00
map00970	Aminoacyl-tRNA biosynthesis	10/294	5/182	1.00
map00250	Alanine, aspartate and glutamate metabolism	10/294	3/182	1.00
map00230	Purine metabolism	9/294	10/182	1.00
map03010	Ribosome	7/294	10/182	1.00