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BEXUS 30 STARDUST – PRELIMINARY RESULTS OF INVESTIGATION OF MICROBES IN THE STRATOSPHERE

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ABSTRACT

The stratospheric microbiome has been investigated several times using the methods of classical microbiology. In this experiment, we have combined them with some novel approaches.. Diversification of methods has helped better describe the collected microorganisms. The results of the experiment may help better understand the mechanisms of survivability of microorganisms in stratospheric conditions.

The microorganisms have been collected in the stratosphere by a sampling system equipped with six filters: three sampling filters and three filters placed between ever-closed valves as control filters. Two of the sampling ones provided the microorganisms for setting up cultures on agar media. Two control filters were treated similarly. The stratospheric microbiome has been compared to the microbiome of air collected from the place of the balloon's start, using an identical, on-ground system. All the parameters of the balloon's flight and the sample collection have been recorded.

The results have shown that the stratosphere is poor in microorganisms in comparison to the regular, tropospheric air. Most of the airborne bacteria in the stratosphere are gram-positive. Thick layers of peptidoglycan in their cell walls may protect them from the harsh conditions.

The experiment was launched on board BEXUS30 balloon sent within REXUS/BEXUS programme.

1. OBJECTIVES

The objectives of the Stardust project was to investigate the stratospheric microorganisms qualitatively and quantitatively, to examine properties of the cultivable strains from the stratosphere and to find new species or strains of bacteria.

2. INTRODUCTION

The first investigation of stratospheric microbiome took place in 1936 in the USA. It was done by Rogers and Meyer with a stratospheric balloon, floating at altitudes between 11 and 21 km a.s.l. Since then, many findings have been done with the use of balloons, high-altitude aircrafts and sounding rockets.

The studies conducted so far have confirmed the presence of the following microorganisms:

- 5 *Bacillus sp.* (Rogers and Meyer, 1936)
- *Micrococci* and spore-forming rods (Greene et al., 1964, Bruch, 1976)
- *Mycobacterium sp*., *Micrococcus sp.* (Imshenetsky et al., 1976)
- 5 *Bacillus sp.*, *Staphylococcus sp*. (Wainwright et al., 2003, Suresh et al., 2004)
- 2 *Bacillus sp.*, *Micrococci*, *Microbacteria, Staphylococcus sp., Brevibacterium sp.* (Griffin, 2005)
- 3 *Bacillus sp., Paenibacillus sp*. (Yang et al., 2008)

The majority of attempts to isolate microorganisms from stratospheric samples have been unsuccessful, showing that most of the cells are viable, but non-culturable. The stratosphere is an extreme environment for life even for microorganisms, it requires adaptation to low temperatures, low pressure and high exposure to ultraviolet radiation. Microorganisms obtained from the stratosphere must have the ability to survive the physical stresses of these environments and abolish their transfer to the conditions prevailing on the ground [2].
Because of difficulties with sampling, is still little known about the upper limit of the biosphere

Future collections should expand our understanding of the diversity, distribution and movement of microbes in the Stratosphere [3].
The authors have previously conducted a test mission with a small, two-filtered version of the system, in order to only isolate and sequence the total DNA from the samples. The results have not been published before. They suggested that the dominant genera of bacteria in the lower stratosphere are Enterococcus, Bacillus and Staphylococcus, which, however, does not mean that the collected cells were alive. Choosing metagenomics as the only method of investigating an environmental microflora may be misleading since the obtained DNA may come from either alive or dead microorganisms, though it may be helpful in discovering uncultivable strains [4][5].

3. EXPERIMENT SET-UP

The experiment was built from a sampling system, Valve Control Units (VCU), the main frame, insulation, an electronic compartment with its external parts and interfaces with the BEXUS30 gondola. The Sampling System was equipped with six filters (⌀25mm PES membrane with 0,2 µm pore diameter, sterile), each of which was controlled by two valves (inlet / outlet). Each element of the Sampling System was connected with silicone tubing (⌀6/10mm). The inlet pipes of the Sampling System were installed on the outside of the Stardust gondola, supported with a foam pipe insulation to keep them oriented horizontally. Three filters were used as sample collectors and the rest as controls. Two BOXER 3KD vacuum pumps were used to generate the air flow through the system. The valves were autoclavable. Two-way valves were used to secure the filters and a three-way valve was used for controlling the airflow through the whole Sampling System.

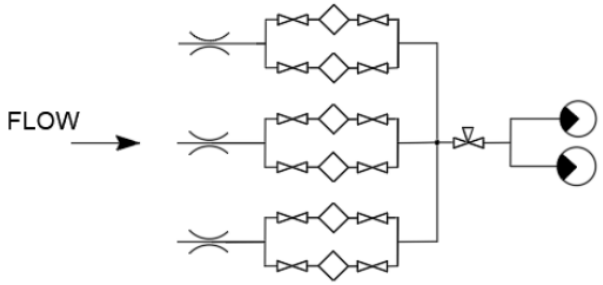
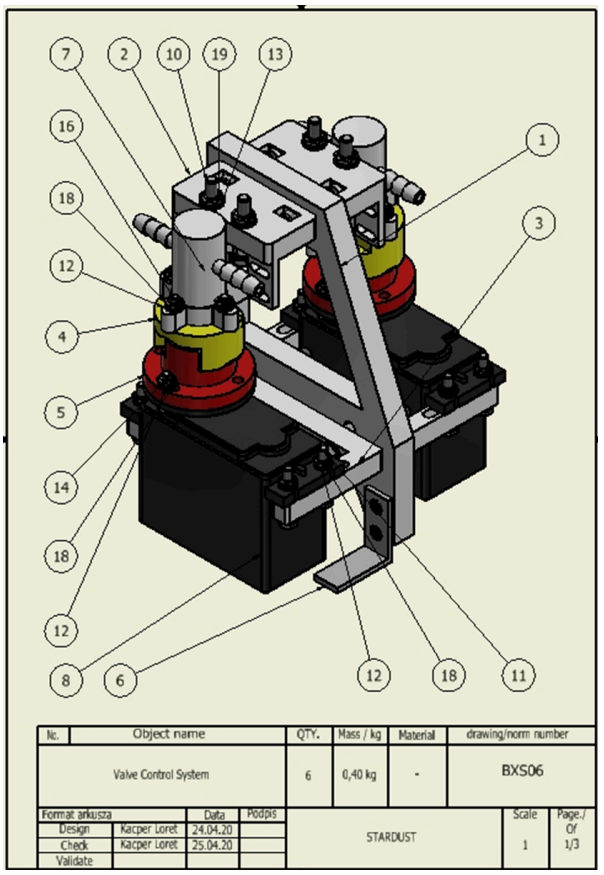
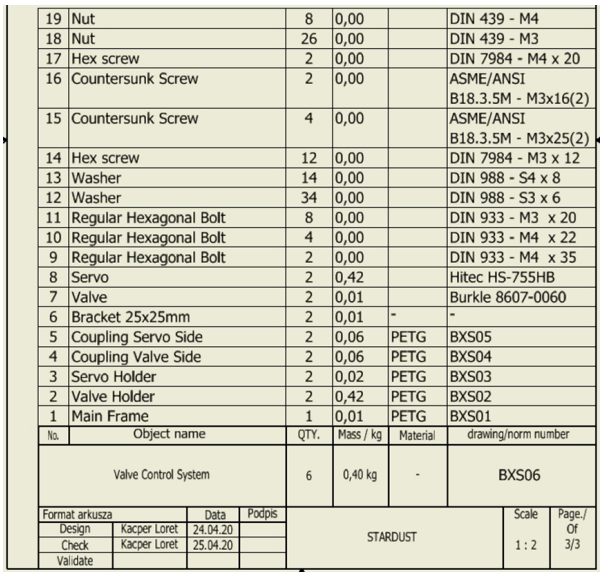


Fig. 1. Sampling System Scheme

To control the state of the valve, Hitec HS-755HB servos were used. The servos were connected with valves using couplings 3D printed from PETG. A VCU beside a servo, consisted of 9 3D printed parts made of PETG material (one main frame, two connectors, two servo mounts, two valve mounts) and was screwed as a whole to the floor of the experiment box. To the main frames of the VCUs there were attached end-stops that were pushed by the turning valve taps and giving signals of reaching an open state by the valves. The parts were connected using standard screws, washers and nuts. The main part of the structure of the Stardust experiment box was made of aluminium profiles and a floor made of an aluminium sheet. The insulation material of the gondola was a wall made of BasoTect material, 50 mm thick, thanks to which the temperature inside the box was stable and the experiment was safe in case of a shock.





No.	Object name	QTY.	Mass / kg	Material	drawing/norm number
19	Nut	8	0,00		DIN 439 - M4
18	Nut	26	0,00		DIN 439 - M3
17	Hex screw	2	0,00		DIN 7984 - M4 x 20
16	Countersunk Screw	2	0,00		ASME/ANSI B18.3.5M - M3x16(2)
15	Countersunk Screw	4	0,00		ASME/ANSI B18.3.5M - M3x25(2)
14	Hex screw	12	0,00		DIN 7984 - M3 x 12
13	Washer	14	0,00		DIN 988 - S4 x 8
12	Washer	34	0,00		DIN 988 - S3 x 6
11	Regular Hexagonal Bolt	8	0,00		DIN 933 - M3 x 20
10	Regular Hexagonal Bolt	4	0,00		DIN 933 - M4 x 22
9	Regular Hexagonal Bolt	2	0,00		DIN 933 - M4 x 35
8	Servo	2	0,42		Hitec HS-755HB
7	Valve	2	0,01		Burkle 8607-0060
6	Bracket 25x25mm	2	0,01	-	-
5	Coupling Servo Side	2	0,06	PETG	BXS05
4	Coupling Valve Side	2	0,06	PETG	BXS04
3	Servo Holder	2	0,02	PETG	BXS03
2	Valve Holder	2	0,42	PETG	BXS02
1	Main Frame	1	0,01	PETG	BXS01
	Valve Control System	6	0,40 kg	-	BXS06

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Fig. 2. Valve Control Unit

The experiment was controlled by a custom electronic system based on the Arduino platform. Arduino Mega (AtMega 2560) microcontroller was used as the on board computer (OBC). It implemented two modes of operation - manual and autonomous. Communication with ground was established via Swedish Space Corporation's proprietary E-Link system. The OBC controlled 9 servos, two pumps and heating elements attached to each pump and servo.
During flight, the OBC collected data from a suite of sensors (pressure, temperature, humidity of air) inside and outside of the experiment gondola. The data was saved on a SD card as well as transmitted to the ground via the E-Link system. The data received on the ground was displayed on a ground station computer.

Based on the data, ground operators could make decisions to control the experiment in flight. Commands sent from the ground station could turn the pumps, valves and heaters on and off as required. The use of heater prevented the actuators from freezing in case of lower than expected temperatures. In case of loss of connectivity during flight, the OBC used altitude and pressure values to conduct a pre-programmed flight plan.

The same set up in another experiment box was prepared for an on-ground reference experiment that was run at the launch pad and was given the same commands at the same time.

4. COURSE OF THE EXPERIMENT

The experiment's main challenge was to collect the stratospheric microorganisms avoiding any contamination. To fulfil this task, specific procedures had to be kept.

4.1. Contamination risk mitigation and control

A flight model of the Sampling System was tested for its sealness and integrity in both positive and negative gauge pressure values, with positive results. The test was carried out in an aquarium filled with stained water with a surfactant addition. No liquid got inside the system and no air bubbles were visible.
Another test was done on the Sampling System in order to verify if it met its microbiological requirements. The system was running 75 min on ground and after that the filters were examined for presence of microorganisms. The sampling filters yielded multiple microbiological colonies while the controls remained microbiologically pure.
Silicone pipes, valves and PP joints were washed with 70% isopropanol solution that had been filtered through sterile PES membranes of 0,2 μm pore diameter. Subsequently, the components were sterilised in a TINGET Class B STE-18L-D steam steriliser set to 134 °C, 4 min, 2,1 Bar gauge pressure. After sterilisation, they were transferred safely to a prepared laminar flow cabinet, together with the sterile filters. The Sampling System was assembled without any contact between the joint areas and any surfaces that might have been not sterile, by a person wearing sterile gloves. The packaging of the filters were disinfected with the 70% isopropanol and carefully opened by a second person. After the assembly, the inlet pipes were secured by sterile safety caps, the valves were closed, the joints were secured by zip ties and the system was secured inside a disinfected box until it was mounted inside the experiment box before the launch of the balloon.
The BEXUS30 gondola walls were washed with a disinfecting agent and dried in a disinfected tumble dryer. The walls were stored in a disinfected box until they were mounted. Since the opening of the box, the curtains did not have any contact with human skin or any other undisinfected body.
An hour before the lift-off, the entire BEXUS30 gondola was sprayed in and out with 70% isopropanol.

The experiment included three control filters: two for controlling contamination by living microorganisms and one for DNA contamination. The air did not flow through them. For an additional control, samples from the BEXUS30 balloon surface were taken using sterile swabs.
The groundbox helped compare the stratospheric samples with the airborne microorganisms living in the lower troposphere.

4.2. Stratospheric mission

The launch of the BEXUS30 stratospheric balloon took place on the 30th September 2021 from the Esrange Space Center in northern Sweden, within the REXUS/BEXUS Programme. The REXUS/BEXUS programme is realised under a bilateral Agency Agreement between the German Aerospace Center (DLR) and the Swedish National Space Agency (SNSA). The Swedish share of the payload has been made available to students from other European countries through a collaboration with the European Space Agency (ESA). EuroLaunch, a cooperation between the Swedish Space Corporation (SSC) and the Mobile Rocket Base (MORABA) of DLR, is responsible for the campaign management and operations of the launch vehicles. Experts from DLR, SSC, ZARM and ESA provide technical support to the student teams throughout the project. REXUS and BEXUS are launched from SSC, Esrange Space Center in northern Sweden. The balloon took off at 6:55am CEST. At 7:42, after the balloon reached 15 km a.s.l., the valves opened and the pumps switched on to start the air filtration. The floating phase began at 8:30 and ended at 11:53 due to the previously planned balloon cut-off. At 11:44 the valves closed, so the filtration lasted 244 min. The minimum volume of the air filtered was 3562,2 dm^3, since the minimum airflow through the Sampling System according to the airflow test result was 14,6 dm^3/min. The gondola landed on a parachute reaching the ground at 12:29. The experiment box was dismounted from the gondola and brought back to the laboratory by helicopter. The scientific team received the box with samples at 16:30. The sampling system was checked for its integrity, secured inside a small vacuum chamber together with the reference system from the groudbox and kept in a refrigerator.

4.3. Samples transport

The samples inside the Sampling Systems were transported to the Gdańsk University of Technology on the 3rd of October inside a vacuum chamber with 0,1 Bar absolute pressure inside. Before it was open on the 4th October, it was kept in a refrigerator.

4.4. Setting up microbiological cultures

After unpacking the filtering systems, one of the control filters was disattached from the system, which made it invalid as a contamination control. The rest was sprayed with 70% isopropanol in order to disinfect the critical surface before dismounting the filters. The filters were opened one by one by cutting their frames with pincers sterilised by immersing them in pure isopropanol and sliding through a flame of a burner. The membranes were taken out with sterilised tweezers, stamped on the surface chocolate agar media and put on them next to the stamps so the surfaces stuck to each other. The media had been described. The cultures were divided: the first half was grown in T=20 °C (later in 25 °C), p=1 Bar and the second half was kept in T=20 °C (later 25 °C), p=0,1 Bar. The growth of microorganisms was observed systematically. Each strain that appeared as a colony was streaked in order to obtain pure cultures.

The swabs with the samples from the BEXUS30 balloon's surface were used to make microbiological smears and were divided and kept in the same manner.

The bacterial pure cultures were subsequently transferred onto Tryptone Soy Agar media and later onto LA media. The fungal strains were transferred onto Sabouraod Agar media.

The pure cultures have been suspended in sterile 25% glycerine solution and frozen in -80 °C for preservation in a biobank.

4.5. Gram-staining

Each purified strain that appeared as a bacterial colony was suspended in sterile 0,9% NaCl solution and underwent staining after the Gram method with staining the gram-negative cells with fuchsin [4] in order to specify their main cell characteristics. The microscopic slides were observed under 100x magnification.

4.6. MALDI-TOF

The bacterial strains were examined through MALDI-TOF mass spectrometry on a Bruker MALDI Biotyper according to a standard protocol. The obtained spectra were compared to Bruker's database.

4.7. DNA Analysis

From the bacterial strains DNA was isolated with a use of BLIRT S.A. Bacterial DNA Isolation Kit according to its protocol, with doubled incubation times and with an addition of lysozyme. The isolated DNA samples underwent a successful PCR amplification with a 16S rRNA-coding-gene-specific primer pair
(R: CGTATTACCGCGGCTGCTGG;
F: AGRGTTTGATCMTGGCTCAG).
After the PCR, the samples were purified and their purity and concentration was measured. Afterwards, the samples were frozen and sent for the gene sequencing.

5. RESULTS

The results of the conducted research show that the applied research system is capable of obtaining microorganisms from the stratosphere efficiently. Table 1. is listing all the cultures obtained either from the groundbox (GB) or from the main experiment (FB - flightbox). The order in the table indicates the speed of growth of the microorganisms.

The strains marked with yellow colour were growing at the edges of the media, which indicates that they are a result of improper media storage. They were not present in the filters.

The stratospheric samples yielded 8 colonies. Each of them started to grow in microaerobic conditions (0,1 Bar). The stratospheric samples kept in 1 Bar did not yield any microbiological colonies. 7 microaerotolerant bacterial strains and one mold strain were obtained from the stratosphere. No gram-negative bacteria were found in the samples from the stratosphere.

For a comparison, the reference samples provided 6 aerobic strains (4 molds, 1 yeast, 1 gram-positive bacterium) and 5 microaerobic ones (4 molds, 1 gram-negative bacterium).

Table 1. List of microbiological cultures overall growing on the agar media

Culture symbol	Origin	Bacterium/ Fungus	Gram stain	First passage date
α	GB/Sample/ 1Bar	Mold	N/A	11.10.2021
β	GB/Sample/ 1Bar	Mold	N/A	11.10.2021
γ	GB/Sample/ 0,1Bar	Mold	N/A	11.10.2021
δ	GB/Sample/ 0,1Bar	Mold	N/A	11.10.2021
ε	GB/Sample/ 0,1Bar	Mold	N/A	11.10.2021
ζ	GB/Sample/ 1Bar	Yeast or bacterium	Mix (+/-)	11.10.2021
η	GB/Sample/ 1Bar	Mold	N/A	14.10.2021
ι	FB/Sample/ 0,1Bar	Mold	N/A	18.10.2021
κ	FB/Sample/ 0,1Bar	Bacterium	G+	18.10.2021
λ	FB/Sample/ 0,1Bar	Bacterium	G+	18.10.2021
μ	FB/Sample/ 0,1Bar	Bacterium	G+	18.10.2021
ν	FB/Control/0 ,1Bar (the unplugged filter)	Bacterium	-	18.10.2021
ξ	GB/Sample/ 0,1Bar	Mold or yeast	G+ (but not 100%)	18.10.2021
ο	FB/Control/1 Bar	Bacterium	G+	25.10.2021
π	GB/Sample/ 1Bar	Bacterium	G+	25.10.2021
ρ	GB/Sample/ 1Bar	Bacterium	G-	25.10.2021
σ	FB/Sample/ 0,1Bar	Bacterium	G+	29.10.2021
υ	GB/Sample/ 1Bar	Mold	N/A	20.10.2021
φ	GB/Sample/ 1Bar	Mold	N/A	14.10.2021
χ	FB/Sample/ 0,1Bar	Bacterium	G+	29.10.2021
ψ	FB/Sample/ 0,1Bar	Bacterium	-	29.10.2021
?	FB/Sample/ 0,1Bar	Bacterium	G+	17.11.2021
!	GB/Sample/ 0,1Bar	Bacterium	Mix (G- based)	29.10.2021
Bal1	BEXUS30 balloon	Bacterium	G-	17.11.2021

Generally, the microorganisms coming from the stratosphere started to grow much later than the tropospheric ones. This indicates that the cells living in the stratosphere are well adapted to the harsh stratospheric conditions, so they need a longer time to readapt to the new environment, in which they may grow relatively abundantly.

Gram-positive bacteria have thick polyglycan layers in their cell walls, which gives them a better protection from environmental factors. Also, some of them, like *Bacillus subtilis*, are able to form endospores, which are additionally coated, and therefore, protected [5]. Other bacteria, like *Deinococcus radiodurans*, have excellent DNA-repairing mechanisms [6]. However, this is only an example, and to better understand the mechanisms of survivability of different bacteria that do not form endospores, still more research has to be done.

To precisely identify the obtained microorganisms, MALDI-TOF analysis along with the DNA analysis have been done. However, due to lack of fundings, the prepared DNA samples are still waiting for sequencing. The MALDI-TOF results are shown in table 2.

Table 2. Results of the MALDI-TOF analysis

Strain symbol	Species name
κ	unspecified
λ	Microbacterium oleivorans
μ	unspecified
ν	Bacillus pumilus/unspecified
ο	unspecified
π	unspecified
ρ	Sporosarcina luteola
σ	Bacillus licheniformis
χ	Pseudarthrobacter polychromogenes/ Pseudarthrobacter oxydans/ New species
ψ	unspecified
!	Staphylococcus epidermitis/unspecified
?	Bacillus simplex/unspecified
Bal1	unspecified

The results of the MALDI-TOF analysis are relatively uncertain since the used database was dedicated mostly to pathogenic bacteria.

In addition, our studies indicated that up to 10 new species or strains of microorganisms might have been discovered either in the stratosphere or in the regular air in northern Sweden, and the DNA sequencing analysis will provide final confirmation of these data.

6. REFERENCES

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