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Research article

The Maleth program: Malta's first space mission discoveries on the microbiome of diabetic foot ulcers

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ABSTRACT

The purpose of the Maleth Program, also known as Project Maleth, is Malta's first space program to evaluate human skin tissue microbiome changes in type 2 diabetes mellitus (T2DM) patients afflicted with diabetic foot ulcers (DFU). This was carried out in both ground-based models and spaceflight. The first mission (Maleth I) under this program was carried out to uncover the effects of spaceflight, microgravity and radiation on human skin tissue microbiome samples from six T2DM patients recruited into the study. Each patient human skin tissue sample was split in three, with one section processed immediately for genomic profiling by 16S typing and the rest were processed for longer term ground-control and spaceflight experiments. Ground-control and spaceflight human skin tissue samples were also processed for genomic profiling upon mission re-entry and completion. Maleth I's overall objective was achieved, as human skin tissue samples with their microbiomes travelled to space and back yielding positive results by both standard microbiology techniques and genetic typing using 16S rRNA amplicon sequencing. Preliminary findings of this mission are discussed in light of its innovative approach at DFU microbiome research, and the clinical implications that may emerge from this and other future similar studies.

1. Introduction

The use of spaceflight as a tool to study basic and fundamental science is not new. However, this was the case for Malta in August 2021 when the very first biomedical science mission was ushered to the International Space Station (ISS) for 30 days and returned for analysis. The Maleth Program's first mission focused on diabetic foot ulcer (DFU) research (Figure 1). DFUs arise as a complication of Type 2 Diabetes Mellitus (T2DM) and can prove difficult to manage. T2DM is a major public health concern with 537 million people suffering from this disease worldwide (IDF, 2022). Malta has one of the highest prevalence in Europe, with 11.2% of the population suffering from this disease (IDF, 2021). Coincidentally in 2019, Malta had the highest recorded obesity rates, in both sexes, amongst the EU countries. Obesity is one of the predisposing factors of diabetes and this together with various genetic mutations identified in the Maltese population contribute to the high prevalence of diabetes in Malta (Eurostat, 2021; Cuschieri et al., 2016; Pace et al., 2019, 2021). As diabetic patients are at risk of ischaemia, peripheral neuropathy and an

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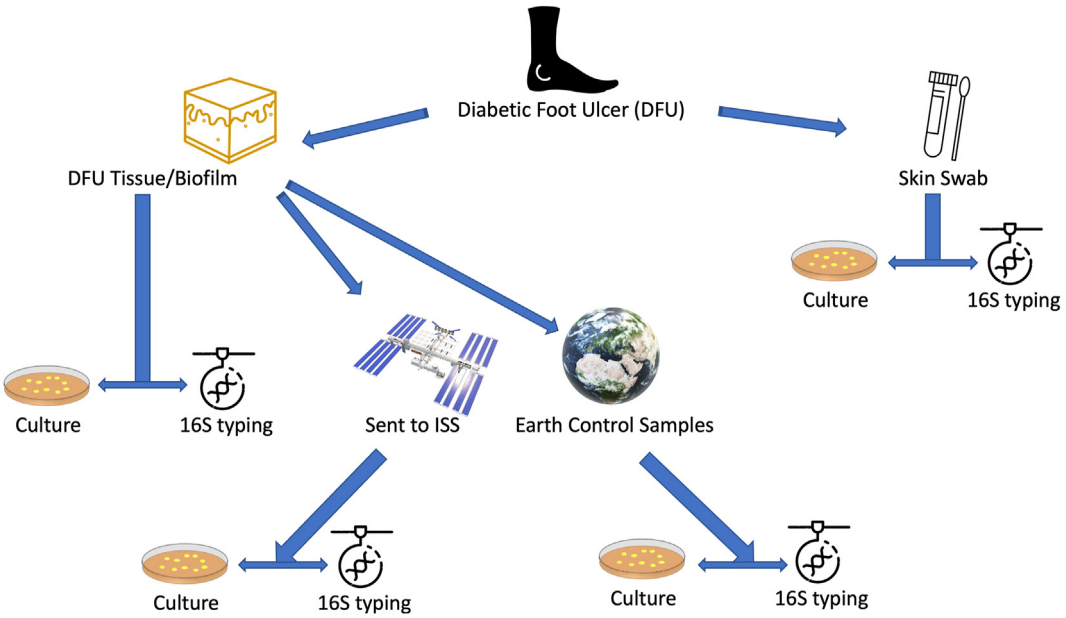

Figure 1. Diagram representing how the different samples taken from patients presenting with diabetic foot ulcers were processed. Tissue samples that were selected for Project Maleth were cut into 4 smaller pieces, one piece was processed for culture, another was processed for 16S rRNA NGS whilst the other two pieces served as either the control sample (which was left on Earth) or the experiment sample (which was sent to ISS).

impaired immunity, 12–25% are at a high risk of developing diabetic foot ulcers (DFUs). These ulcers tend to heal very slowly, becoming chronic and consequently infected, with the patient often requiring hospitalisation (Jneid et al., 2017; Smith et al., 2016). Infections in chronic DFUs have been shown to be polymicrobial with various bacterial communities infecting the ulcer, forming a biofilm, which makes the ulcer impenetrable to the host immune system and systemic antibiotics (Dowd et al., 2011; Gontchorava et al., 2010). 44–68% of infected DFUs continue to develop osteomyelitis often leading to amputations (Jneid et al., 2017; van Asten et al., 2016). Mortality rates of diabetic amputees, within 5 years of major surgery, is 70%. Due to its chronicity, diabetes has a huge impact on the healthcare system. It is estimated that in Malta alone more than 9 million euro are allocated to diabetic patient care. The average hospital stay for T2DM patients in Malta is the highest in Europe with an average of 14.6 days as opposed to 8.4 days in other European countries (NDS, 2016).

Colonisation of a wound is defined as the presence of bacteria without any involvement from the host immune response. On the other hand, an infection occurs when bacteria have overcome the host's immune system and are therefore invading the host. Clinical signs and symptoms normally associated with infection, such as local swelling or induration, local tenderness or pain, local warmth and erythema may not be present or are reduced in diabetic patients. This contributes to a delay in the patient seeking medical help and consequently a delay in treatment (Jneid et al., 2017). This, together with other factors such as immunosuppression, facilitates the formation of biofilms, which is thought to be a major factor that prevents healing in chronic wounds (Dowd et al., 2011).

Biofilms are composed of various microbial populations confined in an extracellular polymeric substance which is attached to a surface such as tissue or bone (Gontcharova et al., 2010). Commensal bacteria, that are normally found on the skin, can become opportunistic pathogens when they find themselves in the nutritious and moist environment of wounds. The commensal bacteria would then form part of the biofilm community and may work synergistically with the pathogenic bacteria to cause chronic infections in DFUs. Additionally, the extracellular polymeric substance would prevent the access of antibiotics as well as the host's immune cells, making these wounds difficult to treat. Furthermore, in mature biofilms bacteria would not be so active and hence would provide challenges for antibiotic treatment since antibiotics target highly active bacteria. Debridement of these wounds is usually performed in order to remove the biofilm. This has been shown to help with ulcer healing, however antibiotics are administered blindly as culture methods can only identify a fraction of the polymicrobial populations present in these DFUs (Dowd et al., 2011; Jneid et al., 2017; Rhoads et al., 2012; Smith et al., 2016).

Conducting space bioscience experiments on T2DM DFU samples and investigating their microbiome's genetic signatures using molecular techniques, such as Next Generation Sequencing (NGS), can be used to identify the types of bacteria present in DFUs and how they adapt to the harsh environment of space, including the stress attributed to spaceflight. The astronaut's skin, as in diabetic patients, gets thinner and drier over time when in space. This makes it itchy and scratching may lead to skin damage and wound formation. This is a very important contributory factor making them prone to infections. Astronauts have also been shown to have delayed wound healing, which is somewhat similar to diabetic patients, the majority of the latter suffering from ischaemia and peripheral arterial disease, preventing immune cells from reaching the ulcer. On a similar level, the astronaut's immune system is perturbed which would make it more difficult for them to fight wound infections more so if the bacteria which are causing the infection may have acquired resistance due to the stresses of space (Farkas and Farkas, 2021; Gardiner et al., 2017; Gary Sibbald and Woo, 2008). The findings may therefore lead to a better understanding of the bacterial diversity found in the DFUs under two very starkly different environmental conditions yet touching on the common field of antibiotic resistance and general adaptation to thrive. Having a better insight of those bacterial communities in these DFUs, as well as their mutations, would undeniably lead to knowledge and data upon which better patient treatment and management can occur, ultimately improving the clinical outcome of these patients as well as shed some light on how the microbiome responds and adapts in space which may be important for future space exploration (Ichijo et al., 2020; van Asten et al., 2016).

Here we show, for the first time, human skin tissue samples obtained from T2DM foot ulcers together with their skin microbiome, being exposed to spaceflight conditions whilst simultaneously running an analogue experiment here on Earth. The objective of this work was to study the human skin microbiome from Maltese T2DM patients and to determine whether any important differences in microorganism species were observed. This article reports on Malta's first ever mission to space, and the molecular genetic studies are limited to 16S rRNA gene

sequencing. Future work will focus on whole metagenomic sequencing and comparative genomics between the biological samples.

1.1. Results

1.1.1. Microbiome signature, diversity is correlated to DFU disease status and tissue type

We aimed to identify if microbiome signature was associated with tissue type. We collected six kinds of samples: 1) Skin swabs taken from the contralateral foot of diabetic patients 2) Diabetic Foot Ulcers (DFUs, also referred to as "Tissues"), 3) DFUs that were sent to the ISS, 4) DFUs that were kept on Earth during the duration of the space mission (the control samples) and analyzed when the ISS samples were returned from orbit, 5) Biofilm samples taken from DFU after debridement, and 6) negative, uninoculated, controls. While the negative controls did have some microorganisms, these were not identified as likely contaminants within our other samples (see *Methods*).

We identified and annotated Operational Taxonomic Units (OTUs) in 16S sequencing data from all sample types and computed the relative abundance of each unique taxonomic annotation by normalizing across sample sequencing depth. Using two different dimensionality approaches (FIG 2A and 2B), we identified discrete differences in overall sample structure between the different tissue types. Skin swabs clustered together in both cases, with other samples demonstrating less tight clustering. For example, samples from the ISS did not appear to group together. The second tightest clustering occurred among the Tissue (DFU) samples.

The alpha diversity and taxonomic richness varied as a function of tissue type (Figure 2C). The skin swabs had the highest Shannon diversity, Simpson diversity, and richness, with statistically significant differences (adjusted p-value <0.05) *via* a Wilcoxon test compared to all other sample types. The ISS Tissue samples additionally had higher average values for all diversity metrics compared to the biofilm samples (adjusted p-value <0.05 in all cases). These samples were in fact higher in diversity on average than all other sample types, except they only were trending towards adjusted significance in comparison with the richness of DFU tissue samples not used for comparison to space flight (i.e., "Tissue", adjusted p-value $= 0.06$) as well as the Simpson diversity Earth control DFU samples that were used for comparison to flight (adjusted p-value $= 0.09$). Finally, the biofilm samples had higher Simpson and Shannon diversity overall than the Tissue samples (adjusted p-value <0.05). The full analysis is available in (Supplementary Table S1).

Analyzing beta diversity *via* Bray-Curtis distance yielded additionally clear structure among the sample types (Figure 2D). Akin to the output of the dimensionality reduction analysis, skin swab samples appeared highly similar to one another based on hierarchical clustering analysis. Most of the earth control samples, biofilms, as well as the T2DM tissue samples ("Tissue") clustered together. The ISS samples appeared to have the most heterogeneity in beta-diversity and interestingly did not cluster with the Earth control samples.

Independent analyses performed using ASAP2 (Tian and Imanian, 2022) provide results on alpha and beta diversity metrics that are in agreement (Supplementary Figure S1).

1.1.2. The prevalence and abundance of specific taxa discriminate between DFU tissue disease status and origin

Having identified distinctive microbiome structure across the different tissue types, we aimed to determine if specific taxa were responsible for such variation. Averaging the abundances of all taxa across each sample type revealed distinct patterns in terms of prevalence and abundance of specific organism groups (Figure 3A). Most phyla were *Firmicutes* or *Proteobacteria* (Figure 3A, bottom bar on heatmap), however the "core" microbiome shared among all samples did not fall into these categories. In some cases, hierarchical clustering revealed members of specific phyla grouping together and association with a specific sample type (as was the case for *Actinobacteria* and "Tissue" samples).

The skin swabs, as we observed in the richness analysis, had the largest number of taxa represented in the greatest prevalence. Conversely, the ISS samples had a very small number of moderately prevalent, pan-phyletic taxa. They did not look similar to any particular sample type, with some taxonomic annotations shared with all other kinds of tissue types.

Observing only the most abundant taxa in each sample group further discriminated between sample types. In most cases, the variation in abundances was so large that the standard deviation on these values was greater in magnitude than the average abundance overall, crossing into negative values. However, both the ISS Tissue samples and the biofilm samples had consistently high abundances of *Pseudomonas*. The ISS Tissue samples had consistently high abundances of *Morganella*. We noted that once again, these ISS samples were characterized best in the sense that they shared specific taxa with all other sample types, yielding a heterogenous microbiome that still fell within the realm of skin and DFU-associated microbes. These observations were in agreement with those reported by ASAP2 analysis (Supplementary Figure S2).

Finally, to explore more than just the high abundance taxa, we executed a Microbiome-Association-Study via a linear mixed modeling approach, reporting the complete results in Supplementary Table S2. Accounting for inter-individual variation by adjusting for patient identifier with a random effect (as certain samples of different tissue types were taken from the same patient), we computed the association between all taxonomically distinct OTUs identified ($N = 220$) and a categorical variable indicating the origin of a given sample. We adjusted for False Discovery Rate (FDR) and separated the coefficients on each of the levels in the categorical variable into separate plots in Figure 4A. We chose the skin swabs as a reference group given 1) their 'healthy' state and 2) their overall consistency in microbiome structure, as observed in Figure 2D.

Overall, these results showed that every sample type was lacking in specific genera relative to the skin samples. These included *Sphingobacterium, Stenotrophomonas, Brevundimonas, Staphylococcus, Enterobacteriaceae, Comomonas,* and *Elizabethkingia*. We also identified small but statistically significant, positive associations with the negative control samples (Figure 4B). We were encouraged to find that these significant taxa did not overlap with the significant taxa for the other samples, further indicating a lack of contamination. As in Figure 3B, the ISS samples were identified as being uniquely abundant in *Morganella*.

2. Discussion

The primary objective of this study was to expose human skin microbiome samples obtained from diabetic foot ulcers to spaceflight, microgravity and high cosmic radiation lasting around 30 days. The human skin tissue samples and microbiomes that formed part of the first mission now form part of an important biobank and sample repository to further test using whole genome sequencing (WGS) on both the tissues and the microbiome present and therefore we may see/have some results with regards to the genetics of these samples as compared to the same ones kept on Earth e.g. changes in resistance mechanisms in the microbiome. Malta has now sent its very first experiment to the ISS, choosing a biomedical science experiment with important clinical relevance to the country. Understanding the role of the human skin tissue microbiome from DFUs is critical to the understanding of the type and quantity of different bacteria present that may be acting as pathogens. Studying the same microbiome under different conditions such as spaceflight, microgravity and a higher solar radiation than that of earth, is one way to probe and test the adaptation and survival of these bacteria to harsh conditions. This is likely to have important implications for both space for space applications, and space for earth applications. In the case of space for space, studying the effects of spaceflight on microbiomes such as in Maleth I assure that missions to the moon, mars and deep space will benefit from greater knowledge on how microorganisms behave over extended and long periods of time in space. Project Maleth also makes a use case of how the International Space Station is used as a test bed for life and health sciences research. In space for earth applications, there is an immediate use-case of using space as an asset for life sciences research. Areas such as cell culture, flow chemistry, and the use of microgravity to create unique 3D cell culture

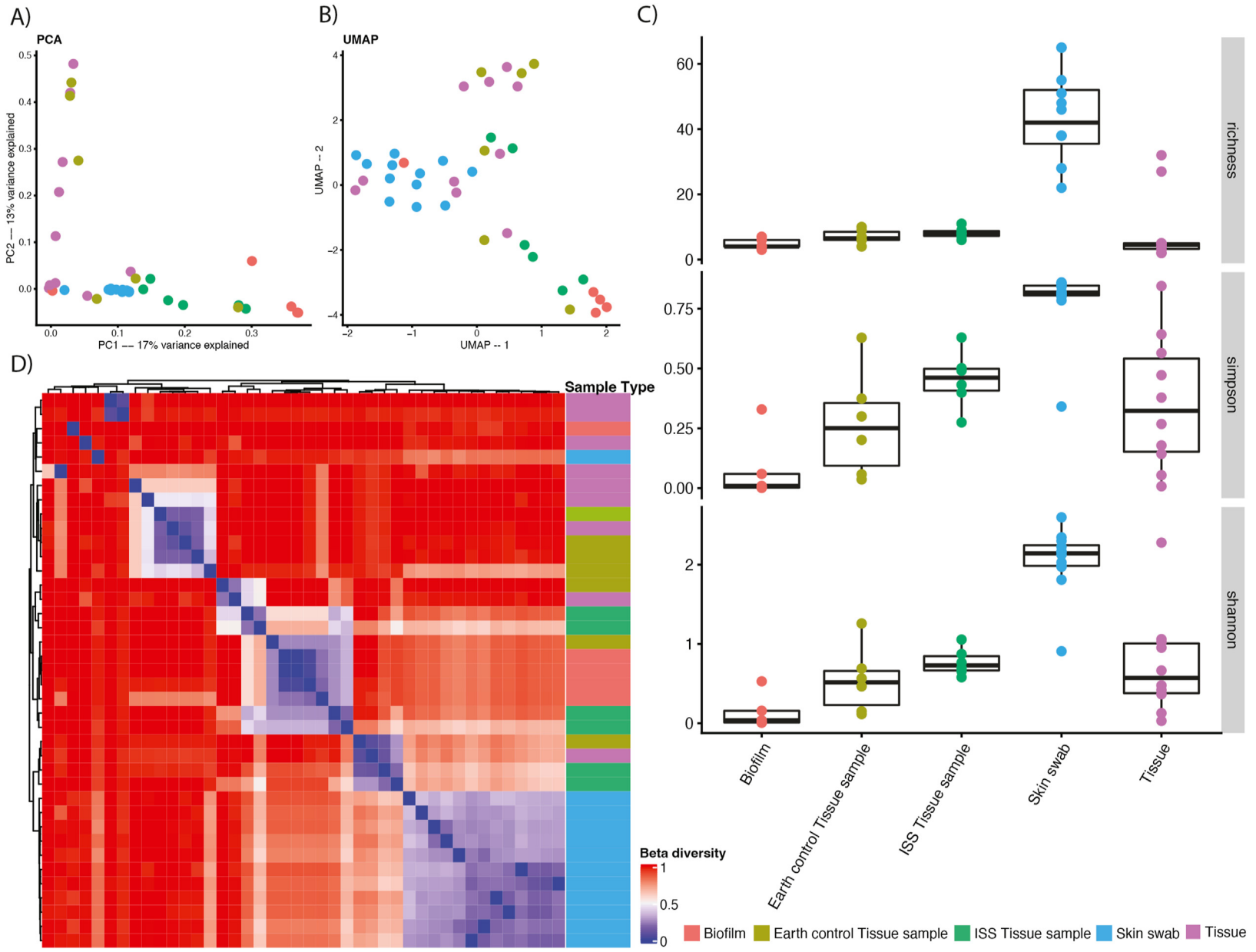

Figure 2. The microbiome structure of DFU tissues in the context of space flight. Colour legend on the bottom of figure. A–B) Principal component and UMAP analysis on the relative abundances of taxonomically distinct Operational Taxonomic Units in our cohort. C) Alpha diversity metrics as a function of sample type. D) Beta-diversity in terms of Bray-Curtis distance across all sample types.

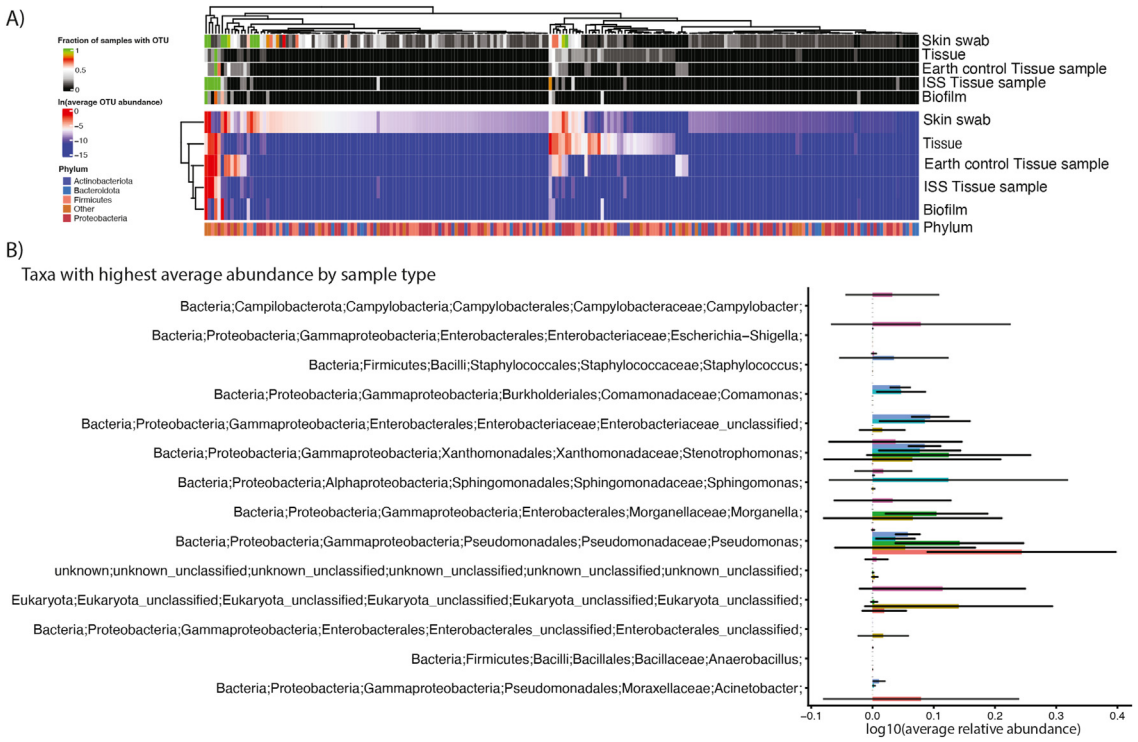

Figure 3. Identifying specific microbes that delineate between sample types. Legend with colours, which correspond to sample types, is on the bottom of figure. A) The top heatmap (mostly black) indicates the fraction of samples a given taxonomy (columns) is present in, by sample type (rows). The bottom indicates the average abundance of each given taxonomy by sample type. Both heatmaps are hierarchically clustered according to the average abundances reported in the bottom heatmap. The lower bar (predominantly purple) indicates the phylum of origin for a given taxon. B) We computed the top five taxa with the highest average abundance by sample type. We report abundance for the union of all of these microbes for all sample types.

models that are not easy to replicate on earth are directly relevant. Project Maleth also stimulates the use of laboratory infrastructure in space.

In our set of experiments conducted as part of Maleth's first mission, gram positive organisms were not cultivated after 30 days for both the ISS and ground-control Earth samples. One possible explanation can be the choice of buffer as a medium. Perhaps the phosphate buffered saline is not selectively good for these microorganisms. There was however one important exception. In patient 9 - *Enterococcus faecalis* which is a gram-positive coccus was isolated from all the samples. Remarkably; Pseudomonas, Stenotrophomonas and *Morganella* were detected in all the samples obtained back from the ISS (Supplementary Table S3). It appears as if the gram-negative rods thrive more. The exact mechanisms and reasons as to why remains yet to be seen. Using 16S typing, and looking at the most abundant genera for our samples; Patients 5 and 9 were consistent all throughout the different mission points of the experiment (Supplementary Table S4). Similar to what was observed using standard microbiology, Pseudomonas was detected for Patient 6 in both earth and ISS samples however not on day 1 of the experiment. For Patients 2 and 8 - both gram positive rods were detected in Earth and Day 1 mission points however this was not observed in the ISS samples. From our results of the Maltese T2DM DFU microbiome mission to ISS, it could be stated that aboard the ISS the gram-positive rods were the least abundant. In Patient 3, Stenotrophomonas (which used to form part of the Pseudomonas genus) was the most abundant in the ISS samples as compared to Day 1 and ground-control.

This very first study served to identify all the bacterial species present in DFUs, and how they adapt or vary when exposed to different environments including harsh ones such as space. Indeed, our results show that certain microorganisms had a higher relative abundance with regards to sample type and in comparison, between earth-bound samples as opposed to space-borne samples. The most common Gram-negative pathogens identified from our samples were *Proteus mirabilis* and *Morganella morganii* amongst others. Such bacteria, when present in higher concentrations, especially at the site of heel injury, ulcers and infections such as those presented by diabetic patients, may become fatal if left untreated or unattended (Ghosh et al., 2009) The behaviour and monitoring of such microorganisms under extreme conditions such as space can yield interesting observations that sheds light on their adaptation mechanisms, and drug-resistant genetic changes. *Proteus mirabilis*, appears to have capabilities of secreting ammonia and other volatile compounds that puts it in a competitive advantage vs. the rest - thus grows more in relation to other bacteria within the same microbiome. Such observations would necessitate further follow-up and research in order to validate the findings and determine whether replicate missions and experiments will yield same or similar results as observed in Maleth I (Juarez et al., 2020) Project Maleth's next objectives shall be looking at the full metagenomic sequencing of the tissue samples, and controls to determine important nucleotide differences if any and whether they affect the resistance to drugs, as well as obtain a full genomic profile of each microorganism present in the samples of the three categories and mission points.

These observations and findings regarding drug- and multiple drug-resistant microorganisms in the context of space travel and exploration play a very critical part of astronaut well-being and overall health. One would not wish to develop multi-drug resistant microorganisms present in the human gut, oral mucosa or skin whilst on deep space missions as the ramifications of medical health and interventions become painfully difficult to muster and control when far away from earth. Therefore, an extensive catalogue of human microbiomes (oral, skin, and gut including others) and their behaviour and adaptation in space is of paramount importance in this decade and others that follow. A thorough understanding of the genetics, and molecular mechanisms of the full microbiomes will benefit both patients on earth for better treatment modalities, and astronaut protection from variant or drug-resistant microorganisms in deep space missions. Such practice must also be carried out and interpreted in the light of other bacterial species that colonise parts of the International Space Station habitats (Bijlani et al., 2021) and healthy astronauts working in space on board the orbital station itself (Voorhies et al., 2019).

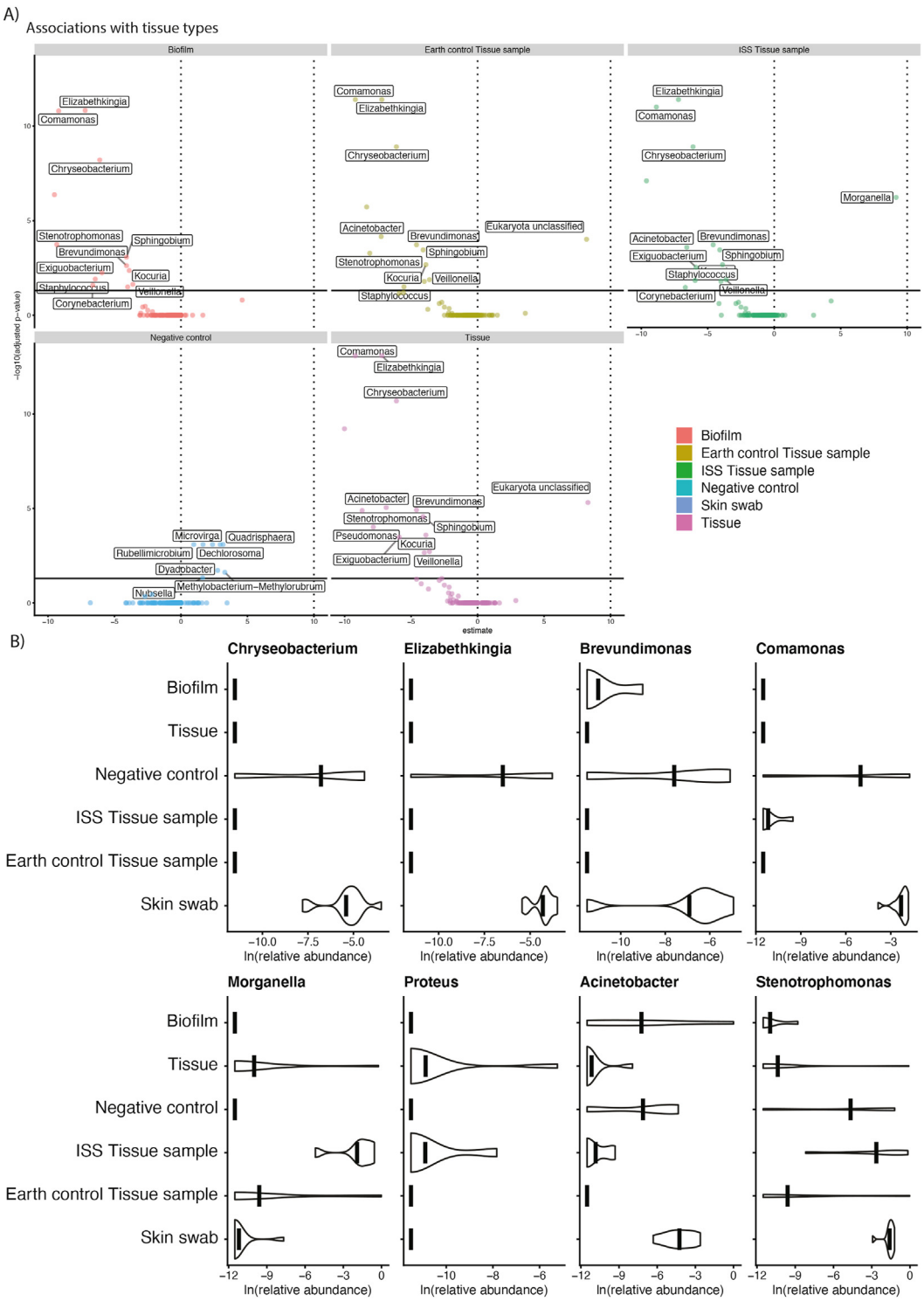

Figure 4. A) A Microbiome-Association-Study, with Control samples as the reference group. X-axes are the beta-coefficients. Y-axes are the negative log10 adjusted p-value. Solid line corresponds to statistical significance (adjusted p-value <0.05). We labelled all taxa with a Genus-level annotation that were statistically significant. Middle dotted line in each plot corresponds to a beta coefficient of 0. B) Violin plots showing the relative abundance of a particular bacteria with regards to sample type.

3. Limitations of the study

An important limitation was the fact that the ISS tissue samples were not snap frozen upon arrival back to earth, but were processed a couple of weeks later until arrival at our laboratories in Malta.

Another limitation was the fact that the experiment on board the ISS only allowed us to test six biological replicates, however this shall also be followed up in subsequent experiments under the Maleth Program's second and third missions to the International Space Station.

Consortia

JB and PM who form part of a consortium of Space Omics Topical Team aims to support the use of omics research among the ESA space

biology community. The members of the Space Omics Topical Team are European affiliated scientists landing at the Space Biology research field as Principal investigators from spaceflight biological experiments or as bioinformatics experts.

Declarations

Author contribution statement

Christine Gatt: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Braden T. Tierney; Pedro Madrigal; Christopher E. Mason; Afshin Beheshti: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Anja Telzerow; Vladimir Benes: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Graziella Zahra: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jurgen Bonett: Kevin Cassar: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Joseph Borg, Ph.D, M.Sc, B.Sc: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Christine Gatt was supported by Università ta' Malta [Government Scholarship].

Data availability statement

Data associated with this study has been deposited to NASA's Gene Lab database (Shayoni et al., 2019) at https://osdr.nasa.gov/bio/repo/data/studies/OSD-487.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at 10.1016/j.heliyon.2022.e12075.

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Appendix B

STAR methods

Key resources table

Reagent or Resource	Source	Identifier
Biological samples		
Tissues from diabetic foot ulcers	Mater Dei Hospital	Tissue
Biofilm from diabetic foot ulcers	Floriana Health Centre	Biofilm
Skin swabs from intact skin of contralateral foot	Mater Dei Hospital	Skin swab
Tissues sent to ISS	Mater Dei Hospital	ISS Tissue sample
Tissues kept on Earth during Project Maleth mission	Mater Dei Hospital	Earth Control Tissue sample
Negative controls	Mater Dei Hospital	Negative control
Chemicals, peptides, and recombinant proteins		
Animal Tissue lysing buffer	QIAGEN	Cat # ID: 939011
Proteinase K	QIAGEN	Cat # ID: 19133
Phosphate Buffered saline	Sigma-Aldrich	D8662-500ML
Critical commercial assays		
QIAamp® DNA Mini QIACube kit (240)	QIAGEN	Cat # 51326
KAPA HiFi HotStart ReadyMix PCR kit	Roche	Cat # 07958935001
4202 NEXTflexV1V3 kit	PerkinElmer	NOVA-4202-04
Agencourt AMPure XP Magnetic Beads	Beckman Coulter	A63881
Deposited data		
GLDS-487 https://osdr.nasa.gov/bio/repo/data/studies/OSD-487		
Oligonucleotides		

(continued on next column)

(*continued*)

Reagent or Resource	Source	Identifier
Biological samples		
Forward CTCTTTCCCTACACGACGCTCTTCCGATCT Reverse CTGGAGTTCAGACGTGTGCTCTTCCGATCT	Integrated DNA Technologies (IDT)	
Software and algorithms		
Amplicon Sequence Analysis Pipeline	https://github.com/tianrenmaogithub/asap2 (Tian and Imanian, 2022)	ASAP v2.2
Scripts to analyze 16S rRNA amplicon sequencing data	https://github.com/Project-Maleth	

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author Prof Joseph Borg (joseph.j.borg@um.edu.mt).

Experimental model and subject details

10 tissue samples were collected from patients presenting with severe diabetic foot ulcers at Mater Dei Hospital. These samples were collected aseptically during amputation surgery. 5 biofilm samples were also obtained from patients presenting with less severe diabetic foot ulcers at the Floriana Health Centre, which is a primary Health Care Facility. The biofilm samples were collected aseptically during ulcer debridement as part of the required treatment. 12 skin swabs were obtained from the abovementioned patients, either on the day of debridement or a day after the amputation surgery. The swab was moistened with Phosphate Buffered Saline (PBS) and an area of the healthy skin from the contralateral foot was swabbed. The inoculated swab was then placed in PBS and transported to the lab for processing. Three patients were not swabbed as they discharged themselves the day after surgery.

In total 15 patients were sampled, 8 males and 7 females with ages ranging from 52-84 years. Out of the 10 tissue samples collected, 6 were chosen for Project Maleth. Sample collection was approved by the University of Malta, Faculty Research Ethics Committee (FREC) of the Faculty of Health Sciences. Informed consent was obtained from all patients.

Method details

Processing of samples for DNA extraction

The tissues and biofilm samples were cut aseptically into several pieces in a Biosafety cabinet. One piece was placed in a Griffiths tube homogenizer and mashed to extract the bacteria and tissue juices. Several agars were inoculated, namely two blood agars (where one was incubated anaerobically and the other was incubated in 7% CO_2 conditions), a MacConkey agar which was incubated in air and a Neomycin agar which was anaerobically incubated. Enrichment media were also inoculated i.e. Cooked Meat Medium (CMM) and Tryptone Soya Broth (TSB). These were incubated for three days and they were then sub-cultured onto two blood agars (where one was incubated anaerobically and the other was incubated in 7% CO_2 conditions) and a MacConkey agar plate which was incubated in air. The macerated tissue and the rest of the extracted liquid were then placed in a small Eppendorf tube and 180μl of Animal Tissue Lysing buffer and 20μl of Proteinase K were added. This was then placed in an incubator at 56 °C until the tissue/biofilm was completely lysed. This method was performed following the QIAamp DNA Mini Blood Mini Handbook. The culture plates were then observed and all the different bacterial colonies cultured were identified by Matrix Assisted Laser Desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The skin swabs taken from the contralateral foot were vortexed. Several agar plates were inoculated i.e. two blood agar plates (where one was incubated anaerobically and the other was incubated in 7% CO_2 conditions), a MacConkey agar which was incubated in air and a Neomycin agar which was incubated anaerobically. The culture plates were then observed and all the different bacterial colonies that were cultured were identified by MALDI-TOF MS (see Figure 1).

Preparation of samples for Project Maleth

The six tissue samples that were chosen for Project Maleth were treated in the following way. Each original tissue sample was further cut into two other pieces. One piece was placed in a cuvette and immersed in Phosphate buffered saline. This tissue, which served as the Earth Control tissue sample, was kept in an incubator at 30 °C for the whole mission duration. This temperature was equivalent to that found on the ISS. The other piece of tissue was placed in another cuvette and immersed in PBS. The choice of PBS as medium for the microbiomes was made after following the results observed in a previous study by Liao and Shollenberger (2003).

The six cuvettes containing the tissue samples were mounted on the IceCube and sent to Cape Canaveral for a SpaceX CRS23 re-supply mission. These will be referred to as the ISS tissue samples. Once on the ISS, the tissues remained in orbit for thirty days until they returned back on Earth. Upon arrival at our labs in Malta, the ISS tissue samples as well as the Earth Control tissue samples, were processed for culture and lysed for DNA extraction as described above (see Figure 1).

DNA extraction

200μl of the lysed tissue/biofilm or 200μl of PBS from the skin swabs were transferred to an Eppendorf tube and placed in the QIACube for DNA extraction using the QIAamp® DNA Mini kit. After DNA extraction, he DNA concentration was measured with Qubit.

Negative control samples were processed in the same way to determine and exclude any reagent contamination. The three negative controls consisted of uninoculated PBS, uninoculated RNase/DNase free water and an uninoculated swab that was vortexed in PBS.

16S rRNA gene sequencing

After DNA extraction, the samples were processed for 16S rRNA gene sequencing. The first PCR was performed by taking 10μl of the 20ng of gDNA and performing the following thermal cycling conditions (98 °C for 2 min, then 30 cycles of 98 °C for 10s, 65 °C for 20s and 72 °C for 20s. The final extension was at 72 °C for 2 min). The following primers were used; Forward CTCTTTCCCTACACGACGCTCTTCCGATCT; Reverse CTGGAGTTCA-GACGTGTGCTCTTCCGATCT. The Primermix is based on Caparose et al., (2011) targeting the V4 region. The second PCR was then performed using the same thermal cycling conditions however, to each different sample a specific barcode tag was added. The samples were then loaded on 1% Agarose gel to check if the samples were amplified to the right size. The samples were then pooled and sample clean-up was performed by magnetic beads. The DNA concentration was then determined again by Qubit and the DNA library was analysed with Agilent Bioanalyzer to determine the size of the base pairs. The band should be between 300-600bp. Finally, the pooled DNA library was then denatured and diluted to 4pM. The PhiX library was also denatured and diluted to 4pM. The amplicon library and the PhiX Control were then combined and loaded into the cartilage. The flow cell and the loaded cartilage were placed in the Illumina MiSeq Next Generation Sequencer for sequencing.

Processing raw 16S sequencing data

We used Dadasnake, which wraps Dada2, to process our 16S samples (Callahan et al., 2016) This pipeline, which we ran with the default settings, includes removing primers from samples, identifying and taxonomically classifying Operational Taxonomic Units (OTUs), and quantifying the number of reads aligning to each OTU. In a post-processing step, we merged OTUs with the same taxonomic annotation and computed their relative abundance by dividing total reads aligned by the total reads in a sample. We checked for contaminants using the decontam package (Davis et al., 2017) based on the control samples.

Quantification and statistical analysis

All bioinformatic and statistical analysis was executed in R 4.1.1 (Team, n.d.). We used the packages tidyverse, ggplot2, and lme4 for data handling and mixed modeling (Bates et al., 2015.; Wickham n.d.; Wickham et al., 2019). We analyzed the processed 16S data to determine if there were microbiome differences between the different sample types: T2DM DFU human skin tissue taken immediately after sampling, earth-bound tissue for comparison with International Space Station (ISS) treated identically and processed upon return of the ISS samples, ISS-bound tissue, healthy skin swabs, and biofilms from DFUs. We used the umap package for dimensionality reduction analysis (McInnes et al. 2018). For comparison, we additionally used the prcomp () function in base R. We computed Simpson, Shannon, and beta diversity for our samples using the vegan package (Dixon 2003).

For the Microbiome-Association-Study (MAS), we used a linear mixed modeling approach to identify associations between microbial taxon abundance (dependent variable, continuous) and sampling site of origin (categorical, 5 levels as it did not include the negative control). The reference group were healthy skin swabs. We adjusted for repeated measures from the same individual using a variable indicating patient ID. We adjusted for multiple hypothesis testing with Benjamini-Yekutieli correction and considered an adjusted p-value of 0.05 as statistical significance and 0.1 as trending towards significance (Benjamini and Yekutieli 2001).

In parallel we ran ASAP2, a pipeline for amplicon sequence variant (ASV)-based amplicon sequencing analysis automatically and consistently (Tian and Imanian, 2022), with taxonomic classifier weighted Silva 138 99% OTUs full-length sequences (Kaehler et al., 2019) '-c silva-138-99-nb--weighted-classifier.qza' downloaded from https://docs.qiime2.org/2021.8/data-resources/.

Data and software availability

Code availability: https://github.com/Project-Maleth/amplicon-sequencing.

Additional resources

QIAamp DNA Mini Blood Mini Handbook URL: https://www.qiagen.com/us/resources/resourcedetail?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en.

QIAamp DNA Mini kit protocol URL: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiaamp-dna-kits/?catno=51306.

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