A smelly business: microbiology of Adélie penguin guano (Point Thomas rookery, Antarctica)

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Key words: Ammonia, *Jeotgalibaca*, uric acid, *Psychrobacter*, targeted 16S metagenomics, enzyme activity

Abstract

Adélie penguins (*Pygoscelis adeliae*) are the most numerous flightless bird group breeding in coastal areas of Maritime and Continental Antarctica. Their activity leaves a mark on the land in the form of large guano deposits. This guano is an important nutrient source for terrestrial habitats of ice-free Antarctic areas, most notably by being the source of ammonia vapors which feed the surrounding grass, lichen and algae communities. Although investigated by researchers, the fate of the guano-associated microbial community and its role in decomposition processes remain vague. Therefore, by employing several direct community assessment methods combined with a broad culture-based approach we provide data on bacterial numbers, their activity and taxonomic affiliation in recently deposited and decayed Adélie penguin guano sampled at the Point Thomas rookery in Maritime Antarctica (King George Island). Our research indicates that recently deposited guano harbored mostly bacteria of penguin gut origin, presumably inactive in cold rookery settings. This material was rich in mesophilic enzymes active also at low temperatures, likely mediating early stage decomposition. Fresh guano colonization by environmental bacteria was minor, accomplished mostly by ammonia scavenging *Jeotgalibaca* sp. cells. Decayed guano contained 10-fold higher bacterial numbers with cold-active enzymes dominating the samples. Guano was

colonized by uric-acid degrading and lipolytic *Psychrobacter* spp. and proteolytic *Chryseobacterium* sp. among others. Several spore-forming bacteria of penguin gut origin persisted in highly decomposed material, most notably uric-acid fermenting members of the *Gottschalkiaceae* family.

1. Introduction

Pygoscelis penguins are represented by three species of flightless birds and are found almost exclusively in Antarctica (Forcada et al. 2006). The most numerous among them are the Adélie penguins (*Pygoscelis adeliae*) with estimated population numbers around 14-16 million individuals (Southwell et al. 2017). Most of their lives Adélie penguins spend in cold waters of the Southern Ocean, mostly in those covered or adjacent to sea pack ice. They feed primarily on Antarctic krill (*Euphausia superba*), foraging through its dense swarms. The penguins are seen on land mainly during the breeding season, which starts as early as late September and typically ends in February (Ainley 2002). Within that time frame groups of up to 1.5 mil individuals are seen in rookeries on land in coastal areas of Maritime and Continental Antarctica (Borowicz et. al. 2018).

Their activity leaves a mark on the land in the form of large guano deposits. This mass is highly heterogeneous and consists of three phases (fractions). The white fraction contains mainly urates (major end-product of nitrogen metabolism in birds), oxalates and phosphates. The red phase constitutes of carotenoid stained chitin from undigested krill shells. A green fraction which consists of proteins, lipids, cholic acids and undigested algae cells (presumably krill stomach content) is also present but rarely seen, most often when adult penguins have a prolonged period of fasting during egg incubation (Myrcha and Tatur 1991; Korczak-Abshire – personal communication).

One of the best studied places where the Adélie penguins breed is the Point Thomas rookery, located on the Western shore of Admiralty Bay, King George Island, Antarctica (Trivelpiece and Trivelpiece, 1990; Chwedorzewska and Korczak 2010; Korczak-Abshire et al. 2016; Sierakowski et al. 2017; Hinke et al. 2018). Breeding phenology at this site differs slightly from season to season but is roughly realized as follows: egg laying – October/November, chicks hatching – November/December, chicks gathering in créches (nurseries) – December/January, adult and young penguins leave the rookery – February (Trivelpiece and Trivelpiece, 1990; Ciaputa and Sierakowski 1999; Korczak-Abshire – personal communication). Adélie penguin numbers increase in the colony when the chicks hatch, consequently so does the amount of guano being deposited. The height of this deposition occurs in late January, when chicks reach their target weight but are still fed by parent birds. The rookery at this point gives off a strong odor, mostly due to ammonia vapors. This phenomenon called "ammonia shadow" is responsible for the unique flora of the rookery characterized by lush *Deschampsia antarctica* (Antarctic grass) communities, extensive *Prasiola crispa* (alga) growth and colorful lichen assemblages comprising endemic *Caloplaca* and *Xanthoria* species (Tatur et al. 1997). This peculiar ammonia cloud is presumably derived from the ammonification of uric acid, present in the guano deposit (Lindeboom 1984). Furthermore, a very chemically aggressive liquid leaching from the decomposing guano reacts with the underneath loam and gravel, creating ornithogenic soils, providing plants with one of the few suitable habitats in those mostly barren areas (Myrcha et al. 1985). However, the mechanism behind this decomposition process or major players involved in it are still debated (Zhu et al. 2011).

The aim of this research was to investigate the bacterial community associated with deposited Adélie penguin guano at the height of the breeding season (maximal fresh guano deposition rate) but also after penguin departure from the rookery (advanced guano decay) to

elucidate the nature of processes involved in guano decay and its potential as a microbial habitat. Our hypothesis states that the decomposition of guano starts immediately after its deposition and is mainly driven by psychrophilic saprotrophs. We thus conducted a thorough microbiological survey of guano samples using direct assessment methods such as 16S targeted metagenomics and a culture based approach.

2. Materials and Methods

2.1 Sites, sampling, pH and water content analysis

Guano samples were collected during the Austral summer of 2016/2017 in December, January and March, once a month from three randomly picked sites within the Point Thomas penguin rookery (Western Shore of Admiralty Bay, King George Island, South Shetland Archipelago, Antarctica) (Table1). Guano was collected aseptically into sterile plastic bags and kept at -20°C until laboratory analysis. A total of nine bags (three per month) were filled with guano, 0.5kg each (4.5kg in total). Temperature during sampling was measured at 1 cm above guano but also of the guano itself with an Elmetron 410 multifunction meter (ELMETRON, Poland). Geographical coordinates were gathered with the use of a Garmin GPSMap 64s device. pH measurements were done according to guidelines provided by Minasny et al. 2011. Briefly: 1 g of guano was carefully dispersed by vortexing in 1 mL of ddH_2O or 0.01 M $CaCl_2$ solution (flocculent). The guano suspensions were then centrifuged at 5000 rpm for 5 min and pH was measured at 22°C (room temperature) with a Hanna HI 9025 microcomputer pH meter. Measurements were done in triplicate. Dry weight was determined after 24 h at 65°C in a dry box with circulating air (Dowgiałło 1975).

2.2 Bacterial cell extraction

Approx. 1 g of fresh guano was weighted and placed in a 50 mL conical tube containing 20 mL of sterile and cool (4°C) dilution liquid composed of 1% (w/v) glycerol, Tween 80 (10 ppm) and 0.1% tetrasodium pyrophosphate (further termed GTP). The

suspension was then shaken for 30 min in a Tornado™ Vortexer at 2000 rpm at 4°C. The tubes were then placed in a VWR Ultrasonic Cleaner USC-TH filled with chilled water and sonicated for 60 sec. The tubes were vortexed afterwards for 30 sec to suspend detached cells. After a brief centrifugation (1 min.; 1000 rpm; 4°C) the suspension was submitted to further analysis. This procedure was adapted from Lunau et al. 2005 and Elliot and des Jardin 1999. This procedure was done in triplicate from each sampling site resulting in 27 suspensions for analysis.

2.3 Cell count

Microbial cell abundance was assessed as in Grzesiak et al. 2015. TCs (total counts) were determined by epifluorescence microscopy using 40,6-diamidino-2-phenylindole (DAPI) on black Nuclepore polycarbonate 0.2-µm-pore-size filters (Porter and Feig 1980), under a Nikon E-200 microscope with a 100 W Hg lamp and 100x CFI 60 oil immersion objective, with a digital DS Cooled Camera Head DS-5Mc-U1, and a filter block of wavelengths: EX 330-380, DM 400, BA 420. Images of fields were analyzed in Nikon NIS Elements BR 3.1 and MultiScan v. 14.02 (computer scanning systems). A minimum of 400 cells in 20 fields per sample were counted automatically in the image analysis system. Percentage contribution of active bacteria with intact membrane (MEM+) was assessed by the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes-Invitrogen, UK). Samples of guano were used in triplicate for enumeration of alive bacteria (Schumann et al. 2003). For 1 mL of the aforementioned suspension a mixture of two BacLight Kit stains – SYTO 9 and propidium iodide was added (1:1 ratio, both dyes final concentration: 0.15%) then incubated for 20 min at room temperature in the dark, filtered through a 0.2 µm pore-size, black polycarbonate membrane filter (Millipore GTBP) and enumerated by epifluorescence microscopy (Dunalska et al. 2012). The percentage contribution of MEM+ bacteria was calculated as a ratio of MEM+ to the sum of MEM+ and MEM− bacterial cells.

126 *2.4 DNA extraction*

127 DNA was directly extracted from guano using the PowerSoil® DNA isolation kit
128 (QIAGEN, Germany) according to manufacturer protocol. An approx. 0.2 g of guano per
129 sampling bag was used in triplicate and pooled. DNA solutions were kept at 4°C until further
130 analysis. Template was quality checked by DNA electrophoresis on 0.7% agarose gel. DNA
131 from pure bacterial strains was extracted from a single colony. The procedure involved
132 suspending the colony with sterile toothpick in 100 µL sterile MiliQ Water containing 5% of
133 Chelex® 200 resin (Sigma) and 5% of garnet sand Lysing Matrix A (MP Biomedicals). The
134 1.5 mL tube with the bacterial suspension was shaken in a Qiagen Retsch TissueLyserII for 5
135 min at 39Hz – 1800 oscillation per min. The tubes were then placed in a thermoblock and
136 heated for 5 min at 99°C. Then the tubes were centrifuged for 3 min at 12000 rpm. Fifty
137 microliters of the clear supernatant was taken and used for PCR amplifications.

138 *2.5 Sequencing*

139 For Illumina 16S targeted amplicon sequencing pooled environmental samples from
140 each month were examined. Phylogenetic study was performed by sequencing and analysis of
141 prokaryotic 16S ribosomal RNA gene. A fragment of the16S rRNA gene containing the V3
142 and V4 variable regions was amplified using gene-specific primers: 16S_V3-F and 16S_V4-R
143 positions 341-357F and 785-805R, respectively, according to *Escherichia coli* 16S rRNA
144 gene reference sequence (Klindworth et al. 2013). Illumina Nextera XT overhang adapter
145 nucleotide sequences were included in addition to the 16S rRNA gene-specific sequences,
146 which allowed sample indexing and pooling. Each PCR amplification was done in triplicate
147 using KAPA HiFi PCR kit (Roche) in a final volume of 20 µL per reaction according to the
148 manufacturer's instructions. Obtained PCR products were pooled in equimolar ratio and
149 indexed using Nextera XT barcodes (Illumina, San Diego, USA). Amplicon libraries were
150 pooled and sequenced on Illumina MiSeq instrument (Illumina, San Diego, USA) in the DNA

151 Sequencing and Oligonucleotide Synthesis Laboratory (Institute of Biochemistry and
152 Biophysics, Polish Academy of Sciences). Sequencing was done in paired-end mode (2 × 300
153 bp) with the use of a v3 (600 cycles) chemistry cartridge which allowed generation of long
154 paired reads fully covering 16S V3–V4 amplicons.

155 Amplification of 16S rRNA gene fragment from pure strains was performed using
156 universal primers 27F and 1492R (Lane 1991). PCR amplification reaction conditions were as
157 follows: 1 min of 95°C initial denaturation followed by 30 cycles of 95°C for 15 sec, 55°C
158 annealing for 15 sec and elongation 72°C for 1min and 30 sec, using DreamTaq polymerase
159 (Thermo Scientific-Fermentas). Obtained PCR products (~1500 bp for 16S rRNA gene
160 fragment) were checked on 0.8% agarose gel and purified using Exonuclease I/Alkaline
161 phosphatase mix (Thermo Scientific—Fermentas). 16S rRNA gene amplicons were
162 sequenced using internal 16S rRNA gene primers: 341F, 518R and 928F (Weidner et al.
163 1996) with the use of BigDye Terminator v.3.1 chemistry and ABI3730xl genetic analyzer at
164 the DNA Sequencing Laboratory (Institute of Biochemistry and Biophysics, Polish Academy
165 of Sciences).

166 *2.6 Activity assessment*

167 Fluorescein diacetate hydrolysis assay (FDA) was adapted from Jiang et al. 2016.
168 Fluorescein diacetate hydrolysis is mediated by a variety of enzymes (proteases, lipases,
169 esterases) present in environmental samples and is therefore considered mostly as indicator of
170 microbial activity in soils (Green et. al. 2006). 15 mL of each guano suspension was amended
171 with 0.3 mL of FDA stock solution (0.2 g powder FDA in 200 mL acetone). One set of
172 suspensions (n=27) was incubated at 4°C (rookery temp.), the other (n=27) at 40°C (penguin
173 gut temperature - Ropert-Coudert et al. 2000). After the addition of the FDA stock solution
174 the guano suspensions were mixed thoroughly and immediately 1.5 mL was removed, mixed
175 with 0.2 mL of acetone to stop the hydrolysis, centrifuged at 5000 x g for 5 min and the

supernatant absorbance was read at 490 nm in a Varioscan plate reader (Thermofisher Scientific) in 8 wells per sample containing 100 µL each. Measurements were done every hour for 6 h. Fluorescein concentrations released by the hydrolysis was calculated based on a standard curve prepared according to Jiang et al. 2016.

Respiration activity by tetrazolium dye reduction was performed in Biolog EcoPlates. Guano suspensions were centrifuged at 6000 rpm for 5 min at 4°C, suspended in sterile, cool 0.9% saline and were adjusted with sterile 0.9% saline to optical transmittance of 0.9. 100 µL aliquots of each suspension were added to each well of Ecoplate microplates (Biolog Inc., Hayward, CA, USA). The plates were incubated in darkness at 4°C, the color development was measured at 590 nm with a microplate reader (OmniLog) and cellular respiration was measured kinetically by determining the colorimetric reduction of tetrazolium dye. Data were collected approximately twice a week over a >65 day period. The Ecoplate Biolog assays assess the ability of a mixed microbial community to utilize any of 31 carbon compounds as the sole carbon source (+ one control well with no-carbon). Microbial communities were characterized for their ability to catabolize 10 different carbohydrates, 9 carboxylic and acetic acids, 4 polymers, 6 amino acids and 2 amines (Weber and Legge, 2009). Absorbance data from the different reading times were first blanked against the time "zero" reading and then OD 590 values, from a defined plate and reading time, were blanked against the respective control well containing no-carbon source

2.7 Culture-based analysis

Colony forming unit (CFU) enumeration was done using the drop plate method optimized by Herigstad et al. 2001. Each agar plate was divided into quadrants. One quadrant was dedicated for one dilution. Fifty µliters of a decimally diluted guano suspension was dispersed in five evenly spread 10 µL drops onto the same quadrant. The plates were carefully transferred and stored face up for approx. 30 min at 10°C to let the agar absorb the liquid.

CFU numbers in a range of temperatures was examined in aerobic conditions on R2A agar (Biocorp). Plate replicas were incubated at 4, 10, 18, 22, 28 and 37°C in Liebher thermostat cabinets for 6 weeks. CFU numbers of copiotrophic microbes (tolerating high nutrient concentrations) were assessed on Rich Medium (Atlas, 2010) at 4°C in aerobic atmosphere. Culturable anaerobe abundance was examined on Thioglycollate Agar (Conda). Plates were incubated at 4°C in anaerobic conditions (bioMerieux Genbag Anaer System). Cultivable penguin gut bacteria were enumerated on BHI agar (Difco) at 40°C in microaerobic conditions (bioMerieux Genbag Microaer System).

Strains for morpho-physiological analysis were cultivated using the traditional spread plate method. 100 μL aliquots of the decimal dilutions of the aforementioned suspensions were plated on pre-cooled R2A agar plates. The plates were incubated at 10° C for 6 weeks to assure complete colony development. The strains were picked randomly, 100 per sampling month and purified by re-streaking onto fresh agar plates. All strains were sub-cultured on R3A broth (Atlas, 2010). Pure bacterial strains were tested for catalase and oxidase activity using 3% H_2O_2 and Biomerieux Oxidase Reagent, Gram reaction by 3% KOH lysis and cell shape morphology (crystal violet staining). Further characteristics were assessed on agar plates by placing a 5μL drop of strain cell suspension onto an appropriate medium followed by a 2 week incubation at 10° C in aerobic conditions. Proteolytic activity of guano derived strains was examined on Skim Milk Agar (Atlas, 2010). Clearing around bacterial growth was scored as positive for protein degradation. Chitin agar was prepared according to Atlas 2010. 5 g of chitin powder from crab shells (Sigma) was dissolved in 200 mL of cooled 37% HCl. The resulting liquid was poured into 1.5 L of cooled deionized water, mixed thoroughly and let sit overnight to precipitate colloidal chitin. The chitin was washed 5 times with deionized water by repeated centrifugation and resuspension in fresh dH_2O and finally suspended in 1 L of deionized water. pH was adjusted to 7.2 using 3% NaOH solution. Then mineral salts were

added to make a mineral salts medium containing per L: Na_2HPO_4 1.1 g, KH_2PO_4 1 g, NH_4Cl 1 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $CaCl_2$ 0.05 g, Yeast extract 0.05 g, Fe-citrate 0.01 g, $NaMoO_4 \cdot 2H_2O$ 0.005 g and agar 15 g, autoclaved and poured into Petri dishes. Clear zone formation around bacterial growth was scored as positive for chitinolysis. Strains' lipolytic abilities were tested with a modified lipoidal emulsion agar method. The medium consisted of two components: base agar (in g/L: Tryptone - 5, Yeast extract – 2.5, Agar – 15) and lipoidal emulsion (in g/L: Tween 80 – 2, Rapeseed oil – 200). After being separately autoclaved 970 mL of the base agar was amended with 30 mL of the lipoidal emulsion and poured onto petri dishes. After inoculation and a 2 week incubation at 10°C, the agar plates were flooded with a 5% $CuSO_4$ solution. The resulting bright blue halo (fatty acid copper salts) around bacterial growth was scored as positive for lipid degradation. Uric acid agar for uric acid utilization assay was prepared using the double layer technique mentioned by Bravo et al. 2015. A bottom layer of base mineral agar identical to the mineral salts medium described earlier was poured first. After it solidified a second, thinner layer of the same agar was poured but containing 10 g/L of suspended uric acid. After inoculation and a 2 week incubation at 10°C a clearing around bacterial growth was scored as positive for uric acid utilization. Calcium oxalate agar for oxalate degradation assay was prepared using the double layer technique mentioned by Bravo et al. 2015. After inoculation and incubation a clearing around bacterial growth was scored as positive for oxalate utilization. Pure bacterial strains were tested in terms of phosphate solubilizing abilities on the NBRIP medium (Nautiyal 1999). Acetate utilization was assessed on a Mineral Base Medium with Acetate (Atlas, 2010). Sodium acetate utilization by bacteria was scored as positive when the medium turned blue due to rise in alkalinity.

Jeotgalibaca sp. growth was tested on mineral salts liquid medium and R3A broth supplemented with nitrogen sources that are produced during uric acid degradation (Ramazzina et al. 2006): allantoin (0.73 g/L), urea (0.56 g/L) and ammonia (NH_4Cl -1 g/L);

nitrification: nitrites (KNO_2 – 1.58 g/L) and nitrates ($NaNO_3$ – 1.58 g/L) and also with an amino acid mix (Casamino acids – 1 g/L). Nitrogen content was calculated to be equal as in 1 g of NH_4Cl for each nitrogen source. Growth was measured as absorbance at 600 nm on a Varioscan device in a microtiter plate. *Jeotgalibaca* sp. growth enhancement experiments involved drop plating a suspension of *Jeotgalibaca* sp. cells on R3A agar in increasing distances from a colony of uric acid degrading *Psychrobacter* sp. growing on either a plug with uric acid agar or on R3A agar itself. Appropriate control were done with no *Psychrobacter* sp. inoculation. Additionally *Jeotgalibaca* sp. was grown the same way in vicinity of an agar cut well containing an NH_4Cl solution (3 g/l).

2.8. Data analysis

Obtained Ilumina reads were quality checked using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Andrews 2010). Raw sequencing data were cleaned, aligned and classified automatically by the EzBioCloud platform using the PKSSU4.0 database (Yoon et al. 2017). Illumina reads were deposited in the NCBI Sequence Read Archive (SRA) as BioProject PRJNA566128.

Strain sequence chromatogram files were analyzed using FinchTV ver. 1.4.0 (Geospiza, Akron, USA). Consensus sequences were obtained with Seqman Pro ver. 9.1 software (DNAStar, Madison, USA). 16S rRNA gene fragments sequences were aligned against 16S reference sequence database GenBank using BLAST (Altschul et al. 1990). Multiple sequence alignments were performed using ClustalW program. Phylogenetic trees were constructed using MEGAX software. Sequences were deposited in GenBank under accession numbers MN480548-MN480558.

Data compilation, basic statistical analysis and graphs were done using Excel (MS Office 2016 for Windows.

3. Results

276 *3.1 Sampling site description and physicochemical factors.*

277 Table 1 contains a brief description of conditions at the Point Thomas penguin rookery
278 with temperature data for air and guano *in situ*. Guano gathered in December had the highest
279 temperature measured during the sampling period (av. 11.8°C) with average temperature 1cm
280 above guano deposits equating 6.7°C, while air temperature measured by the meteorological
281 station was 2.8°C. Guano temperatures in January were slightly lower than the ones registered
282 in December (av. 8.4°C) with comparable temperature 1cm above guano (av. 6.1°C) but with
283 higher air temperature (4.6°C). Guano sampled in March was the coldest, averaging 3.8°C,
284 with temperatures 1 cm above guano 5.6°C on average and temperature registered by the
285 meteo-station was 2.1 °C.

286 Guano pH in December was slightly acidic (6.7-6.9 in ddH_2O and 6.6-6.8 in 0.01M
287 $CaCl_2$) while guano in January had a slight alkaline pH (7.1-9.0 in ddH_2O and 7.1-8.8 in
288 0.01M $CaCl_2$) with pronounced variations between samples. Guano sampled in March had a
289 uniformly alkaline pH (8.1-8.6 in ddH_2O and 7.7-8.0 in 0.01M $CaCl_2$).

290 *3.2 Direct cell count*

291 Bacterial total counts (TC) were similar in December and January. December-sampled
292 guano counts ranged from 1.15 to $8.06x10^{10}$ cells per gram dry weight (av. $3.42x10^{10}$), while
293 January counts were in the range of 1.7 to $8.14x10^{10}$ cells per g d.w. (av. $3.26x10^{10}$). A 10-
294 fold increase in bacterial cell abundance was noted in March with $2.93x10^{11}$cells per g d.w.
295 (1.75-3.54x10^{11} cells/g d.w.) (Fig. 1).

296 Percentage of cells with intact membranes ("alive" MEM+) was the highest in March-
297 sampled guano reaching 99% (98.1% on av.) with minor discrepancies between sampling
298 sites. More than half of cells in guano sampled in January were "dead" (MEM-) although
299 major differences between sites were noted (40-65%). The least "alive" cell contribution was

300 found in December-sampled guano (27.5%) also with high variation between sites (18-45%)
301 (Fig. 2).

302 *3.3 Phylogenetic affiliation*

303 Valid reads numbers obtained from the samples were as follows: December - 73336,
304 January – 71552, March – 73237. Phylum rank bacterial community composition is presented
305 in Fig. 3 and family rank sequence contribution in Table 2. Sequences belonging to the
306 Firmicutes phylum were the most numerous in all investigated samples (December – 70%,
307 January – 44%, March - 53%). Within this phylum the family *Gottschalkiaceae* displayed a
308 steady presence in all samples in the range of 18-19%. A fairly high percentage of sequences
309 was maintained also by sequences belonging to the *Bacillaceae* (mean 4.8%),
310 *Carnobacteriaceae* (10.23%), *Eurysipelotrichaceaea* (4.17%) and *Tissierellaceae* (6.27%).
311 Bacteroidetes sequences were found to be the second numerous group (on av.) in guano
312 samples (December – 10.6%, January – 37.6%, March – 25.4%). Two families that displayed
313 high abundances (based on sequence percentage) were the *Flavobacteriaceae* (av. 11.97%)
314 and *Porphyromonadaceae* (10.35%). The *Flavobacteriaceae* sequences were most abundant
315 in January-sampled guano (26.27%) whereas the *Porphyromonadaceae* in March-sampled
316 material (15.56% of all sequences). Proteobacterial sequences contribution (December –
317 13.38%, January – 9.93%, March – 18.62 %) were mainly due to the presence of sequences
318 belonging to two families: the *Moraxellaceae* (av. 7.12) and *Pseudomonadaceae* (av. 3.63).
319 The *Moraxellaceae* contribution was the highest in December sampled guano whereas the
320 members of *Pseudomonadaceae* family were the most numerous in March sampled material.
321 Lastly, the phylum Actinobacteria (December – 4.9%, January – 6.9%, March – 2%) were
322 represented mainly by the families *Intrasporangiaceae* (av. 1.08%) and *Nocardiaceae* (av.
323 1.45%). Unaffiliated bacterial sequences and belonging to other phyla comprised less than 1%
324 in each sample.

Eleven strains were chosen as representatives of groups that emerged in clustering by marpho-physiological features (data not shown) and subjected to identification based on 16S rRNA sequence similarity. Seven strains were affiliated to genus *Psychrobacter*, two to *Jeotgalibaca* and two to *Chryseobacterium*. *Psychrobacter* strains isolated from December (D1, D4) and January-sampled guano (J3) had the highest similarity based on 16S rRNA gene sequence coverage to *Psychrobacter adeliensis* (type strain) DSM15333 (98-100%) while *Psychrobacter* strains derived from March-sampled material displayed greater similarity to *Psychrobacter faecalis* (type strain) Iso-46. Discovered only on plates inoculated with guano sampled in December and January were members of the genus *Jeotgalibaca*, which displayed enough low similarity (96-97%) to the sequence of its closest described species *J. dankookensis* to be considered a new species. *Chryseobacterium* strains were isolated only from March-sampled guano and displayed closest similarity (by 16S rRNA gene sequence) to *Ch. antarcticum* type strain AT1013 (Fig. 4).

3.4 Enzyme and respiration activity in P. adelie guano

Fluorescein release in December and January samples displayed considerable discrepancies between incubation temperatures. A 6.5-fold increase of fluorescein release (in mg/kg guano d. w. h^{-1}) in December samples was noted at 40°C compared to 4°C. January material exhibited a similar response with an almost 4-fold increase. March-sampled guano releases were fairly similar in 4 and 40°C (32.7 and 47.6 mg/kg guano d. w. h^{-1} respectively) (Fig. 5).

Substrate-dependent microbial community respiration measured as colorimetric responses in Biolog Ecoplates and displayed in Omnilog Arbitrary Units were (on average) lower in December and January samples (16.19 and 32.9 respectively) compared to March samples (163). Carbon sources that triggered greatest responses were: L-Asparagine (291), α-

D-Lactose (266), D-Cellobiose (257), L-Arginine (244), L-Phenylalanine (232), L-Serine (221), Tween 40 (214), α-Cyclodextrin (199) and Putrescine (197) (Fig. 6).

3.5. Bacterial cultivation

Colony forming unit (CFU) enumeration in different temperatures revealed major discrepancies between samples from different months. December samples displayed highest CFU counts ($8.5x10^6$) in 22°C (moderate mesophile range) while the lowest values were recorded at 18°C (psychrotolerant range). January-sampled guano CFU's ranged from $1.75x10^5$ to $2.36x10^6$, displaying highest and lowest values at 37°C and 10°C respectively. March sampled guano CFU's showed considerable variations between incubation temperatures (from $1.32x10^5$ in 37°C to $3.79x10^9$ in 18°C) (Fig. 7).

Psychrophilic (4°C) oligotrophic (R2A medium) CFU count was the highest in March-sampled guano reaching $8.00x10^8$ CFU/g d.w. followed by December ($6.57x10^6$) and January counts ($1.05x10^6$). Psychrophilic copiotrophic (RM medium) counts in March-sampled guano exhibited the highest CFU per g. d. w. of all conditions and media examined ($1.89x10^{10}$). December and January sampled guano also displayed high values ($4.9x10^7$ and $1.5x10^7$ CFU/g d.w. respectively). Psychrophilic anaerobic count in December and March samples was comparable ($1.91x10^7$ and $2.88x10^7$ CFU/g d.w.), lower in January – $7.7x10^5$ g d.w. Simulated penguin gut conditions (BHI agar, microearobic atmosphere) yielded uniform results in all samples (December – $7.02x10^5$, January – $5.95x10^5$, March – $6.02x10^5$ CFU/g d.w.) (Fig. 8). Cultivability (CFU/TC%) of guano-derived bacteria was extremely low, especially in January (0.000-0.039%) and December samples (0.001-0.145%). Relatively high cultivability was achieved in March samples, most notably on Rich Medium (RM) reaching 6% (Table 3).

Bacterial strains derived from December-sampled guano overall had the widest range of properties involved in guano decomposition albeit only a small number of strains displayed

those features (uric acid degradation – 27%, calcium phosphate solubilization – 7%, chitin degradation – 2%, proteolysis –6%, calcium oxalate decomposition – 1%, acetate utilization – 11%). 15% of December strains displayed 2 traits, 20% - one trait and 65% displayed no traits considered as useful in guano utilization. Strains derived from January samples displayed even less properties (uric acid degradation – 10%, calcium phosphate solubilization - 4%, proteolysis – 2%. Only 4% of January strains displayed more than 1 property, 85% showed no potential involvement in guano decomposition. A majority of strains isolated from March-sampled material displayed guano-decomposing properties. Uric acid degradation and lipolysis was exhibited by 42 and 44% of strains respectively. Proteolysis and acetate utilization was displayed by 31% and 23% of strains respectively. 16% of those strains displayed more than three guano-degrading features, 24% displayed two, 44% - one and only 16% had none of the examined traits (Fig.9).

Growth of *Jeotgalibaca* sp. J13 was visibly enhanced in vicinity of uric acid degrading *Psychrobacter* sp. J3 growing on an uric acid agar plug (Fig. 1S A). Similar enhancement was observed in vicinity of an agar-cut well containing a concentrated NH_4Cl solution (Fig. 1S B). There was no enhancement of *Jeotgalibaca* sp. S13 growth around uninoculated uric acid agar plug (Fig. 1S C) and only slight enhancement around *Psychrobacter* sp. S3 growing directly on R3A agar (Fig. 1S D).

On the premise that *Jeotgalibaca* sp. J13 growth was enabled due to catabolism products of uric acid degradation, a variety of compounds were tested as nitrogen sources. Organic uric acid degradation products (allantoin and urea) were negative in promoting growth on mineral (minimal) and full nutrient medium (R3A). So were nitrates and nitrites and surprisingly also Casamino acids. Only ammonia (final nitrogen-bearing product of uric acid degradation) in the form of NH_4Cl was able to support growth on R3A but not on minimal mineral medium with glucose (Table 1S).

4. Discussion

Presented data highlight microbial community characteristics of recently deposited and decayed Adélie penguin guano at the Point Thomas rookery in Maritime Antarctica. Diversity, activity and abundance of resident bacterial cells have been assessed by direct and culturable approaches to examine the fate of deposited guano *in situ*.

4.1 Early stages of decomposition

Recently deposited guano (December and January samples) was characterized by relatively high bacterial numbers, although the percentage of injured cells (MEM-) was substantial. 16S rRNA gene amplicon sequencing revealed that those samples were dominated by members of the phylum Firmicutes, including mesophilic, anaerobic bacteria belonging to the *Gottschalkiaceae, Erysipelotrichaceae, Peptostreptococcaceae* and *Tissierellaceae* families (Poehlein et al. 2017, Verbarg et al. 2014, Slobodkin 2014, Alauzet et al. 2014). These bacteria, presumably of Adélie penguin intestine origin, may have been prone to damage by oxidative stress and freeze thaw cycles occurring at the rookery site (Plenzler et al. 2019). Noteworthy here is the family *Gottschalkiaceae*, which consists currently of only three species: *Gottschalkia acidurici*, *Gottschalkia purinilytica* and *Andreesenia angusta*, all of which are strictly anaerobic, spore-forming, uric acid fermenters (Poehlein et al. 2017). Sequences clustering within this family have been recovered from bird-associated materials, most notably from penguin nesting sites (Aislabie et al. 2009a, Kim et al. 2012, Banks et al. 2009), suggesting their major involvement in guano decomposition. However, their optimum growth temperature is noted to be 30-37°C, which is considerably higher than the measured guano temperature *in situ* in this study, suggesting negligible activity after deposition (Poehlein et al. 2017). Therefore, ammonia released during active breeding period measured by several researchers (Mizutani et al. 1985, Theobalt et al. 2013, Riddick et al. 2016) did

presumably originate from uric acid degradation occurring before rather than after defecation with ammonia being released from the deposited guano largely by physical or chemical processes (Zhu et al. 2011).

Microbial respiration on different organic substrates serving as electron donors was minor at low temperatures (4°C) as measured by tetrazolium salt reduction in Biolog Ecoplates. Moreover, cultivability of bacterial cells was also very low, despite using a range of different media, temperatures and oxygen concentrations. Minor cultivability of bacteria in fresh Adélie penguin guano has also been observed by Zdanowski et al. 2005. Culture techniques recover mostly opportunistic microbes (Siegler and Zeyer, 2004; Zdanowski et al. 2013), those that are known to start decomposition processes of a variety of organic compounds (Mooshammer et al. 2014; Naeem et al. 2000). Low respiration rates and cultivability (especially at low temperatures) suggests negligible colonization by psychrophilic bacteria at this stage of decomposition. High deposition rates might be one of the causes of this phenomenon, where psychrophilic decomposer bacteria derived in moderate quantities by various means are being constantly buried and/or diluted by ongoing guano supply. This is apparent when comparing strain activities derived from December and January sampled guano. Percentage of psychrophilic guano decomposers in December is considerably higher, probably due to lower fecal deposition rates (smaller chicks are fed less frequently) and therefore higher chance of cold-adapted saprotroph population establishment. However, CFU numbers are comparable, suggesting some other factors might also exert influence, like high concentration of ammonia, amine compounds or even phytoplankton-derived acrylic acid (Thouzeau et al. 2003; Sieburth 1959, Sieburth 1960). pH does not seem to have the potential to influence bacterial colonization greatly, as it falls within a “safe” physiological range.

Interestingly, the FDA assay confirmed the mesophilic nature of enzymes present in December and January sampled guano which displayed considerable activity in 40°C

(penguin gut temperature) compared to 4°C (Struvay and Feller 2012; Ropert-Coudert Y et al. 2000). This suggest they may be of penguin and/or penguin gut bacteria origin. Surprisingly, their activity at 4°C was comparable to enzyme activity in March sampled material (advanced stage of decomposition), where psychrophilic enzymes were present (similar activity at 4 and 40°C), which points towards them still being active in recently deposited guano *in situ*, implying the possibility of guano components degradation without active microbial participation (Myrcha et al. 1985). This would explain low percentage of strains bearing guano decomposing traits isolated from December and January sampled material. Rather than degrading complex molecules, primal colonizers scavenge the already digested nutrients (Ghosh et al. 2017).

One such scavenger has been reveal in this study. The recovered *Jeotgalibaca* sp. represents an interesting case of an ammonia scavenger. Its growth was very poor when no ammonia was present. Growth was greatly enhanced when ammonia salts were added or ammonia producing bacteria grew next to it. *Jeotgalibaca* sp. also required complex media to grow, further confirming its auxotrophic and scavenging life style. Colonization by *Jeotgalibaca* sp. presumably occurs after deposition as the isolated strains were psychrophilic with their growth being hampered in media above room temperature (data not shown). And there are no reports of it forming heat resistant endospores (Lee et al. 2014; Zamora et al. 2017). Reports of its sister genus *Trichococcus* say this group is associated with penguin guano deposits, however there is no mentioning of it requiring NH_3 for growth (Pikuta et al. 2006).

Despite having some similar traits, the bacterial community composition of December and January samples differed even at the phylum level. January sampled guano had a considerable amount of Bacteroidetes sequences. This might be connected to the age of young penguins and the state of their intestine flora. Barbosa et al. (2016) observed such differences

474 in fecal samples from chicks and adults of chinstrap penguins (*Pygoscelis antarctica*), where
475 adult birds tended to have more bacteria of the phylum Bacteroidetes, whereas chicks
476 harbored more Firmicutes.

477 *4.2 Late decomposition stage*

478 March samples represent guano in an advanced decomposition stage as there has been
479 no guano deposition for at least a month at this point. In those samples colonization by
480 psychrophilic saprotrophs was apparent. FDA assay shows similar enzyme activity at 4°C and
481 40°C pointing towards psychrophilic enzyme presence (Struvay and Feller 2012). Elevated
482 microbial numbers compared to December and January samples indicate bacterial
483 proliferation, implying also new niche establishment, similarly as observed by Zdanowski et
484 al. (2005). Furthermore, a great majority of cells had intact membranes, suggesting their
485 adaptation to prevalent conditions. Microbial respiration rates at 4°C were considerably
486 higher than in early decomposition stages confirming microbial activity *in situ*. However,
487 penguin gut derived bacteria like the *Gottschalkiaceae* remained at similar abundance level as
488 in recently deposited guano. This suggests adaptation to harsh Antarctic conditions or what is
489 more plausible presence of inactive endospores as these bacteria (formerly in genus
490 *Clostridium*) have been proven to possess this ability (Olguín-Araneda et al. 2015; Poehlein et
491 al. 2017). This scenario could also be applied to several other penguin gut related spore
492 formers persisting in decayed guano (Verbarg et al. 2014, Slobodkin 2014, Alauzet et al.
493 2014). Not so, for the *Porphyromonadaceae* family members, all of which are strict non-spore
494 forming anaerobes (Sakamoto 2014). Their contribution increases in later stages of
495 decomposition, which suggests their proliferation on site therefore implying anaerobic
496 microhabitat establishment. The *Porphyromonadaceae* in Antarctic settings have been
497 enriched in media at anaerobic conditions from wind dispersed cryoconite material
498 (Zdanowski et. al. 2017), hinting toward their ability to survive oxic atmosphere conditions.

Frequently associated with penguins are bacteria belonging to the *Carnobacteriaceae* (Firmicutes) and *Moraxellaceae* (Proteobacteria) families (Yew et al. 2017; Kim et al. 2012, Dewar et al. 2013). Several genera within *Carnobacteriaceae* family have been found in association with penguin-on-land activity, including *Carnobacterium, Atopostipes, Trichoccoccus* and discovered in this study *Jeotgalibaca* (Shekh et al. 2009; Aislabie et al. 2009a, Pikuta et al. 2006). Besides the latter, their role in guano and guano derived materials (ornithogenic soils) have not been elucidated. However, their source seems to be environmental rather than intestinal due to their psychrophilic/psychrotrophic nature and no ability to produce heat-resistant spores (Lawson and Caldwell 2014). Belonging to the *Moraxellaceae* family *Psychrobacter* spp. have been found as an integral part of penguin and other birds' excrements (Romaniuk et al. 2018; Dewar et al. 2017). It's main role is that of an aerobic uric acid degrader as seen in December and January samples. However, *Psychrobacter* strains isolated from March-sampled guano also had the ability to degrade triglycerides (lipolytic activity). This implies depletion of the aforementioned digested nutritional compounds and colonization by active saprotrophic degraders rather than scavengers. This is further confirmed by the cultivation of proteolytic strains of the genus *Chryseobacterium,* found only in decayed guano. Very little chitinolytic strains have been recovered from guano which is consistent with some of the previous observations (Aislabie et al. 2009b; Zdanowski et al. 2004, Pietr et al. 1983). Zdanowski et al. 2005 found that 40% of chitin present in guano after deposition remains even after 42 days of decay in rookery conditions, while CFU numbers of aerobic chitin degrades were low (10^5 g d.w.). Presumably chitin decomposition is mediated by gut derived chitinases in recently deposited guano and if in later stages chitin degradation still occurs unculturable and/or anaerobic microbes may be responsible. Aerobic, cultivable chitinolytic bacteria may occur in detectable quantities in even later stages of guano decay, as they have been found to live in ornithogenic soils

(Aislabie et al. 2009a). Several analyzes suggest that in these stages scavengers also occur. Acetate and putrescine, common waste products of bacterial metabolism (Wolfe 2005; Pircher et al. 2007), were catabolized by bacteria in March gathered samples, which implies that a different group of scavenging microbes thrived on the end products of saprotrophic guano degradation. Despite that penguin guano is said to be rich in mostly insoluble phosphates (Tatur and Barczuk 1985), a minor number of phosphate solubilizing bacteria have been recovered. This could imply either that the guano has enough labile phosphorus sources (dissolved phosphates, organic P) or that the solubilization of phosphate minerals might be releasing toxic substances like fluorine or heavy metals and is therefore unfavorable (Zdanowski et al. 2005; Romaniuk et al. 2018). Cellulose and lactose consumers were also present, as hinted by the EcoPlates. Lactose metabolism might be explained by the presence of lactic acid bacteria of the aforementioned family *Carnobacteriaceae* (Lawson and Caldwell 2014). Cellulose as catabolic substrate may occur due to colonization of the guano by the ornithocoprophyllic alga *Prasiola crispa* (Smykla et al. 2007) and due to remnants of microscopic algae deposited with the guano as krill stomach content (Myrcha and Tatur 1991).

5. Conclusions

Psychrophilic saprotrophs rather slowly colonized recently deposited guano, while the bulk of bacteria at that time was of penguin gut origin. Enzymes present in fresh guano were also mostly of gut origin but had the potential to contribute to guano decomposition *in situ*, at least for some period of time. This set the stage for simple compound scavenger colonization. One of the first psychrophiles to colonize the guano were ammonia scavengers of the *Jeotgalibaca* genus, which fed on dissolved ammonia produced by anaerobic bacteria in penguin guts that was being released after deposition. Psychrophilic, aerobic uric acid decomposers like *Psychrobacter* sp. colonization was apparent but negligible. Their presence

was more noticeable in later stages while a specific succession occurred within this genus with a broadening of utilized substrate spectrum. Decayed guano also harbored proteolytic *Chryseobacterium* sp., acetate and putrescine scavengers and also cellulose and lactose consumers. Several spore forming intestinal bacteria persisted throughout the season in guano material but their activity *in situ* was questionable.

This is the first study that showcases the severe changes that naturally occur within the Adélie penguin guano microbial community throughout the summer season. Due to the variety of methods used it was possible to conclude, that large bacterial groups that show up in high throughput amplicon sequencing data might be dead or inactive, especially in cold polar region settings. The cultivable approach provided information on how to increase the cultivability of guano associated bacteria as well as pinpointed the timeframe in which to prospect for bacteria bearing certain traits that are of value for biotechnological application. Nonetheless, a more detailed investigation is needed, preferably spanning a whole year of sampling to gain a more precise insight into the microbiological interactions within this unique habitat.

Acknowledgements

This work was supported by statuary funding of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. Samples and data were obtained thanks to the scientific facility of the Polish Antarctic Station ARCTOWSKI. Authors declare no conflict of interest.

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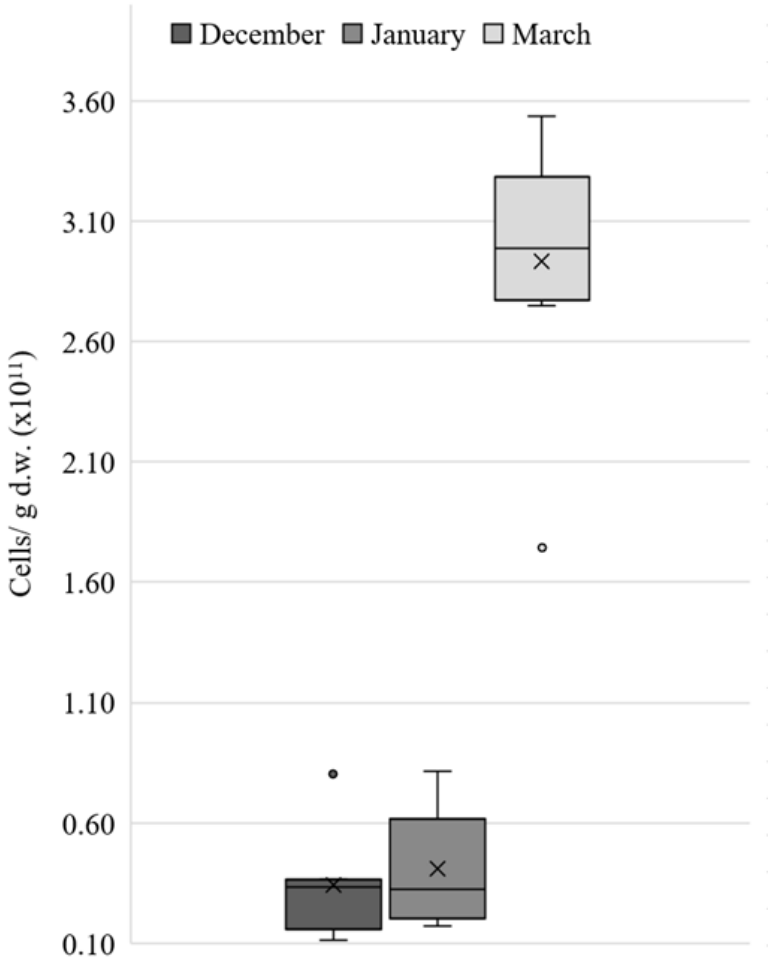
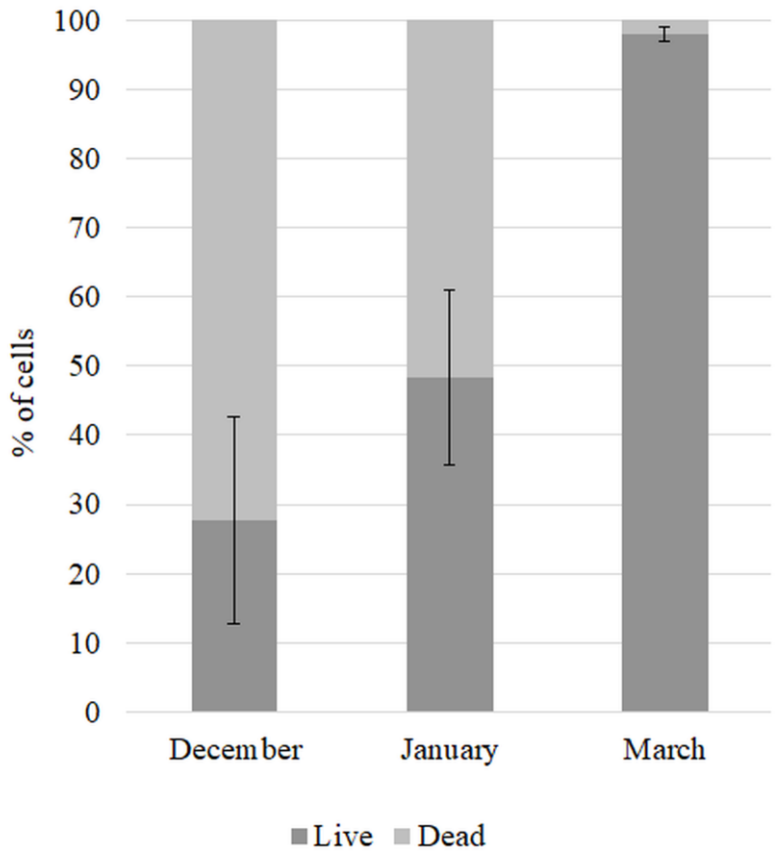


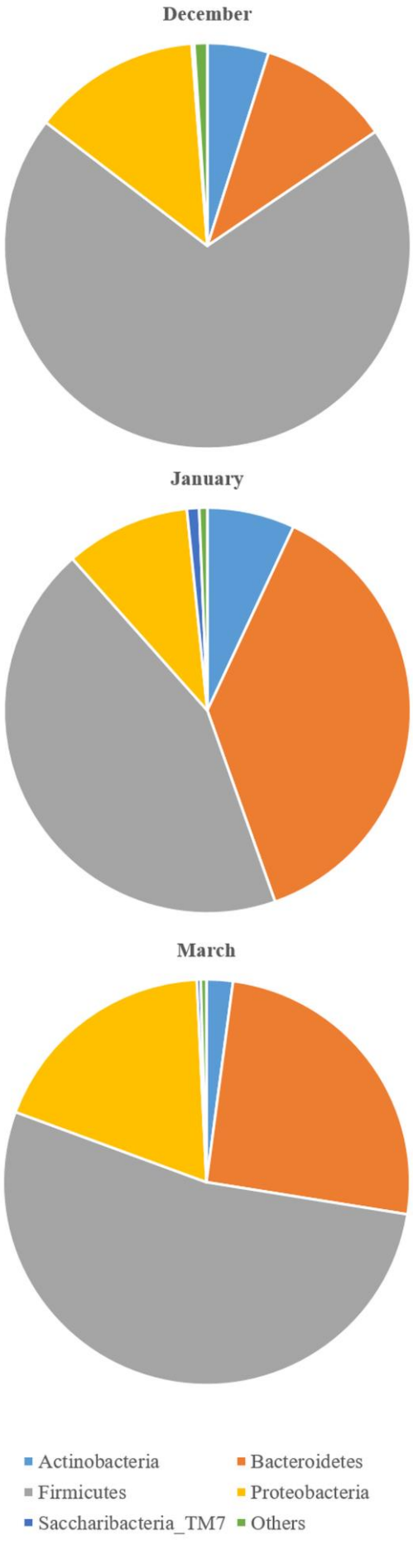
Fig.1. Bacterial total count in penguin guano. Each boxplot represents the distribution of cell numbers per gram dry weight (g. d. w.) of guano in each sampling month. X- mean value.



867

868 **Fig.2. Percentage of bacterial cells with intact (Live) and disrupted membranes (Dead)**

869 **within guano samples from each month. Mean values with standard error.**


December
January
March
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Saccharibacteria_TM7
Others

870

Fig.3. Pie charts with sequence percentage contribution identified at phylum level for pooled samples from each month obtained by 16S rRNA gene fragment amplicon sequencing.

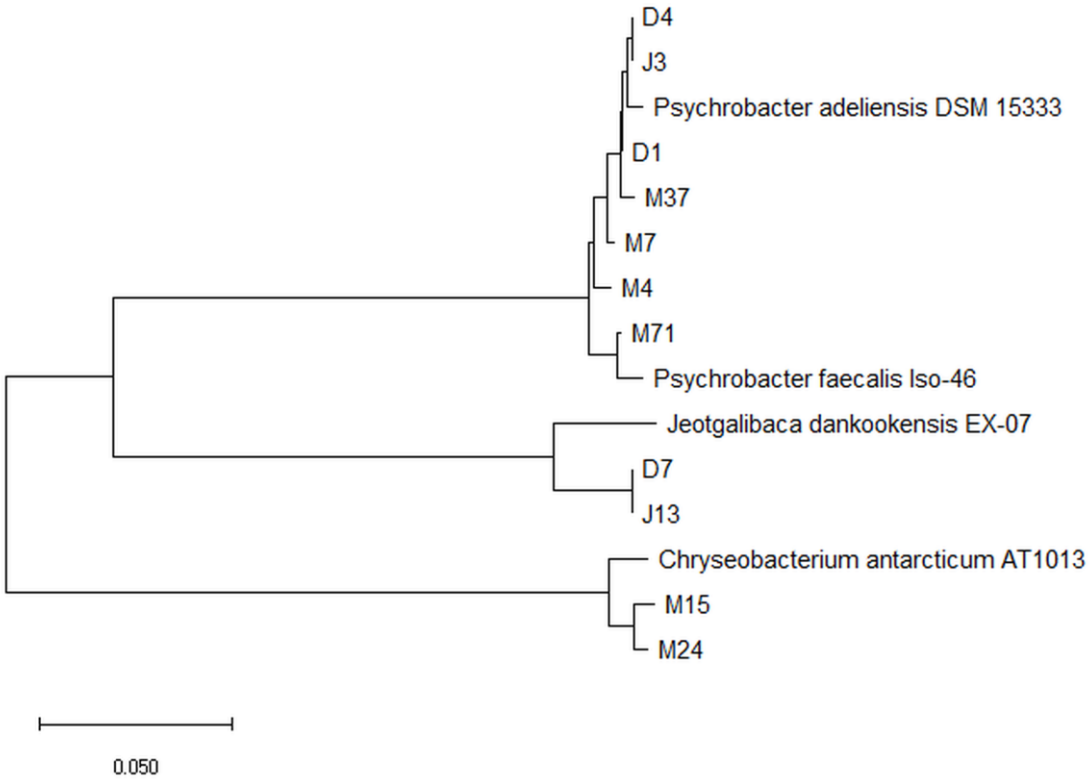
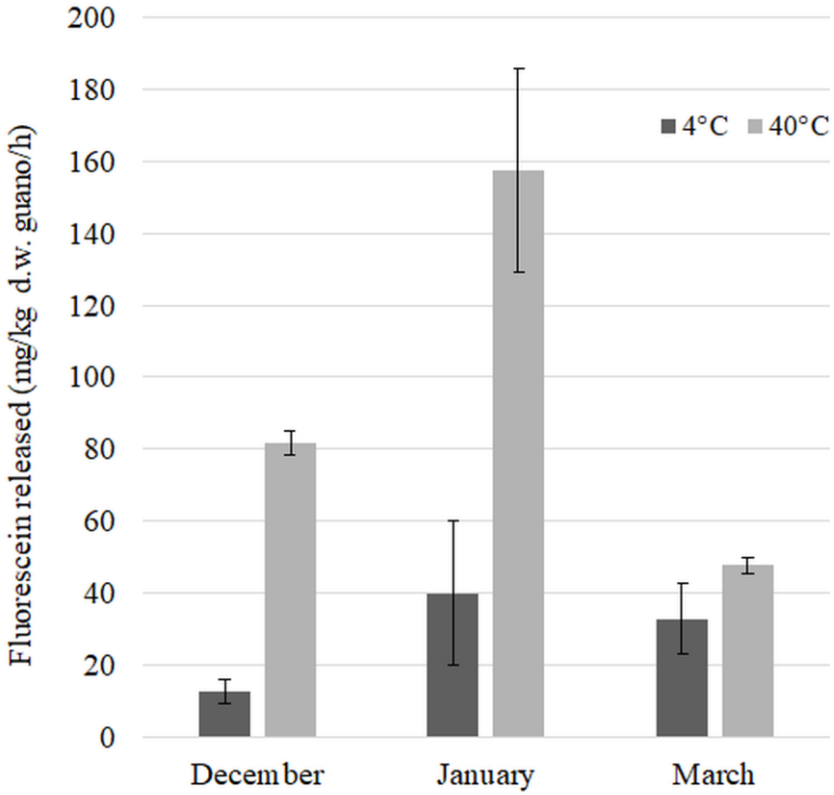


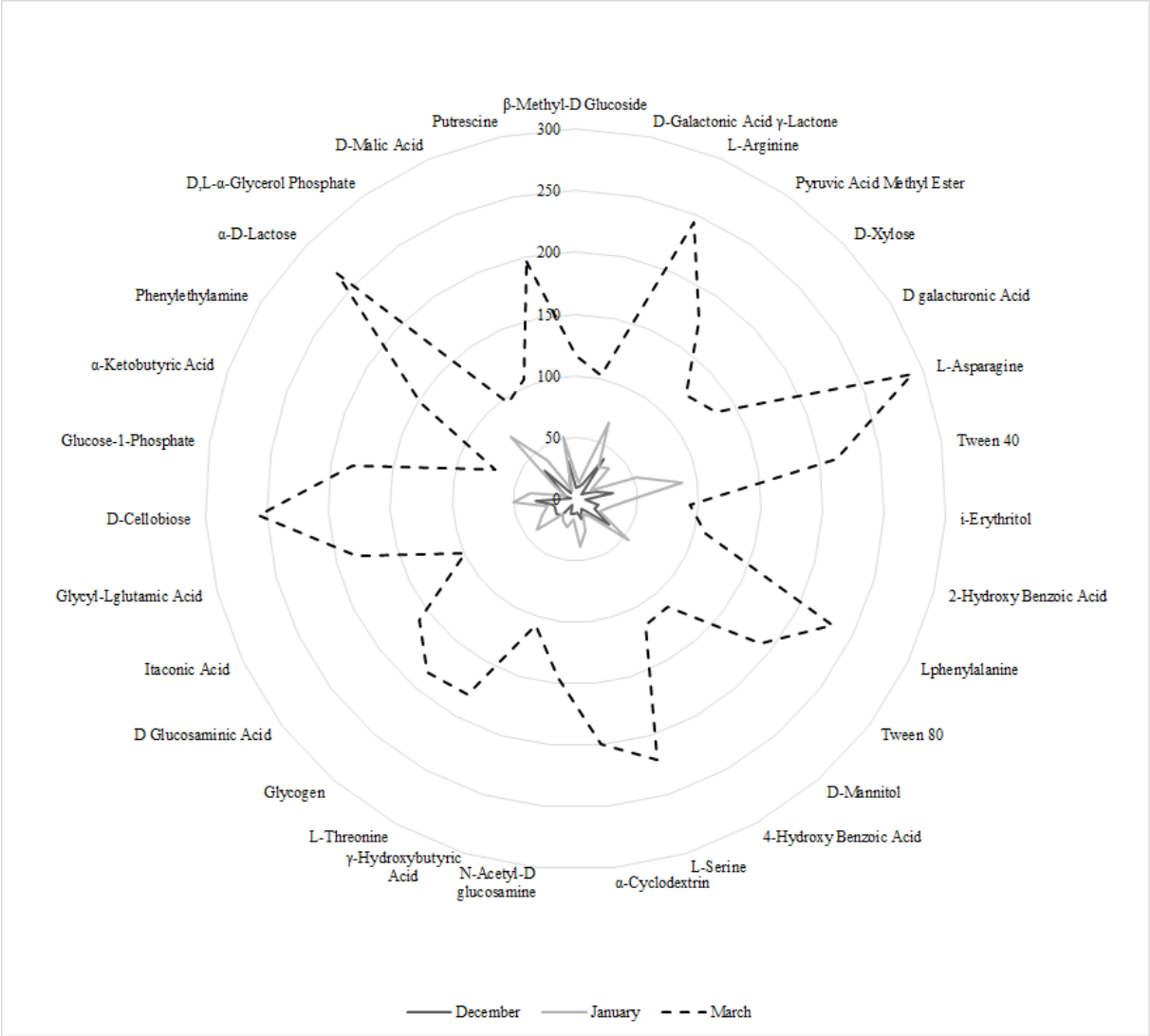
Fig.4. Phylogenetic tree constructed using partial 16S rRNA gene sequences of guano-derived bacterial isolates. Designations indicate sampling month: D-December, J-January, M-March. The tree was built using the neighbor-joining method.



882

883 **Fig. 5. Enzymatic activity in guano samples as fluorescein release rate in mg of**

884 **fluorescein per kg of guano dry weight per hour. Mean values with standard error bars.**



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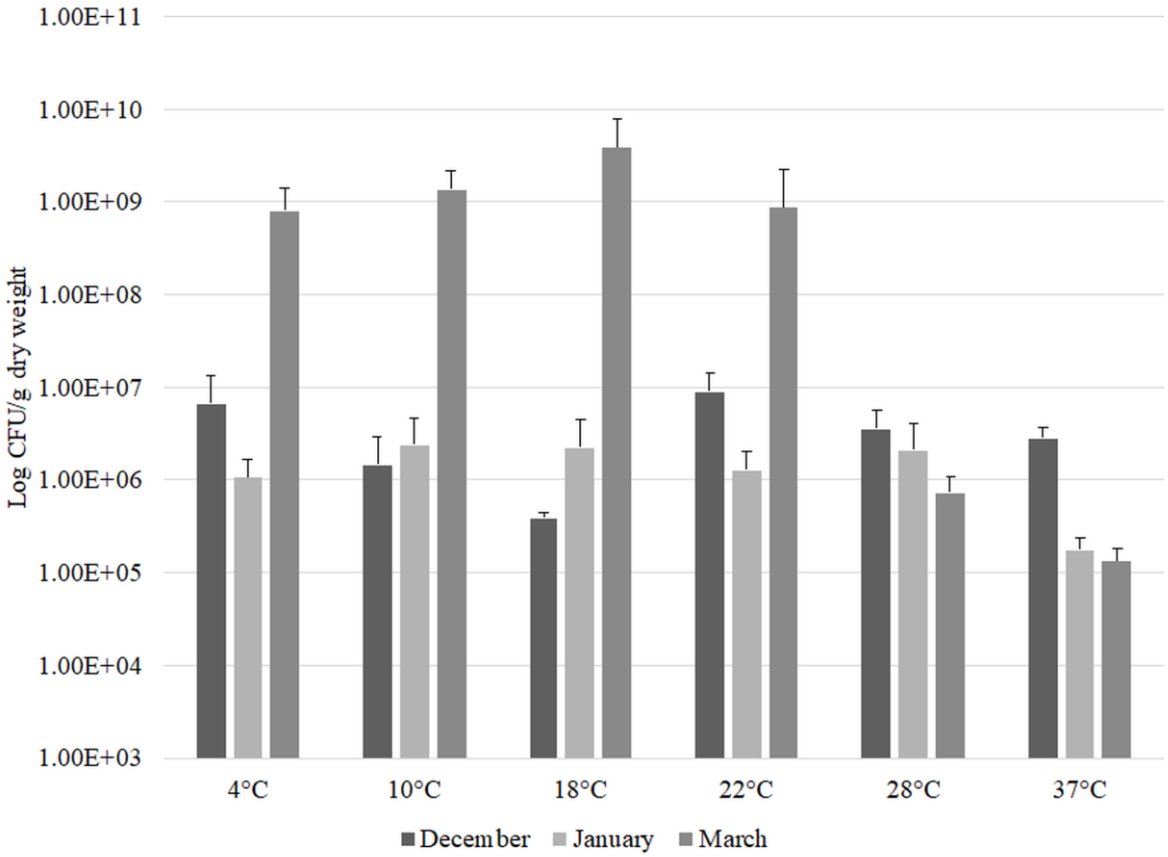
886 **Fig. 6. Radar chart displaying microbial community responses in Biolog Ecoplates.**

887 **Mean values are given in Omnilog Arbitrary Units, n=9.**



888

889 **Fig.7. Aerobic colony forming unit (CFU) count on R2A agar after incubation in given**

890 **temperature expressed as log CFU per gram dry weight (g.d.w.). Bars represent mean**

891 **values with standard error, n=9.**

892

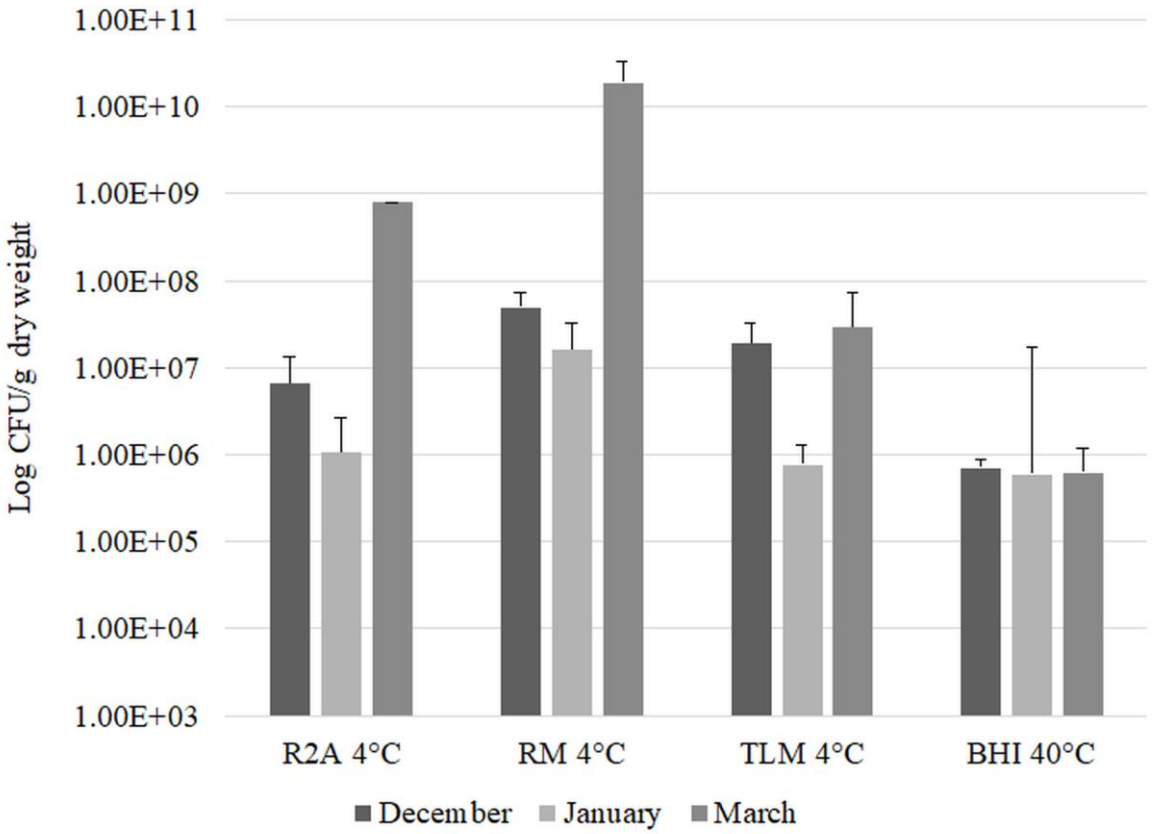


Fig.8. Colony forming unit (CFU) count on different media and conditions expressed as logCFU per gram dry weight of guano. Bars represent mean values with standard error, n=9. R2A 4°C – R2A agar medium at 4°C in aerobic conditions; RM4°C – RM agar medium at 4°C in aerobic conditions; TLM 4°C – Thioglycollate agar medium at 4°C in anaerobic conditions; BHI 40°C – Brain Heart Infusion agar medium at 40°C in microaerophilic conditions.

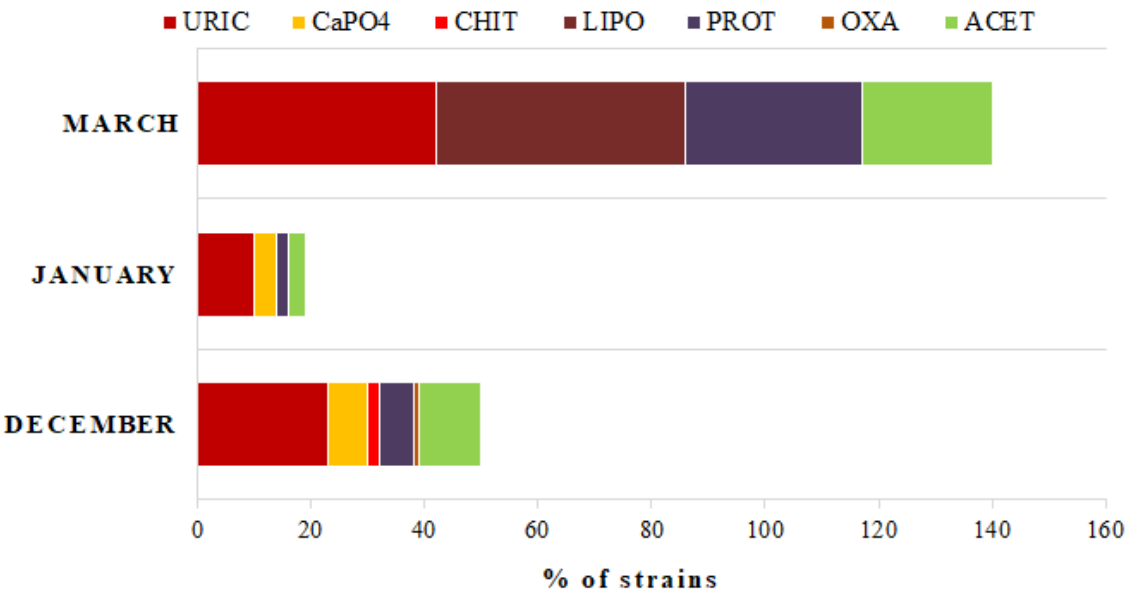


Fig. 9. Percentage of strains expressing guano decomposing abilities isolated from material sampled in each month. URIC-uric acid decomposition; CaPO4-calcium phosphate solubilization; CHIT-chitin degradation; LIPO-lipolytic activity; PROT-proteolytic activity; OXA-oxalic acid utilization; ACET-acetate consumption.

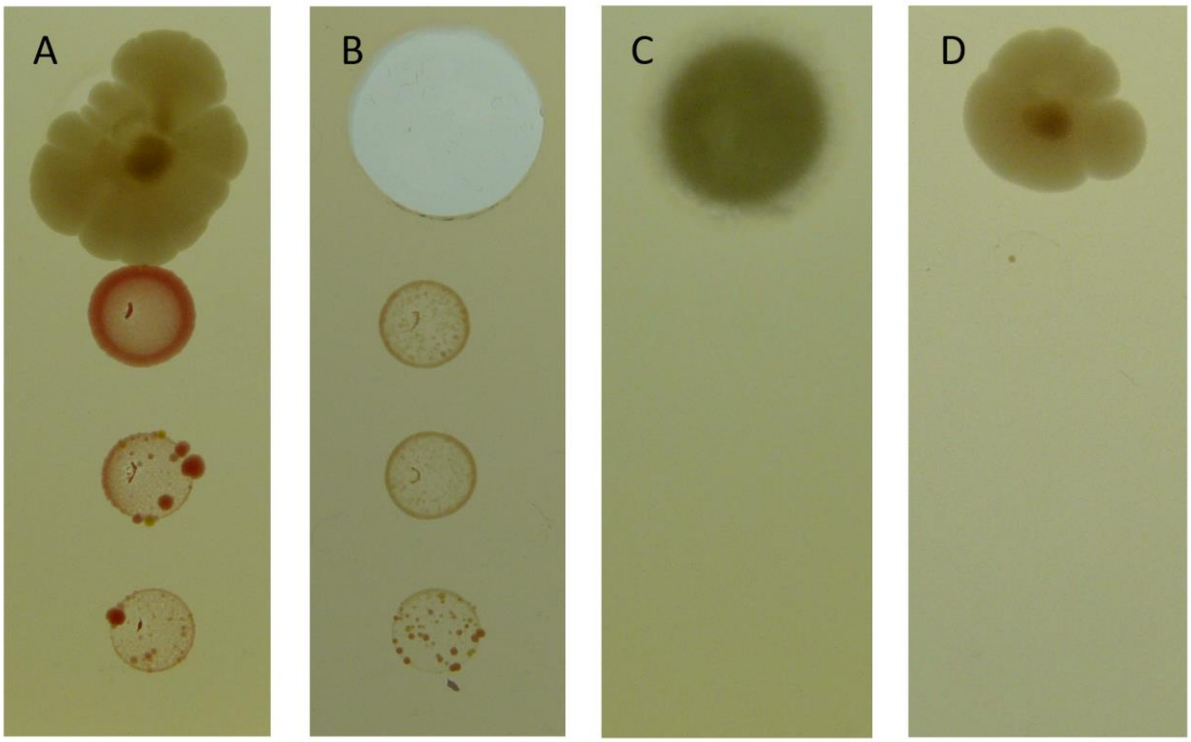


Fig. 1S. Growth of Jeotgalibaca sp. S13 inoculated as 5µl liquid culture to the surface of R3A agar medium. A – growth stimulation of Jeotgalibaca sp. S13 near an irregular colony of uricolytic Psychrobacter sp. growing on uric-acid agar plug. B - growth stimulation of Jeotgalibaca sp. S13 near ammonium chloride solution filled agar-cut well. C – control without Psychrobacter sp. inoculation. D – control without uric-acid agar plug underneath Psychrobacter sp. growth.

Sample ID	Coordinates	Guano temperature	Air temperature (1cm above sampling site)	Atmosferic conditions during sampling*	Breeding site description
D1	62 09.811 S, 58 27.762 W	10.8°C	6.0°C	Temperature: 2.8°C; Humidity: 63%; Wind: 3.6 m/s; Air pressure: 999 hPa	P. adeliae chics gathered in nurseries, begin to shed feathers.
D2	62 09.816 S, 58 27.698 W	13.5°C	7.1°C		
D3	62 09.821 S, 58 27.688 W	11.2°C	6.9°C		
J1	62 09.811 S, 58 27.762 W	9.2°C	7.0°C	Temperature: 4.6°C; Humidity: 85%; Wind: 0.2 m/s; Air pressure: 992 hPa	Most chics with new feather coat, begin to leave the colony for sea shore; colony begins to depleet in adult penguins.
J2	62 09.816 S, 58 27.698 W	8.2°C	5.7°C		
J3	62 09.821 S, 58 27.688 W	8.0°C	5.7°C		
M1	62 09.811 S, 58 27.762 W	3.8°C	5.7°C	Temperature: 2.1°C; Humidity: 73%; Wind: 7m/s; Air pressure: 993 hPa	Rookery devoid of penguins since early February.
M2	62 09.816 S, 58 27.698 W	4.5°C	6.3°C		
M3	62 09.821 S, 58 27.688 W	3.3°C	4.8°C		

Tab. 1. Site description, guano temperature and atmospheric conditions during sampling. Sample abbrev.: D – December samples, J – January samples, M – March samples. *Data obtained from the meteorological station at Arctowski Polish Polar Station.

	December	January	March
Bacillaceae	6.35	3.18	5.00
Carnobacteriaceae	14.93	6.70	9.05
Clostridiaceae	2.99	1.47	2.44
Crocinitomicaceae	0.01	0.81	1.19
Erysipelotrichaceae	5.55	3.12	3.84
Flavobacteriaceae	2.09	26.27	7.55
Gottschalkiaceae	18.57	18.40	19.28
Intrasporangiaceae	0.95	1.99	0.28
Lachnospiraceae	2.12	0.94	4.79
Moraxellaceae	10.90	5.25	5.21
Moritellaceae	0.00	0.74	1.66
Neisseriaceae	0.00	0.90	0.00
Nocardiaceae	1.89	1.51	0.94
Peptostreptococcaceae	11.27	1.21	0.67
Planococcaceae	1.41	2.57	0.60
Porphyromonadaceae	8.00	7.51	15.56
Pseudomonadaceae	0.39	0.30	10.20
Sphingobacteriaceae	0.07	1.37	0.26
Tissierellaceae	6.03	5.85	6.94
Xanthomonadaceae	1.01	0.87	0.28

Tab.2. Heatmap displaying percentage contribution of family-rank affiliated sequences derived from pooled guano samples within each sampling month obtained by 16S rRNA gene fragment amplicon sequencing.

Culture conditions	December	January	March
R2A 4°C	0.019%	0.003%	0.273%
R2A 10°C	0.004%	0.006%	0.463%
R2A 18°C	0.001%	0.005%	1.293%
R2A 22°C	0.026%	0.003%	0.297%
R2A 28°C	0.010%	0.005%	0.000%
R2A 37°C	0.008%	0.000%	0.000%
RM 4°C	0.145%	0.039%	6.443%
TLM 4°C	0.056%	0.002%	0.010%
BHI 40°C	0.002%	0.001%	0.000%

Table 3. Cultivability of guano-derived bacteria expressed as contribution percentage of average colony forming units for particular culture conditions in each month bacterial total count for that month. Color intensity indicates highest values within sampling month for temperature range and within culture condition for the remaining data.

Medium	***Jeotgalibaca* sp. D7**	***Jeotgalibaca* sp. J12**
MM	-	-
MM+NH_4Cl	-	-
MM+Urea	-	-
MM+KNO_2	-	-
MM+KNO_3	-	-
MM+Allantoin	-	-
MM+Casamino acids	-	-
R3A	-	-
R3A+NH_4Cl	+	+
R3A+Urea	-	-
R3A+KNO_2	-	-
R3A+KNO_3	-	-
R3A+Allantoin	-	-
R3A+Casamino acids	-	-

990

991 Table 1S. Growth characteristics of Jeotgalibaca sp. Isolated from P. adeliae guano on
992 different media supplemented with various nitrogen sources. MM- mineral medium devoid of
993 a nitrogen source, R3A – R3A medium. + growth, - no growth.

994