Microgravity impact on the skin microbiota – A bacterial analysis of ISS internal surfaces samples

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INTRODUCTION

The International Spatial Station (ISS) is a hot and humid confined environment submitted to microgravity and cosmic radiations. The temperature and humidity are controlled inside the cabins through a control system (Environmental Control and Life Support System). Temperature regulated at 20.5 +/- 3.0°C and hygrometry level at 60% in average enable microbiological growth, development and even invasion by biofilms inside the compartments of the station. Living beings in the ISS receive a dose of 1 millisievert per day, which is equivalent to the dose received from radiation on Earth for 1 year [1]. The level of microgravity ranges varies from 10^{-3} to 10^{-6} g [2]. Focus on the microgravity impact is due to the fact that this factor represents the major influence on bacterial growth kinetics and bacterial cell behavior during short orbital flights [3].

Despite appropriate precautions, the main source of contamination of the intern surfaces of the ISS remains the astronauts themselves or more precisely their microbiota. This is leading to potential microbiological hazard as the ISS internal surfaces are contaminated by the astronauts' microbiota released by natural desquamation which brings around 30 bacteria by squama [4]. Inside the compartments, biofilms formation, metal corrosion but also health issues are risks for the space crew and this risk is increased by the growing duration of the space expeditions. In this context, the question arises of understanding the microgravity, most impactful factor of stress in the ISS, on skin bacteria and their virulence [5] .

The bacteria present in these conditions are subjected to various stresses. Microorganisms undergo certain genetic and physiological changes to adapt to stress conditions [6]. These modifications can induce an increase in biofilm formation [7], in virulence [8] or in antibiotic resistance [9]. These modifications can also in some cases affect the production of secondary metabolites. The production of secondary metabolites may increase, decrease, or remain unchanged in microgravity [5]. There is no universal bacterial response to microgravity. All these modifications can cause risks for the health of astronauts, so it is essential to study bacteria, and their evolution due to microgravity.

In order to assess the difference of bacterial response during and after submission to microgravity, we fortunately had the opportunity to work on swab samples from the internal surface of the Russian compartment sluice of the ISS. From those swabs, we targeted skin microbiota species by metagenomic identification and revivified few bacteria by cultural methods. Here, we decided to investigate the responses of these bacteria still submitted to the microgravity condition and their response after a re-acclimatisation to the terrestrial gravity.

MATERIAL AND METHODS

Study material

Swabs from the Russian ISS access sluice obtained from collaboration with ESA partners at the Moscow University were submitted to culturomic analysis based on previous metagenomic results. Four aerobic bacterial strains were revivified, isolated, identified and immediately cryogenized at -80°C in cryotubes at T0 and labelled as R0. They were subcultured for 40 times every 24 hours to let then reacclimate to terrestrial gravity and environment and cryogenized again as R40. Their physiology (growth, resistance to antibiotics, cytotoxicity, inflammatory potential) was characterized immediately at T0 (R0) and after 40 generations of reacclimating to terrestrial conditions (R40). The strains were grown in Tryptic Soy Broth (TSB) liquid medium or Tryptic Soy Agar (TSA) solid medium at 37°C for 24 hours.

Identification

After biochemical orientation tests (catalase, oxydase) and Gram coloration, the strains were submitted to metabolic identification using API® strips (BioMerieux, Craponne, France): ID32 Staph for Staphylococcaceae. The same isolates were also submitted to sequencing of the 16S ribosomal RNA by DNA sequencing (Sanger sequencing services, Genewiz). DNA was extracted from an overnight culture in tryptic soy broth (TSB) at 37°C with a genomic extraction kit (GeneJET genomic DNA purification kit, catalog number K0721; Thermo Scientific) following the supplied procedure, with pretreatment using a lysis solution (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 1.2% Triton X-100, and 20 mg/ml lysozyme).

Bacterial growth studies

Bacterial growth curves were assessed by spectrophotometry using the Spark Multimode Microplate Reader TECAN (Research Triangle Park, NC, USA). The bacteria were brought to an optical density (OD) of 0.16 and then seeded in a 96-well microplate with TSB medium. The OD at 580nm was then measured every 15 minutes for 24 hours. To determine

the equivalence of OD in Colony Forming Unit (CFU)/mL, the OD of strains ISS-A, ISS-8, ISS-17 and ISS-20 at R0 and R40 were measured. Once OD 0.8 is reached, a cascade dilution is performed to obtain dilutions of 10^{-3} to 10^{-6}. These dilutions are inoculated on TSA agar with a duplicate rake. Then, the operation was repeated for the OD 1 and OD 1.2. The boxes are incubated at 37°C, then counted after 24 hours.

Antibiotic sensitivity

The antibiotic susceptibility test was performed following the disc diffusion method in triplicate. For each strain, an inoculum at a concentration of 10^7 CFU/mL was seeded on Mueller-Hinton agar. The antibiotics used included Linezolid 10 µg; Cefoxitin 30 µg and Erythromicin 15 µg. These antibiotics were selected according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and according to the availability of the laboratory. The bacterial strains were cultured in TSB and brought to 0.5 MacFarland, then were spreaded on Muller Hinton (MH) agar plates. Sensitivity test discs (ThermoFisher Scientific, Eugene, OR, USA) were placed on the MH agar (3 per dish) then incubated at 37°C. The diameter of the inhibition zones around the antibiotic discs were measured after 24 hours of incubation.

Cells culture

The human epidermal keratinocyte HaCaT cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, USA) supplemented with 20% fetal bovine serum (FBS; Sigma-Aldrich, USA) and 100 mg/mL of penicillin, and streptomycin (Sigma-Aldrich, USA) at 37 °C with 5% CO2 and 95% humidity. For experimental assays (cytotoxicity), the cells were seeded at a density of approximately 10^5 cells/cm^2 in 24-well tissue culture plates with the medium regularly changed and incubated until 80% of confluence.

Cytotoxicity

The cytotoxicity of the four strains ISS-A, ISS-8 was assessed on the keratinocyte cell line HaCaT. After 1 hour and 16 hours of incubation of the cells with the bacteria at 37°C, the supernatant from each well was collected and the LDH activity was determined using the CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Eugene, OR, USA), and strictly following the manufacturers' instructions. Control wells were prepared with HaCaT cells alone (negative control) or with 1% Triton X-100 (positive control). The percentage of cytotoxicity was determined following the formula provided with the LDH assay kit and then converted to percentage of relative cytotoxicity.

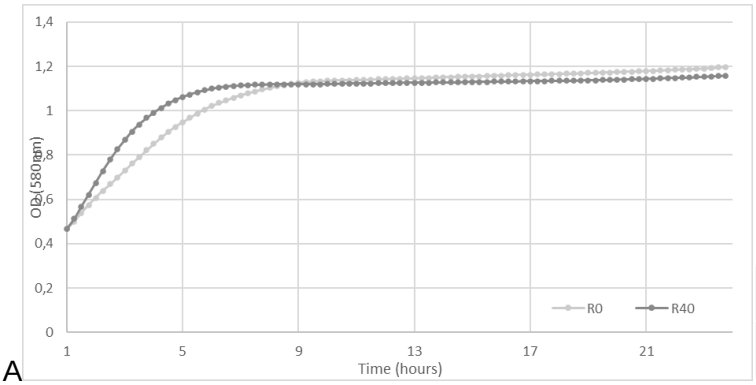
Statistical analysis

Statistical analysis was realized using Microsoft Excel and GraphPad QuickCalcs Software. The results were fitting with a normal distribution law so the significance of all other data was tested using the *Students' t-test*.

RESULTS

As expected, human skin microbiota bacteria (*Staphylococci*, *Cutibacteria*, *Corynebacteria*, and other rarer microorganisms) were identified on swabs. Revivification was successful and four aerobic strains were isolated and identified as different strains of *Staphylococcus epidermidis* (ISS-A and ISS-20) and *Staphylococcus aureus* (ISS-8 and ISS-17).

A



A	Generation time (hours)
R0	3,02 +/- 0,22
R40	1,66 +/- 0,24

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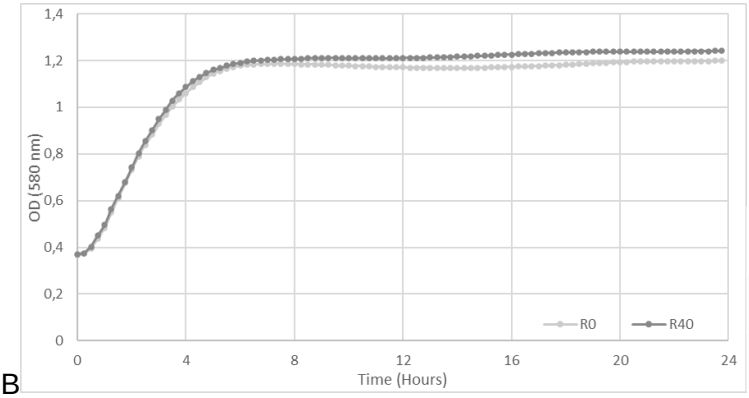
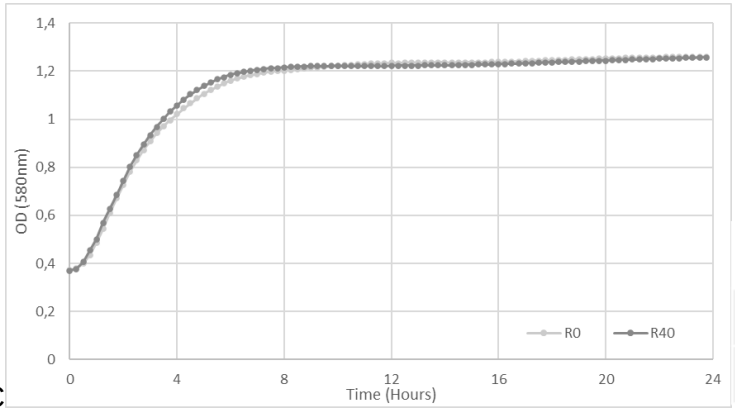
B



B	Generation time (hours)
R0	1,31 +/- 0,08
R40	1,32 +/- 0,02

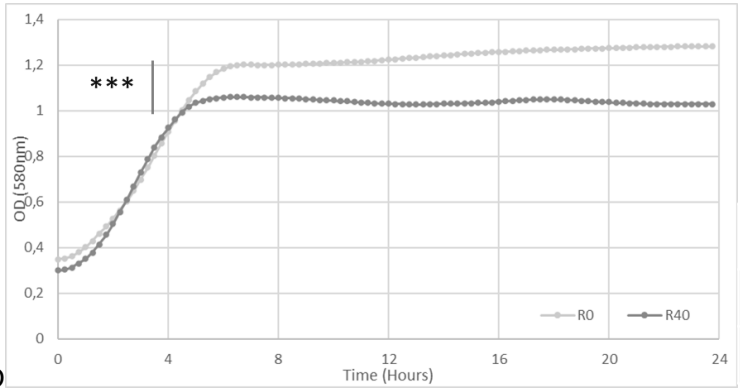
NS

C



C	Generation time (hours)	
R0	1,33 +/- 0,05	NS
R40	1,34 +/- 0,03	

D



D	Generation time (hours)	
R0	1,73 +/- 0,09	NS
R40	1,56 +/- 0,08	

Figure 1: 24 hours growth curves of four ISS bacterial aerobic strains at 37°C

Values are mean and standard deviation based on triplicate data (n=3). *Staphylococcus epidermidis* strain ISS-A (**A**), *Staphylococcus aureus* strain ISS-8 (**B**), *Staphylococcus aureus* strain ISS-17 (**C**) and *Staphylococcus epidermidis* strain ISS-20 (**D**) (** *P*<0.01; NS: non statistically significant)

No difference of growth kinetics was observed between R0 and R40 cultures for the two strains of *Staphylococcus aureus (***Figure 1B and 1C***)*. Strains ISS-8 and ISS-17 have similar growth curves, with a very short exponential phase and a stationary phase reached in average at a mean OD_{580nm} of 1.20. On the other hand, the results were different between R0 and R40 cultures for the two strains *Staphylococcus epidermidis*. ISS-A strain showed a higher generation time at R40 culture than at R0 culture (**Figure 1A**). After the re-acclimatisation to the terrestrial gravity, the strain has almost doubled its generation time. This could be a proof of a stress du to the microgravity but further investigations are needed to assess the link between mechanical effects on the bacteria and its capacity to divide and proliferate. For ISS-20, another characteristic was modified between R0 and R40: the stationary phase was reached at a mean OD_{580nm} of 1.20 +/- 0.01 for the R0 culture whereas for the R40 culture, it was reached at a lower mean OD_{580nm} of 1.05 +/- 0.03 (**Figure 1D**). We emitted two hypotheses: one being that the bacterial cells were bigger and the other one that their number increased. Further investigations are needed to confirm or invalidate one or other of these hypotheses such as performing flow cytometry to assess a difference of bacteria sizes or number.

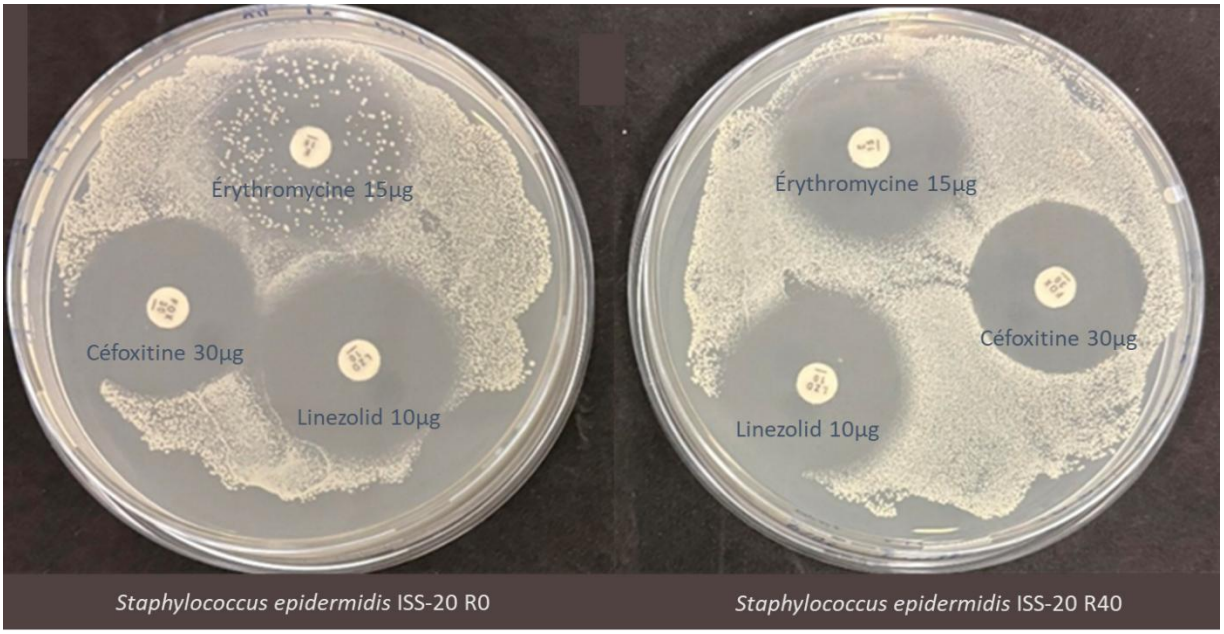
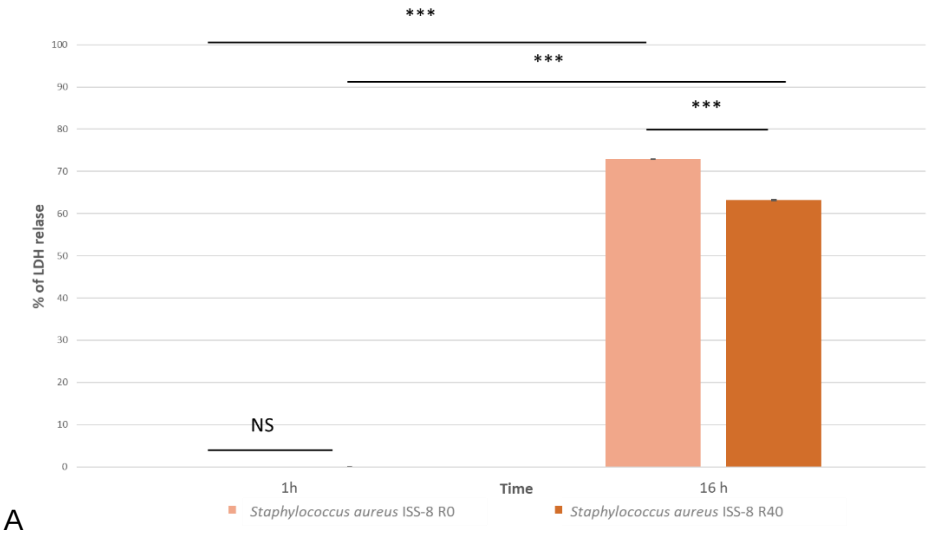


Figure 2 Antibiotic sensitivity of Staphylococcus epidermis ISS-20 at R0 and R40 Test were conducted in three separated replicates (n=3) which showed the same pattern.

After the inhibition halo measurements, the four strains were found sensitive to linezolid, and cefoxitin antibiotics according to EUCAST guidelines. Strains ISS-A and both *Staphylococcus aureus* strains ISS-8 and ISS-17 were resistant to erythromycin with a total absence of inhibition halo (data not shown). Based on a previous study of bacterial isolates collected from the ISS, most staphylococci were found resistant to erythromycin, and the data from this study confirm the transfer of resistance to erythromycin [10]

Remarkably, the antibiotic sensitivity profile of the *S. epidermidis* strain ISS-20 showed significant modifications at R40. Strain ISS-20 at R40 is sensitive to this antibiotic, as halos of inhibition appeared in triplicate (2.91, 2.72 and 2.81 cm). However, at R0, there were colonies present in the inhibition halo (**Figure 2**). This suggests that the strain has acquired some resistance to erythromycin under space station conditions and that this acquired resistance is reversible after submission to terrestrial conditions. It is possible that the plasma membrane of the bacteria undergoes modifications due to microgravity mechanical stress, these modifications leading to resistance by preventing the cytoplasmic penetration of the antibiotic and thus its action against protein synthesis. Further studies are needed to conclude on this hypothesis.



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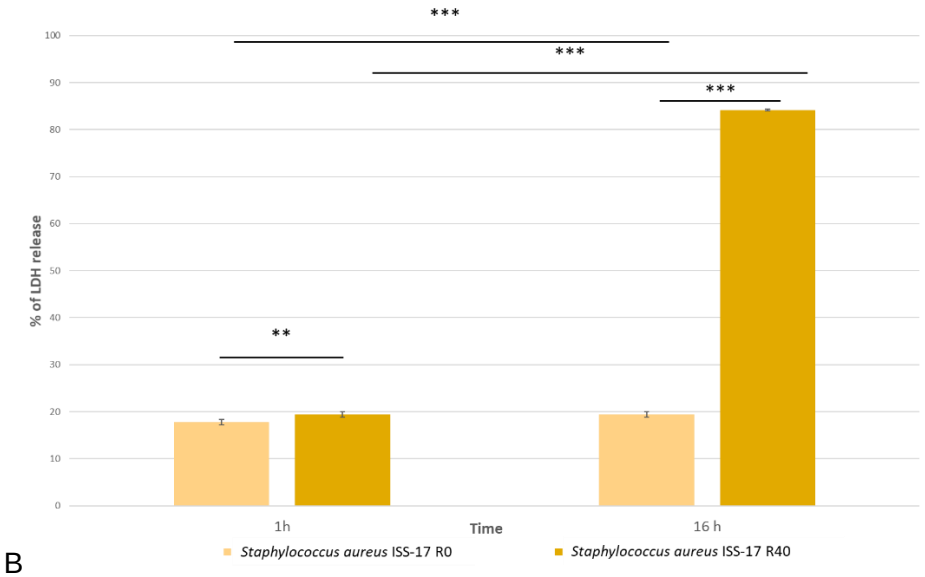

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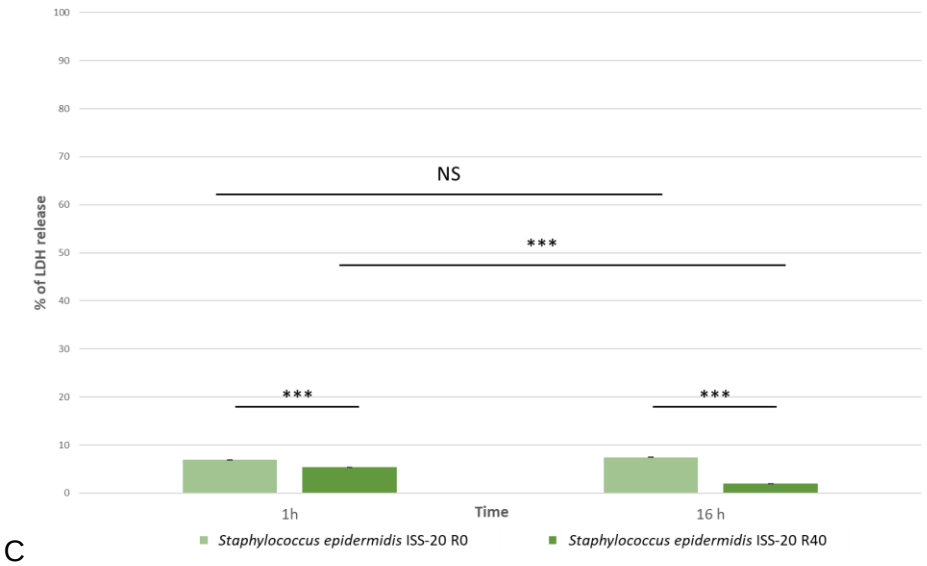

C

Figure 3 Levels of cytotoxicity for human keratinocyte cells (HaCaT) after 1 hour and 16 hours of infection with the three tested strains. The cytotoxicity levels are determined by LDH

release. The data shown include the cell viability measures. Cell total lysis was determined to calculate the ratio. Data for the three tested strains *Staphylococcus aureus* ISS-8 (**A**) and ISS-17 (**B**) and *Staphylococcus epidermidis* ISS-20 (**C**) are shown. Cells treated with 2% Triton-X 100 (v/v) and DMEM media were used as positive and negative controls, respectively. Data are pooled from three independent assays (n=3) and compared using student's t-test. (*** *P*<0001; ** *P*<0.01; * *P*<0,05; NS: non statistically significant)

Cytotoxicity levels were assessed for three of the four strains due to technical problem and short timeline so the results are presented for ISS-8, ISS-17 and only one of the *Staphylococcus epidermidis* strain, ISS-20 (**Figure 3A, 3B and 3C**). All strains demonstrated higher rate of relative cytotoxicity at 16 hours of contact than at 1 hour which is expected for a longer exposure and depending of the initiation start of the cells lysis process following infection. Interestingly, a difference is observed for all the strains between R0 and R40 cultures at 16 hours of contact. This difference is also observed for ISS-17 and ISS-20 strains at 1 hour of infection. ISS-8 strain showed such a low cytotoxicity that no significant difference between R0 and R40 was observed. This same strain showed a more remarkable result at 16 hours of contact with the HaCaT. Indeed, the strain is more cytotoxic at R0 than at R40 (respectively 72% and 63% in average) meaning that the microgravity conditions leaded to a statistically significant increase of its cytotoxicity. Moreover, this characteristic was reversible. Strain ISS-17 (*Staphylococcus aureus*) is the one with the highest percentage of relative cytotoxicity: after one hour of contact, it already reached an average rate of 19% (**Figure 3B**). Though it remains a low cytotoxicity especially for a *S. aureus* strain. At 16 hours of infection, the strain is four times more cytotoxic at R40 culture (average mean at 82%) than at R0, indicating that the reacclimatized strain is more cytotoxic than the one grown under the conditions of the space station. For both of these strains, further investigations are currently processed to understand the link between microgravity stress and modification of the cytotoxicity with a focus on the metabolites production. Strain ISS-20 (*Staphylococcus epidermidis*) had very low cytotoxicity (<10%) both at short and long contact with the HaCaT cells. This result was not surprising as it is consistent with the literature. Previous study reported that *Staphylococcus aureus* strains released a level of higher LDH than *Staphylococcus epidermidis* strains [11].

DISCUSSION

Among the bacteria still present and cultivable, four strains, anaerobic species, typical of the skin microbiota, have been identified. As demonstrated here, only one of the characteristics of one strain could appear and a different one depending, not on the species but rather on the strain. Influence on the generation time, on the antibiotic resistance and on

cytotoxicity were shown and are quite concerning for the safety of the space crew. This study reveals that space can impact both the bacterial resistance to antibiotics and their virulence. Moreover, we are currently investigating the microgravity impact on their response to CGRP and the first data suggest potential modifications of skin-microbiota interactions during space flights. For health safety reasons and with a view to long-term space travel, it is important and much needed to understand the impact of space conditions (microgravity, space radiation, confined environment, etc.) on these bacteria and their virulence factors. Particularly, it raises concern as micro-organisms are identified as a major source of material corrosion [12], [13] and biological hazard [14], [15].

These results have provided more insight into the behavior of microbiota strains under microgravity, but more analyses are needed. It would be interesting to carry out additional tests such as biofilms adhesion and formation. The ability of bacteria to make biofilms is an important virulence parameter, it has been shown that simulated microgravity influences the physiological processes that trigger bacterial fixation and biofilm formation [7]. Purple crystal staining or the use of confocal microscopy would make it possible to observe the dynamics of these strains within a biofilm. HaCaT Inflammation in absence and presence of the four strains is currently assessed as an indirect marker of cytotoxicity. For *S. aureus* ISS-8, the cytotoxicity on HaCaT keratinocytes showed variations in the absence or presence of the skin neuromodulator Calcitonin Gene Related Peptide (CGRP). The impact of spatial conditions on the inflammatory properties of the four revivified strains are in progress. These results already reveal that large transversal studies should be realized to determine the mechanisms involved in the effect of microgravity on skin microbiota homeostasis and anticipate potential dermatological health issues and thus dermo-cosmetics solutions for long-distance space flights.

The reversibility of the antibiotic resistance and the cytotoxicity is interesting as a tool to understand the link between microgravity and these modified characteristics. Currently, it is still not possible to conclude on the reversibility of these parameters. This could be highly enlightening for understanding the impact of microgravity-modified microbiota on its human astronaut host. Especially for the skin microbiota, as it was demonstrated that its responses are also affected by the presence of released chemical compounds from textiles [16] and in the presence of cosmetic products. In a perspective of long-term spatial missions, it brings a lot of interrogations on potential under-estimated hazards and thus, concern.

Keywords: Skin microbiota, microbiome evaluation, internal and external stress.

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