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High antagonistic activity and antibiotic resistance of flavobacteria of polar microbial freshwater mats on King George Island in maritime Antarctica

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This is the first study to demonstrate a relationship between antagonistic interactions with antibiotic resistance within flavobacterial strains, a component of polar-region microbial mats. These strains were derived from ephemeral freshwater reservoirs, i.e. ponds and streams of the periglacial zone of Ecology Glacier (King George Island, maritime Antarctica). The study demonstrated the strains' surprisingly high phylogenetic diversity, with 20 species among 50 isolates. Flavobacteria were characterised by different patterns of antagonism and sensitivity to antimicrobials. 29 strains produced substances inhibiting the growth of other isolates, with 21 strains being sensitive to such compounds; 34 strains were multidrug-resistant (MDR). The antibiotic resistance index (ARI) demonstrated a significantly higher proportion of MDR strains and ARI ≥ 0.2 in stream mats (87%) as compared to the strains derived from pond mats (55%). A strong correlation was observed between the strains' antagonistic potential and antibiotic resistance. An important role in these phenomena is accomplished by the "super bacteria" strains that effectively accumulate numerous traits associated with antagonistic potential and can be involved in the potential transfer of these traits. The results of the study demonstrate that there are individual patterns of antagonistic interactions and antibiotic resistance among the biotic components of mats.

Keywords Flavobacteria, Antagonistic activity, Antibiotic resistance, Microbial Mats, Antarctica

Polar regions are characterised by extreme environmental conditions, including low temperatures, high UV intensity, limited nutrient availability and the pressure from frequent freezing and thawing[1]. Due to the selectivity of these factors, only microorganisms with high adaptive capacities thrive in these environments.

Flavobacteria in natural environment

Flavobacteria are widely distributed in nature and characterised by high molecular, metabolic and physiological plasticity[2,3]. Currently (September 2024), over 400 species are classified in the genus *Flavobacterium*, including strains described as occurring in natural environments and relevant to veterinary medicine, agriculture, medicine and biotechnology[4]. This taxon's widespread occurrence, diversity and environmental potential have aroused strong interest among researchers in both cognitive and applied terms. Flavobacteria are among the most commonly isolated cold-adapted microorganisms of polar-region environments. These include organisms indigenous to these regions, e.g. *F. antarcticum. F. glaciei*, *F. fryxellicola*, *F. degerlachei and F. sinopsychrotolerans*[1,5–11]. The characteristic features of psychrotolerant and psychrophilic flavobacteria include an adaptive strategy for producing pigments (mainly carotenoids), proteins that neutralise reactive oxygen species as well as antifreeze and cold shock proteins[12]. Flavobacteria colonise diverse ecosystems of Arctic and Antarctic polar environments, including glaciers, lakes, streams, soils and plant rhizosphere zones, as well as microbial mats[1,3,6,13].

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Polar microbial mats

Polar-region freshwater microbial mats contain a particularly diverse microbiota that mainly comprises *Cyanobacteriota*, *Chloroflexota*, *Pseudomonadota*, *Actinomycetota*, *Bacteroidota*, *Bacillota* and *Archaea*[14,15]. In the structure of cold-environment microbial mats flavobacteria are an abundant component[3,5]. Conglomerates of diverse microbial groups usually comprise aggregated, self-sustaining, autonomous ecosystems. Their distinctive feature is the close link between the primary and secondary production processes and the regeneration of nutrients[16,17]. The dominant portion of the total biomass of the microbiome of polar terrestrial ecosystems is contained within microbial mats indicating the crucial role they play in the functioning of these extreme environments[18,19]. In heterogeneous microbial mat biocenotic systems, the essential factor that shapes the structure of the consortia is the intercellular interaction taking place at the species, functional group, and whole microbiota levels. Positive and negative interactions determine the dynamics of the colonised environment's microbiota[20].

Antagonistic interactions and antibiotic resistance

Antagonistic responses are among the most primeval and essential forms of interactions occurring between microorganisms that make up microbial mats consortia[21,22]. Aggregated microorganisms have developed mechanisms to actively compete through bacteriostatic or bactericidal interactions with rival microbial communities or species[23]. Bacteria, which have a very wide range of different compounds at their disposal, i.e. exotoxins, toxic enzymes, bacteriocins and antibiotics, use them in antagonistic intercellular interactions[24,25]. The consequence of such interactions is the acquisition of resistance to these compounds. Forming an extended antibiotic resistome is particularly important for bacteria. Widespread antibiotic resistance in microbial mat microbiocenoses is the result of two mechanisms, i.e. natural evolutionary processes, which represent important adaptation of bacteria developing in heterogeneous microbiocenoses[26–28], and induced resistance resulting from environmental contamination by commercial antibiotics used e.g. in medicine, agriculture and veterinary medicine[29,30]. At the same time, diverse horizontal gene transfer (HGT) mechanisms lead to rapid and efficient development of drug resistance across the microbiota[31]. These processes result in a pool of strains characterised by multi-antibiotic resistance, representing an important reservoir of resistance genes in the global microbiome[32]. Referring to these phenomena, contemporary research into microbial mats lacks reports describing antagonistic interactions between their biotic components and their antibiotic resistance. The novelty of the current study is that it describes these relationships within flavobacteria, which are ecologically important and abundant in natural environments, including polar environments.

The current study contains a phylogenetic analysis of flavobacterial strains isolated from polar-region microbial mats that develop in small freshwater ponds and streams of the proglacial zone of the Ecology Glacier on King George Island, Antarctica (vicinity of Arctowski Polish Antarctic Station). It also presents the results of investigated antagonistic activity of flavobacterial isolates in the cross-inhibition reaction and their resistance to antibiotics. It was hypothesised that antagonism at the species level in aggregated microbial mat systems is a common phenomenon and plays a significant role in shaping bacterial communities. It was assumed that the antagonistic interactions are linked to the antibiotic resistance of strains, and the intensity of these interactions is determined by the nature of the aquatic environment in which the microbial mat is developing.

Materials and methods

Study site

The Ecology Glacier is situated near the Arctowski Polish Antarctic Station at the western shore of Admiralty Bay, on King George Island, South Shetland Archipelago, maritime Antarctica (Fig. 1).

It is subjected to annual surface snow melt like other glaciers in the vicinity[33]. The study was carried out in the vicinity of this glacier. Samples were taken during the Antarctic summer (March/April) of 2019 at twenty sites from streams (S) and ponds (P). Detailed description of research sites, environmental parameters and physicochemical conditions are presented in Tables 1 and 2.

Microbial mats sampling and isolation of bacterial strains

The mats collected for research were also the subject of other analyses, including metagenomic and phycological analyses. The study showed that the structure of the microbial mats was characterised by the dominance of two main groups of photoautotrophs: cyanobacteria and diatoms[34]. An example of the macroscopic and microscopic (cross-section) structure of the microbial mat of the periglacial zone of Ecology Glacier is shown in Fig. 2. Microbial mats were taken from mineral and organic substrates from proglacial streams and ponds.

Under aseptic conditions, the samples were placed into sterile Petri dishes. Mat samples were collected twice using a flat metal mesh with an area of 5 cm^2. A total of 20 microbial mats were collected. For bacterial cultivation, 1 g of each mat sample was suspended in 20 mL sterile saline (0.85%) in 100 mL sterile flasks with glass beads and shaken gently on a universal shaker (150 rpm, 20 min, 5°C). Suspensions were then stored in the refrigerator (4°C) for 10–20 min to allow larger particles to settle. A decimal dilution series of the supernatant to 10^{-3} was prepared in sterile saline, and then 100 µL of suspension was inoculated on Petri plates with R2A medium in triplicates. The cultures were incubated at 7°C for one month and inspected every third day for colony development and growth. This also enabled observing particular colony types from different sampling points and CFU growing rates. Pure cultures of different bacterial colonies were isolated after transport to Poland. Bacterial colonies that differed in phenotypic traits i.e. macroscopic appearance (the form of colonies, size and pigmentation) were selected for further purification. The obtained single colonies were transferred onto new Petri dishes with R2A to obtain pure cultures. Finally 127 isolates were molecular identified.

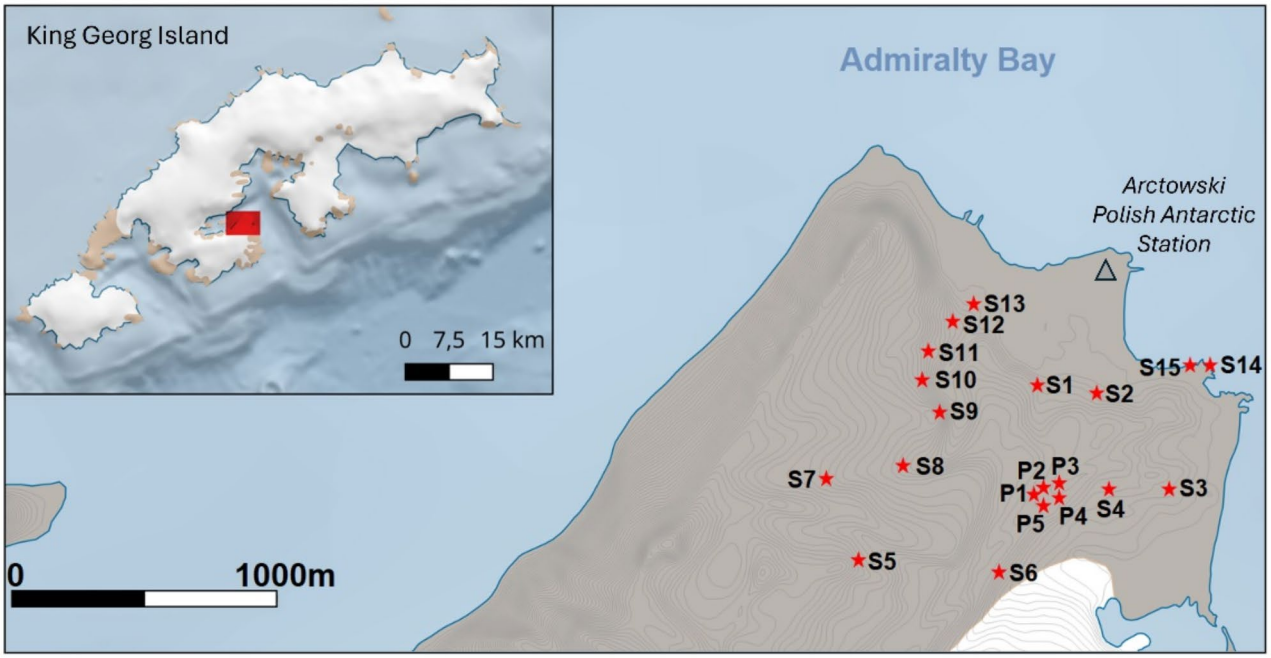

Fig. 1. Location of the sampling area and sampling sites of streams (S1–S15) and ponds (P1–P5) microbial mats in the periglacial zone of Ecology Glacier, King George Island, maritime Antarctica. Characteristic of sampling sites is shown in Table 1 (https://www.npolar.no/quantarctica/; QGIS 3.40 Bratislava, http://www.qgis.org/).

Site	Coordinates	Site description
S1	62.21869 S; 58.35080 W	Stream on the slope of Upłaz moraine
S2	62.16886 S; 58.48086 W	Stream on the slope of Upłaz moraine
S3	62.16700 S; 58.46867 W	Side moraine stream of the Ecology Glacier (south—S)
S4	62.16789 S; 58.46739 W	Side moraine stream of the Ecology Glacier (north—N)
S5	62.16931 S; 58.47725 W	Czech Creek
S6	62.16892 S; 58.47225 W	Czech Creek
S7	62.16675 S; 58.4918 W	Petrified Forest Stream (the highest/beginning point)
S8	62.16681 S; 58.49175 W	Petrified Forest Stream
S9	62.16689 S; 58.48817 W	Petrified Forest Stream
S10	62.16508 S; 58.47811 W	Petrified Forest Stream
S11	62.16789 S; 58.48356 W	Petrified Forest Stream
S12	62.16728 S; 58.48253 W	Petrified Forest Stream
S13	62.16667 S; 58.48094 W	Inflow to the drinking water reservoir (the lowest/ending point)
S14	62.16200 S; 58.46222 W	Tributary area from a penguin colony
S15	62.16219 S; 58.46239 W	Tributary area from a penguin colony
P1	62.16486 S; 58.47025 W	Ponds at the top of the lateral moraine of the Ecology Glacier
P2	62.16572 S; 58.47033 W	Ponds at the top of the lateral moraine of the Ecology Glacier
P3	62.16603 S; 58.47097 W	Ponds at the top of the lateral moraine of the Ecology Glacier
P4	62.16603 S; 58.47067 W	Ponds at the top of the lateral moraine of the Ecology Glacier
P5	62.16608 S; 58.47033 W	Ponds at the top of the lateral moraine of the Ecology Glacier

Table 1. Characteristics of the study sites (S – stream, P – pond).

16 S rRNA gene-sequencing-based taxonomic identification

Bacterial DNA obtained from a single colony was isolated using the Genomic Mini AX Bacteria + kit (A&A Biotechnology, Poland) with additional mechanical lysis of the sample in a FastPrep-24 device using zirconium beads. DNA concentration was measured using the fluorometric method on a Qubit 4 Fluorometer. Primers **27f** (5'-GAG TTT GAT CCT GGC TCA G-3')[35] and **rp2** (5'-ACG GCT ACC TTG TTA CGA CTT-3')[36] were used in the PCR reaction. DNA obtained from the amplification reaction (product length 1260 bp) was purified using

Parameters	Minimum	Maximum	Mean	±SD	CV (%)
Streams					
Temperature, T (°C)	1.3	5.6	3.3	1.42	43
pH	6.50	8.31	7.09	0.54	8
Electrolytic conductivity, EC (μS cm^{-1})	70	1514	268.2	397.52	148
Total nitrogen, TN (mg dm^{-3})	0.70	8.40	2.30	2.22	99
Ammonium, N-NH4 (mg dm^{-3})	0.02	2.30	0.24	0.59	249
Total phosphorus, TP (mg dm^{-3})	0.09	4.30	0.60	1.27	212
Phosphates, P-PO4 (mg dm^{-3})	0.02	1.33	0.19	0.40	210
Ponds					
Temperature, T (°C)	3.7	5.1	4.6	0.55	12
pH	6.98	8.83	7.66	0.72	9
Electrolytic conductivity, EC (μS cm^{-1})	72	350	209.6	120.83	58
Total nitrogen, TN (mg dm^{-3})	0.70	4.10	1.86	1.30	70
Ammonium, N-NH4 (mg dm^{-3})	0.02	2.30	0.24	0.59	249
Total phosphorus, TP (mg dm^{-3})	0.09	4.30	0.60	1.27	212
Phosphates, P-PO4 (mg dm^{-3})	0.02	1.33	0.19	0.40	210

Table 2. Physical and chemical parameters in the streams and ponds in March/April 2019.

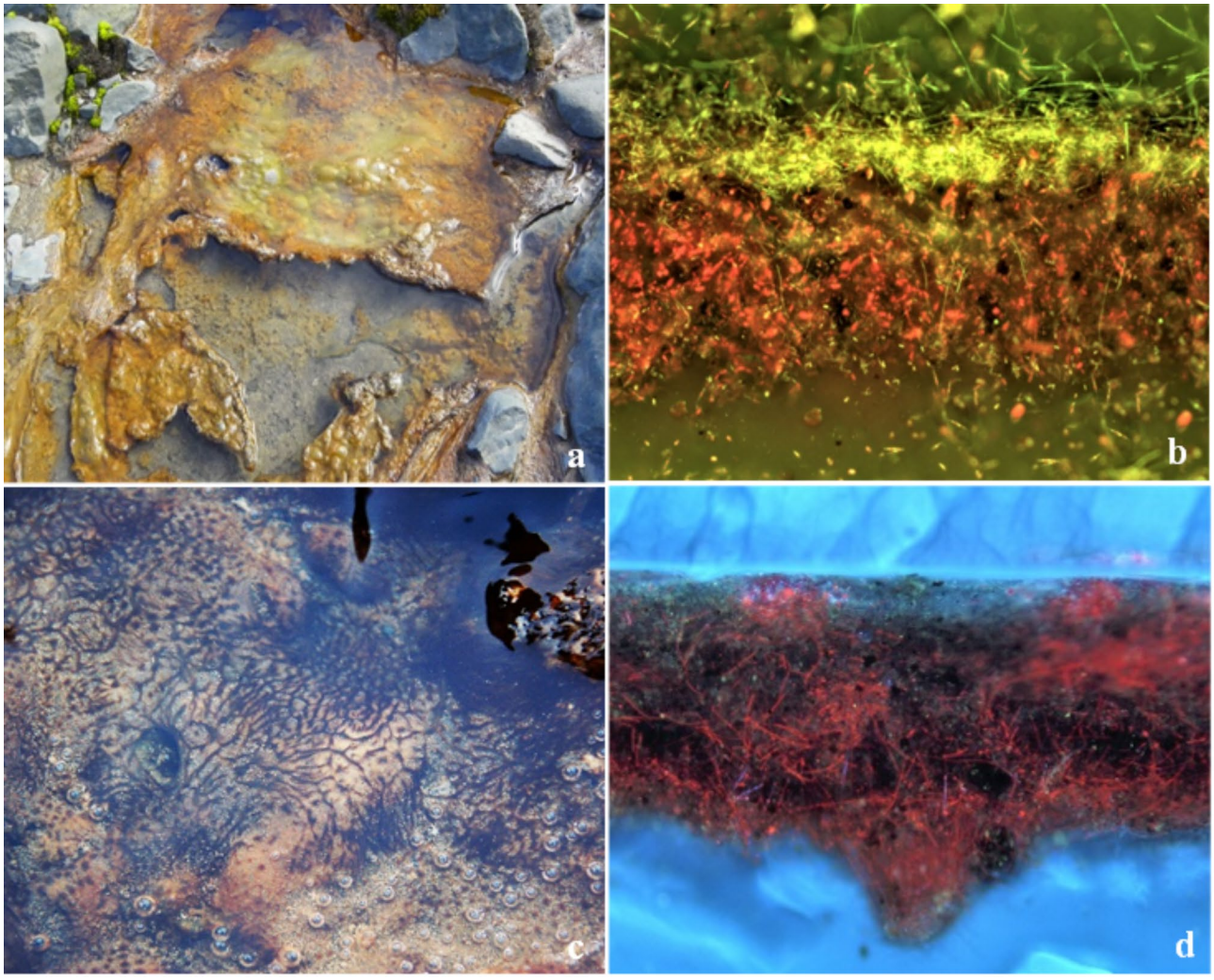

Fig. 2. Macroscopic and microscopic (vertical cross section) structure of the microbial mats of the periglacial zone of Ecology Glacier; stream (**a**, **b**) and pond (**c**, **d**).

the Clean-Up AX kit (A&A Biotechnology, Poland). PCR products, suspended in 10 mM Tris-HCl pH 8.0 buffer and diluted to a concentration of 50 ng/µL, were sent for sequencing to Macrogen Europe BV (The Netherlands).

Antibiotic resistance

According to Bauer et al. (1966)[37], the disc diffusion method was applied using R2A as a replacement for Mueller-Hinton agar to determine the phenotypic antibiotic resistance of flavobacteria strains originating from microbial mats. The 25 antibiotics with three different modes of action, belonging to 12 functional groups, were used (Antimicrobial Susceptibility Discs, Oxoid) (Table 3). The growth temperature of the cultures during the determination of antibiotic resistance and antagonist activity was determined based on previously performed optimization tests (data not shown). For the tested flavobacteria, the optimal growth temperature was 20ºC. The plates were incubated at these temperature for 48–96 h, depending on the growth rate of strains. Antibiotic resistance was considered to be the absence of a zone of inhibition of the strain's growth around the antibiotic disk. The antibiotic resistance index (ARI) was determined according to Krumperman et al. (1983)[38]. Multi-drug resistance (MDR) was determined according to resistance to at least one of each mode of action of antibiotics used[39].

Antagonistic interactions

Taxonomically identified flavobacteria strains were screened for antimicrobial substance production using the spot-on-lawn method described by Prasad et al. (2011)[40]. Each strain was tested against the other strains for cross-inhibition. The cells were washed out from 1 mL of liquid culture of each strain by centrifuging three times (10 min/8000 rpm), each time suspending the cell pellet in sterile physiological saline at 20 °C. The cell pellet was then adjusted to a density of 0.5 McFarland and plated on the R2A medium, treating the inoculated strain as a indicator strain for examining the relationship with other strains. Following this, 10 µL of liquid culture of each of the remaining strains was applied pointwise. The culture was carried out at 20 °C for seven days. After this time, the formation of a growth inhibition zone of the indicator strain around the spotted strains was considered an antagonistic effect.

Statistics

All data were statistically analysed using Statistica version 13.3 (StatSoft Inc.). The assessment of the significance of differences in the data obtained used a multivariate analysis of variance (ANOVA), and for data that failed to meet the normality test, a non-parametric Kruskal-Wallis test was applied. Linear regression analysis (LRA) was used to investigate correlational relationships. The antagonistic relationships associated with antibiotic

Antibiotic	Symbol	Functional group	Mode of action (inhibitors of)	Disk potency (µg)
Ampicillin	AM	β-lactams	Cell-wall synthesis	2
Carbenicillin	PY	β-lactams	Cell-wall synthesis	10
Cefixime	CFM	Modified β-lactams/3rd generation cephalosporine	Cell-wall synthesis	100
Cefotaxime	CTX	Modified β-lactams/3rd generation cephalosporine	Cell-wall synthesis	5
Ceftazidime	CAZ	Modified β-lactams/3rd generation cephalosporine	Cell-wall synthesis	5
Imipenem	IMP	Modified β-lactams/Carbapenem	Cell-wall synthesis	2
Vancomycin	VA	Glycopeptide	Cell-wall synthesis/ RNA synthesis	30
Gentamicin	CN	Aminoglycosides	Protein synthesis	15
Kanamycin	K	Aminoglycosides	Protein synthesis	15
Streptomycin	S	Aminoglycosides	Protein synthesis	30
Chloramphenicol	C	Chloramphenicol/ Aminoglycosides	Protein synthesis	10
Tetracycline	TE	Tetracycline/Polyketide	Protein synthesis	1.25
Clarithromycin	CLR	Macrolide	Protein synthesis	10
Erythromycin	E	Macrolide	Protein synthesis	5
Clindamycin	DA	Lincosamide	Protein synthesis	15
Lincomycin	L	Lincosamide	Protein synthesis	30
Mupirocin	MUP	Monocarboxylic acid	Protein synthesis	5
Nitrofurantoin	F	Nitrofuran Inhibitor of folic acid synthesis	DNA/RNA synthesis	5
Novobiocin	NV	Aminocoumarin	DNA/RNA synthesis	10
Ciprofloxacin	CIP	Quinolones	DNA/RNA synthesis	30
Nalidixic acid	NA	Quinolones	DNA/RNA synthesis	5
Metronidazole	MET	Metronidazole/Quinolones	DNA/RNA synthesis	100
Rifampicin	RA	Rifamycin	DNA/RNA synthesis	25
Trimethoprim	TMP	Trimethoprim	RNA synthesis	5
Cotrimoxazole	SXT	Trimethoprim/Sulfamethoxazole	RNA synthesis	5

Table 3. Characteristics of the antibiotics used in the study.

resistance were illustrated in network graphs using the program Cytoscape 3.1.0[41], and the data were processed in the R Package's in-house scripts.

Results

The current study examined the antibiotic resistance and antagonist interactions of 50 isolates belonging to the genus *Flavobacterium*, representing 39% of all identified strains, originating from microbial mats of freshwater ponds and streams.

Phylogenetic analysis

Twenty species were identified among the 50 strains belonging to the genus *Flavobacterium*, derived from microbial mats of ponds and streams (Fig. 3). Ten species were noted in pond and stream mats, with the more numerous species including *F. aquidurense*, *F. hydatis*, *F. kayseriense*, *F. saccharophilum* and *F. xanthum*. Five species originated from ponds with the *F. antarcticum* strains being the most abundant. Five species were isolated exclusively from stream mats, among which *F. pectinovorum* strains were the most abundant. Sequence data of studied flavobacteria strains have been deposited in the GenBank database. A phylogenetic tree based on 16 S rRNA gene sequences comparing the isolated flavobacteria strains among their closest related species within the genus *Flavobacterium* and the accession numbers are shown in Fig. 3.

Antibiotic resistance

Antarctic flavobacterial strains isolated from microbial mats exhibited a broad spectrum of antibiotic resistance (Fig. 4). Among the isolates under study, 98% exhibited resistance to at least one of the 25 antibiotics applied. Nearly half (45%) of the strains were resistant to 1–5 antibiotics, 20% to 6–10 antibiotics, and 15% to 11–25 antibiotics. A significant percentage among the isolates under study (68%) were multi-drug resistant (MDR) strains, i.e. strains resistant to at least one antibiotic from each of the three groups mode of action, i.e. inhibiting cell wall synthesis (CW), protein synthesis (P), and nucleic acid synthesis (NA). MDR strains accounted for 65% and 70% of isolates in ponds and streams, respectively. 42% of them represented strains resistant to 10 or more antibiotics. Among the strains derived from pond mats, 80% were resistant to at least one antibiotic from the CW group and 65% to antibiotics from the P group. All strains were resistant to at least one antibiotic from the NA group. In the group of antibiotics inhibiting cell wall synthesis (CW), the largest pool was bacteria resistant to ß-lactams, i.e. ampicillin (40%) and carbenicillin (40%), as well as a 3rd generation cephalosporin, i.e. cefixime (45%). Only two strains derived from pond mats, *F. antarcticum* and *F. kayseriense*, were resistant to imipenem. In the group of antibiotics inhibiting protein synthesis (P), 40% were resistant to clindamycin, and 45% to lincomycin. Only one isolate, *F. antarcticum*, was resistant to tetracycline. In the group of antibiotics inhibiting nucleic acid synthesis (NA), all strains derived from pond mats exhibited resistance to at least one antibiotic. Most (70%) of strains were resistant to trimethoprim, 55% to metronidazole, and 50% to mupirocin and ciprofloxacin. Three MDR strains derived from pond mats, i.e. *F. degerlachei*, *F. hibernum* and *F. saccharophilum*, were resistant to 16 of the 25 antibiotics applied. Two strains, i.e. *F. glaciei* and *F. hydatis* exhibited a very low resistance to only one antibiotic. Among the strains derived from stream mats, 87% were resistant to at least one antibiotic from the CW group, 77% to antibiotics from the P group, and 93% to antibiotics from the NA group. Most (87%) represented strains resistant to 6 or more antibiotics. Only 13% of strains derived from stream mats were resistant to 1–5 antibiotics.

In the group of antibiotics inhibiting cell wall synthesis (CW), the largest pool consisted of bacteria resistant to 3rd generation cephalosporins, i.e. cefixime (83%), cefotaxime (73%) as well as ampicillin (70%) and vancomycin (63%). No resistance to imipenem was found among the tested strains. Nearly 80% of isolates were resistant to at least one antibiotic inhibiting protein synthesis (P), 57% were resistant to lincomycin, and 50% to chloramphenicol, erythromycin and clindamycin. The study found the lack of resistance to tetracycline and resistance of only two strains, i.e. *F. hydatis* and *F. saccharophilum*, to gentamycin. In the group of antibiotics inhibiting nucleic acid synthesis (NA), 93% of strains exhibited resistance to at least one antibiotic. Most strains (87%) were resistant to metronidazole, 77% to trimethoprim, 73% to mupirocin, and 67% to nitrofurantoin. Among the MDR strains was the broad resistance of *F. piscis* and *F. flabelliforme* to 19 and 18 antibiotics, respectively, and of three strains: *F. aquidurense*, *F. hydatis* and *F. bizetiae*, to 17 out of the 25 antibiotics applied. Two *F. kayseriense* isolates were resistant only to two antibiotics, i.e. ciprofloxacin and trimethoprim (belonging to the NA group). One strain, *F. pectinovorum*, exhibited no resistance to the antibiotics applied.

Analysis of the antibiotic resistance index (ARI) showed a significantly higher proportion of multi-drug resistant strains (ARI ≥ 0.2) in stream mats (87%) compared to the strains isolated from pond mats (55%) (Figs. 4 and 5). At the same time, the average ARI value for flavobacteria isolated from pond mats (0.27) was significantly higher, compared to the value for the strains isolated from stream mats (0.44). Using a unpaired t-test statistically significantly higher resistance to antibiotics was demonstrated in strains derived from stream mats ($n = 50$, $p < 0.01$) (Fig. 5). In these strains, significantly higher resistance to antibiotics from the group inhibiting cell wall synthesis (CW) ($p < 0.001$), and from the group inhibiting nucleic acid synthesis (NA) ($p < 0.05$), was demonstrated. At the same time, no significant differences were noted in the resistance of the strains under study to antibiotics belonging to the group inhibiting protein synthesis (P).

Antagonistic relations

Antagonistic relations demonstrated among flavobacterial strains derived from polar-region microbial mats showed noticeable differences between strains isolated from stream mats and from ponds (Fig. 4). It was demonstrated that 50% of the flavobacteria derived from pond mats and 63% derived from stream mats produced compounds inhibiting the development of other isolates.

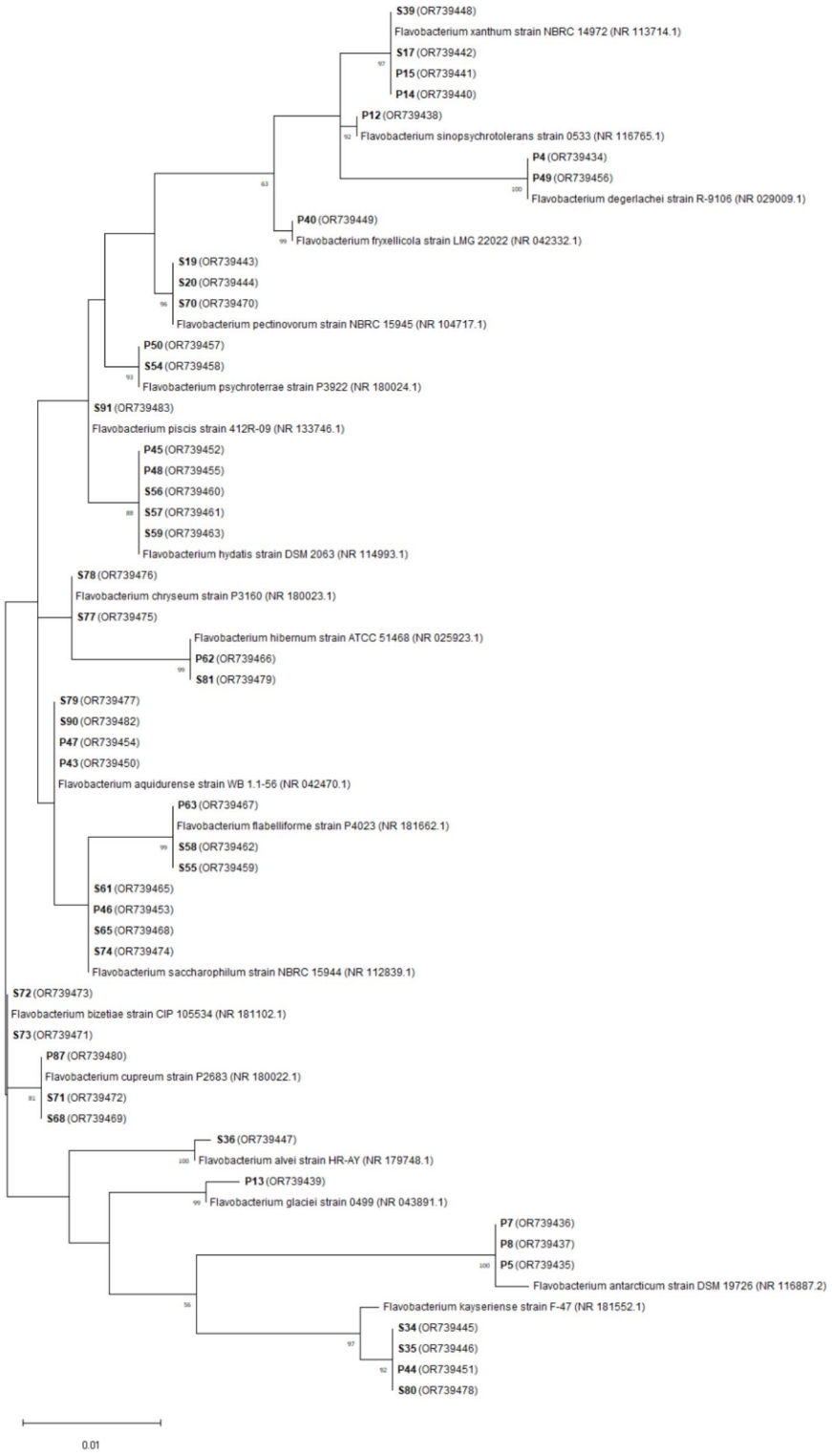

Fig. 3. Phylogenetic tree based on 16 S rRNA gene sequences comparison of the isolated flavobacteria strains among their closest related species within the genus *Flavobacterium*. The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei distance with the gamma model. Bootstrap probability values (percentages of 1000 tree replications) greater than 50% are indicated at branch points. Bar, 0.01 substitutions per nucleotide position. Flavobacterial isolates are marked by a strain code number (S – strim, P – pond) and GenBank accession number (in brackets).

Isolate	Species	Origin	AM	PY	CFM	CTX	CAZ	IMP	VA	CN	K	S	C	TE	CLR	E	DA	L	NV	NA	CIP	SXT	MUP	F	RA	TMP	MET	NAR	ARI	I %	R %	ITP
P5	*F. antarcticum*	Pond																										9	0.36	0	100	R
P7	*F. antarcticum*	Pond																										3	0.12	0	40	SR
P8	*F. antarcticum*	Pond																										4	0.16	0	100	R
P4	*F. degerlachei*	Pond																										16	0.64	0	100	R
P49	*F. degerlachei*	Pond																										4	0.16	0	90	SR
P40	*F. fryxellicola*	Pond																										4	0.16	5	70	PRS
P13	*F. glaciei*	Pond																										1	0.04	20	60	PRS
P12	*F. sinopsychrotolerans*	Pond																										2	0.08	0	90	SR
P43	*F. aquidurense*	Pond																										6	0.24	0	100	R
P47	*F. aquidurense*	Pond																										7	0.28	10	80	PRS
P87	*F. cupreum*	Pond																										10	0.4	0	100	R
P63	*F. flabelliforme*	Pond																										9	0.36	15	100	PR
P62	*F. hibernum*	Pond																										16	0.64	0	100	R
P45	*F. hydatis*	Pond																										9	0.36	10	100	PR
P48	*F. hydatis*	Pond																										1	0.04	5	70	PRS
P44	*F. kayseriense*	Pond																										3	0.12	5	100	PR
P50	*F. psychroterrae*	Pond																										7	0.28	0	100	R
P46	*F. saccharophilum*	Pond																										16	0.64	35	100	PR
P14	*F. xanthum*	Pond																										6	0.24	5	80	PRS
P15	*F. xanthum*	Pond																										3	0.12	5	100	PR
S79	*F.aquidurense*	Stream																										10	0.4	3	100	PR
S90	*F. aquidurense*	Stream																										17	0.68	0	100	R
S68	*F. cupreum*	Stream																										6	0.24	3	36	PRS
S71	*F. cupreum*	Stream																										7	0.28	0	79	SR
S55	*F. flabelliforme*	Stream																										18	0.72	16	100	PR
S58	*F. flabelliforme*	Stream																										17	0.68	30	100	PR
S81	*F. hibernum*	Stream																										7	0.28	10	100	PR
S56	*F. hydatis*	Stream																										14	0.56	0	89	SR
S57	*F. hydatis*	Stream																										17	0.68	16	100	PR
S59	*F. hydatis*	Stream																										12	0.48	23	100	PR
S34	*F. kayseriense*	Stream																										2	0.08	0	74	SR
S35	*F. kayseriense*	Stream																										2	0.08	0	79	SR
S80	*F. kayseriense*	Stream																										17	0.68	13	100	PR
S54	*F. psychroterrae*	Stream																										8	0.32	7	100	PR
S61	*F. saccharophilum*	Stream																										10	0.4	0	100	R
S65	*F. saccharophilum*	Stream																										16	0.64	10	53	PRS
S74	*F. saccharophilum*	Stream																										15	0.6	13	79	PRS
S17	*F. xanthum*	Stream																										10	0.4	0	95	SR
S39	*F. xanthum*	Stream																										7	0.28	0	68	SR
S36	*F. alvei*	Stream																										7	0.28	3	89	PRS
S71	*F. bizetiae*	Stream																										8	0.32	0	79	SR
S72	*F. bizetiae*	Stream																										17	0.68	16	100	PR
S77	*F. chryseum*	Stream																										10	0.4	7	100	PR
S78	*F. chryseum*	Stream																										15	0.6	3	100	PR
S19	*F. pectinovorum*	Stream																										4	0.16	3	100	PR
S20	*F. pectinovorum*	Stream																										9	0.36	7	95	PRS
S70	*F. pectinovorum*	Stream																										0	0,00	3	37	PRS
S91	*F. piscis*	Stream																										19	0.76	0	100	R
S60	*Flavobacterium sp.*	Stream																										15	0.6	10	100	PR
S89	*Flavobacterium sp.*	Stream																										16	0.64	0	100	R
	% Resistance	Total	58	44	68	52	44	4	52	12	12	10	34	2	28	38	46	52	38	4	40	30	64	50	4	74	74					
		Pond	40	40	45	20	25	10	35	15	15	10	10	5	15	20	40	45	15	5	50	15	50	25	5	70	55					
		Stream	70	47	83	73	57	0	63	7	10	10	50	0	37	50	50	57	50	3	33	40	73	67	3	77	87					

Fig. 4. Map of the antimicrobial resistance profiles (orange – resistant; grey – sensitive) and antagonistic activity data of flavobacteria isolates from the microbial mat of ponds and streams in the periglacial zone of Ecology Glacier. NAR – number of antibiotic resistance; ARI – antibiotic resistance index; I % – percentage of inhibited strains; R % – percentage of resistance to antagonistic interactions; ITP – interactive type profile: P – production of antibacterial compounds; R – resistance to antibacterial compounds; S – sensitivity to antibacterial compounds. Characteristics of antibiotics are listed in Table 3.

Four groups of strains were distinguished among the possible types of interaction, with all types of interaction occurring among isolates from both pond mats and stream mats. The following strains were distinguished: PR – where the isolate produces antibacterial compounds but is also resistant to antibacterial compounds produced by other strains; PRS – where the isolate is an antagonist, i.e. produces antibacterial compounds, while being resistant and sensitive to one or more strains; SR – where the isolate produces no antibacterial compounds while being sensitive and resistant to antibacterial compounds produced by other strains; R – the isolate produces no antibacterial compounds but is resistant to the action of these compounds produced by other strains.

Among the isolates derived from the environments under study differences in antagonistic activity were demonstrated. In the pond mats, the PR strains accounted for 25%, with the highest antagonistic activity exhibited by *F. saccharophilum*, which at the same time was resistant to the majority of the antibiotics applied. The remaining strains from this group exhibited low and moderate antibiotic resistance. In the stream mats, the PR strains were the dominant group (43%), with the most antagonistically active strains including *F. flabelliforme*, *F. hydatis* and *F. bizetiae*. These strains also exhibited a very high antibiotic resistance. Moderate and high antibiotic resistance characterised the remaining strains from this group. The PRS strains in pond and stream mats accounted for similar percentages, 25% and 20%, respectively. The PRS strains derived from pond mats were characterised by moderate antagonistic activity and antibiotic resistance. In contrast, *F. glaciei* and *F. hydatis* inhibited the growth of only a few strains while being resistant to just one antibiotic. The PRS

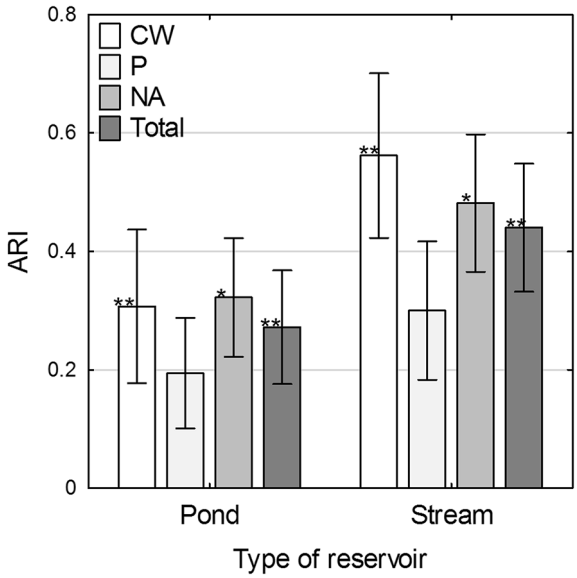

Fig. 5. Antibiotic resistance index (ARI) of flavobacteria to a selected group of antibiotics: inhibitors of cell wall synthesis (CW), proteins (P) and nucleic acids (NA). ARI index with $p < 0.05$ is indicated (*), and $p < 0.01$ is indicated with (**).

strains were characterised by higher antagonistic activity and a broader antibiotic resistance in stream mats. *F. saccharophilum* and *F. pectinovorum* exhibited moderate antagonistic properties while being resistant to many antibiotics.

The SR strains in ponds represented the least numerous group (15%) and were distinguished by a high resistance to antagonistic responses and a low antibiotic resistance (ARI < 0.2). Only *F. antarcticum*, derived from this environment, was distinguished by higher sensitivity to antagonistic interactions than the remaining strains and it displayed resistance to one antibiotic from each of the antibiotic groups applied. Only two SR strains derived from stream mats showed a low resistance to antagonistic interactions and a low antibiotic resistance (ARI = 0.08). The R strains in pond mats represented a dominant group (35%). *F. degerlachei* and *F. hibernum* were characterised by a lack of sensitivity to antibacterial compounds produced by other strains and the highest antibiotic resistance. All R strains from stream mats exhibited similar properties, yet it was the least numerous group isolated from this environment (13%).

Antagonistic activity and antibiotic resistance interactions

The results show a statistically significant correlation between the strains' ability to produce antibacterial compounds and their resistance to antibiotics ($p < 0.05$). In general, strains with high antagonistic activity were characterised by lower sensitivity to antagonistic interactions and higher antibiotic resistance (Fig. 6). Strains from both: pond mats and stream mats, exhibited a statistically significant correlation between the antagonistic potential of the strains, i.e. their production of antibacterial compounds, and their resistance to antibiotics: $r = 0.7$ ($p < 0.05$) and $r = 0.64$ ($p < 0.01$), respectively (Fig. 7a).

These traits were exhibited by *F. saccharophilum* derived from pond mats, and *F. piscis* isolated from stream mats, which were characterised by the highest antagonism and antibiotic resistance values. Furthermore, among strains derived from both environments (ponds, streams), a positive correlation was noted between resistance to antagonistic interactions and their antibiotic resistance: $r = 0.52$ ($p < 0.05$) and $r = 0.49$ ($p < 0.01$), respectively. These properties were exhibited by *F. hibernum* and *F. degerlachei* derived from pond mats, and *F. piscis* derived from stream mats (Fig. 7b).

Discussion

The phylogenetic structure of the collection of flavobacterial strains isolated from the microbial mats of the periglacial zone of the Ecology Glacier on King George Island showed high species heterogeneity, which corresponded, for the most part, to strains found in cold environments, including polar regions. The five species isolated from pond mats: *F. antarcticum*. *F. glaciei*, *F. fryxellicola*, *F. degerlachei* and *F. sinopsychrotolerans* were indigenous to Antarctic lakes[6–10]. The species derived from stream mats, i.e. *F. chryseum*, *F. pectinovorum*, *F. alvei*, *F. bizetiae* and *F. piscis*, are described as occurring in various inland polar ponds, and their origin is also linked to cold-adapted fish pathogens[11,42–45]. Of the nine species found in both pond mats and stream mats, the following eight have so far been described in polar environments: *F. flabelliforme*, *F. hibernum*, *F. hydatis*, *F. kayseriense*, *F. psychroterrae*, *F. saccharophilum*, *F. xanthum* and *F. cupreum*[1,46–51]. One species, *F. aquidurense*, was also isolated from water of temperate zone streams[52,53]. This indicates the cosmopolitan nature of flavobacteria and their role as an integral component of the microbiocenoses of natural environments, including polar environments.

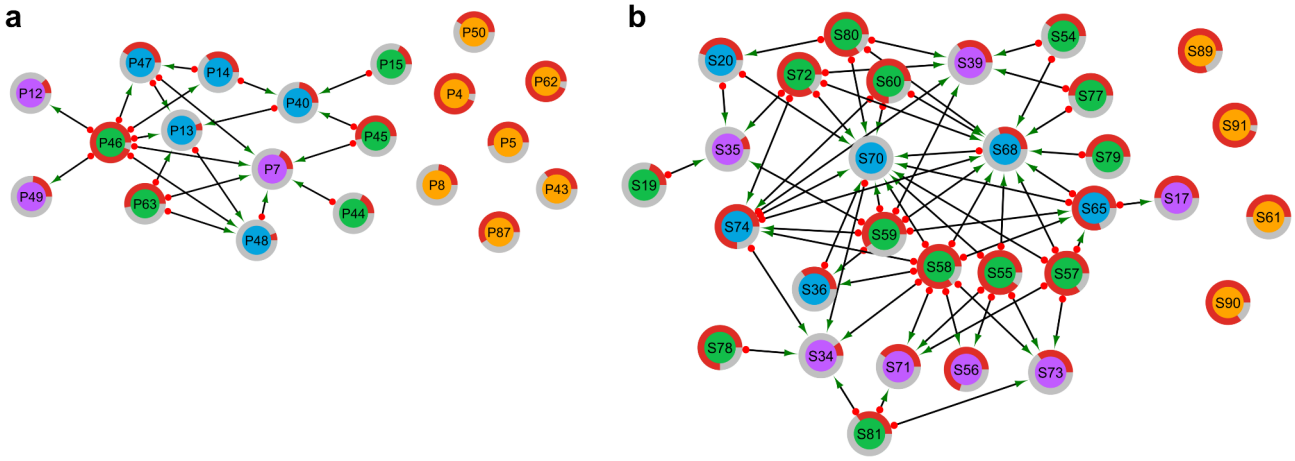

Fig. 6. Network analyses of antagonistic interactions and antibiotic resistance among flavobacteria isolated from microbial mats, (**a**) 20 × 20 array of tests among ponds isolates, (**b**) 30 × 30 array among streams isolates. Each node represents bacterial strains. Strains with the same type of antagonistic activity (ITP – interactive type profile) have the same fill colour: PR – green, PRS – blue, SR – purple, and R – orange. Each line represents an antagonistic interaction from an active strain (red dot) towards a sensitive strain (arrow). The number of red dots indicate range of antagonistic activity of strain, and number of arrows indicate range of sensitivity of strain. The size of the red ring indicates the antibiotic resistance range. The species names of the strains are listed in Fig. 4.

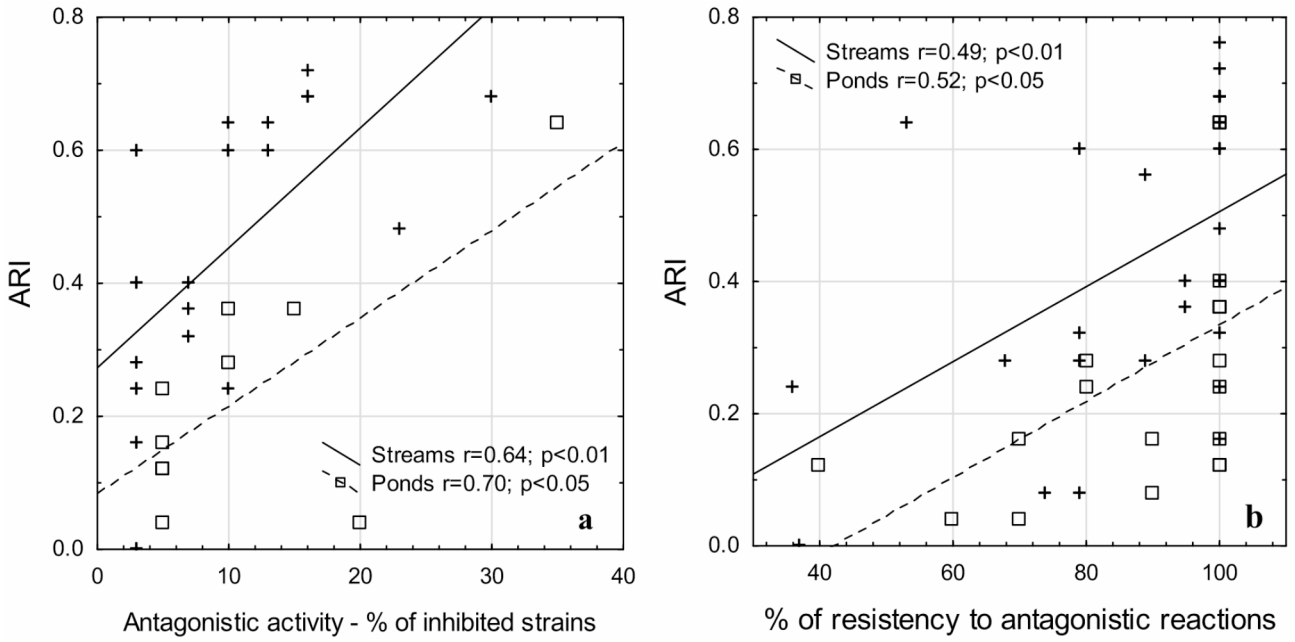

Fig. 7. Correlation between antibiotic resistance index (ARI) and antagonistic activity (**a**) and resistance to antagonistic reactions (**b**) of flavobacteria strains.

The results highlight the importance of antagonistic interactions in forming the structure and functioning of the bacteriocenoses of polar microbial mats. In the available literature, few papers exist on interactions occurring in heterogeneous microbial consortia dependant on the same environmental resources. These relationships are often studied in the context of interactions with test microorganisms, e.g., human and animal pathogens or food contaminants[54]. Chemoecological studies conducted to date have shown the existence of various types of interactions within heterogeneous microbial consortia: amensalism, commensalism and mutualism, which shape the structure of microbial communities[20]. Meanwhile, this phenomenon has been studied, to a limited extent, in primeval ecosystems such as polar-region microbial mats. Antagonism is an important survival strategy for aggregated bacteria, enabling them to gain an advantage over competing microorganisms[20,55–57]. In the current study, over 50% of the strains exhibited the ability to produce substances that inhibit the growth other

Flavobacterium spp. strains, and approximately 40% were sensitive to substances produced by microorganisms inhabiting the same niche. A similar, high antagonistic activity was demonstrated for the heterotrophic bacterial strains originating from saline lake microbial mats (60%), oceanic seston (53.3%), aggregated marine organic matter (54.1%), and the microbiome of Antarctic sponges (62.1%)[20,57–59]. A considerably lower antagonistic activity was demonstrated in the bacteriocenoses of pelagic aquatic environments. Lo Giudice et al. (2007)[56] identified only 15% of antagonistically active bacteria among strains isolated from the Ross Sea waters. A study by Long et al. (2013)[20] demonstrated that the level of bacterial cell aggregation determines the production of antibacterial substances. Over 50% of bacteria aggregated on the seston surface revealed antagonistic activity, while only 5% of bacterioplankton had such an ability. There is no doubt that antagonistic interactions among microbial mat bacteria are complex. This was confirmed by the occurrence of different patterns of antagonism and sensitivity interactions in the flavobacteria isolates under study: PRS, PR, SR and R. These intraspecies and interspecies properties may serve the structural and functional stability of the bacteriocenosis. This suggest that different mechanisms can determine antagonism, which is usually an individual feature of the strain. It is noteworthy that all the flavobacterial strains isolated from microbial mats were resistant to at least one antibacterial agent, and more than half were producers of antibacterial substances. The individualisation of antagonistic properties of individual strains is a rather poorly understood phenomenon, which, however, appears to be of key importance in the formation of the structure of microbial consortia[20,40,57]. The clear difference in the phylogenetic structure and antagonistic activity of the flavobacteria of the microbial mats of ponds and watercourses, demonstrated in the current study, could be determined by various factors. It has been shown in numerous studies that a crucial role in shaping the structure and activity of benthic consortia is played by the dynamics of water flow[60–62]. Ylla et al.[63] emphasise the importance of organic matter availability and assimilability for developing bacterial groupings and their metabolic and physiological activity. The poorer trophic conditions found in streams are usually associated with a quantitative predominance of hardly decomposable plant polymers, mainly celluloses and hemicelluloses. In stagnant water bodies, the organic matter usually accumulates and "ages" gradually. Its structure simplifies, and readily available nutrient substrates are released[63]. Given the above, it is reasonable to assume that the stream environment forces greater competition between bacteria in microbial mats. The ability to produce antimicrobial substances, demonstrated for a significant portion of the isolated flavobacterial strains, may be an example of one of the mechanisms regulating the bacteriocenosis of microbial mats. This is expressed by the high species diversity of flavobacteria found in the mats under study, i.e. 20 species identified among 50 isolates. The current study of flavobacteria suggests that impaired microbial development under suboptimal conditions of an extreme polar environment induces high susceptibility of the bacteriocenosis to even extremely low antagonistic interactions. A study by Grossart et al. (2004)[59] points to the existing correlation between antagonistic activity and the ability of bacteriocenosis to degrade organic matter. The production of antibiotics, bacteriocins and other antimicrobial substances, even in subthreshold quantities, can ensure an advantage over other microorganisms that co-form the bacteriocenosis and inhibit colonisation of the niche by allochthonous microorganisms[55,64].

The high antibiotic resistance of flavobacteria strains observed in the current study, in which over 70% of isolates were MDR strains, was surprising but not unexpected. Broad antibiotic resistance is increasingly being identified in isolated strains and the metagenome of the microbiocenoses of various polar environments, indicating that this feature is common. The presence of multi-antibiotic-resistant bacteria was found, e.g. in different polar terrestrial environments[65–67], marine bottom sediments[68] and glacial ice[69]. Numerous publications on polar areas point to the importance of the active soil layer as a reservoir of antibiotic resistance gene-harbouring bacteria[1,3,32]. Marcoleta et al. (2022)[67], when investigating the resistome within the Antarctic Peninsula soils, demonstrated a very high frequency and diversity of antibiotic resistance genes. Microbial mats are a specific formation in which the interrelationships between the biotic components provide the basis for structural and functional homeostasis[22,26]. Antibiotic resistance in such aggregated microbiocenoses is a common phenomenon confirmed also by the current study of flavobacterial strains. Natural antibiotic resistance in indigenous environmental bacteria is a widespread phenomenon and, like other metabolic traits, has developed over millions of years of evolution. Numerous researchers have noted the importance of microevolutionary processes in the development of natural antibiotic resistance in native environmental bacteria[70,71]. Van Goethem et al. (2018)[32] demonstrated that in natural environments, most antibiotic resistance genes originate mainly from ancient antibiotic-producing species. This supports the concept that phylogeny, i.e. gene transfer transmitted vertically over generations, rather than HGT, drives differences in resistome content in the environment.

The occurrence of antibiotic resistance in bacteria in areas remote from human activities may also be due to horizontal gene transfer from allochthonous bacteria introduced into the environment. Antibiotic resistance genes, identified in polar-region bacteria, are perceived as a biotic contaminant and a significant ecological problem[1,72]. Metagenomic research suggests that the Antarctic microbiota is the source of ancient antibiotic resistance genes, but, at the same time, multi-antibiotic-resistant strains are found in areas of high anthropogenic impact. King George Island is certainly one such region, which explains the resistance also to antibiotics routinely used in modern therapy found in the current study. Evidence indicates that antibiotic resistance genes originate from multiple bacterial sources, showing that the genomes of all bacteria can be considered as one global set of genes, from which most, if not all, bacteria can draw in search of genes necessary for survival[73]. On the other hand, microbial mats can be important reservoirs of resistance to antimicrobial agents[74]. There are very few reports on the resistome of Antarctic flavobacterial strains. As demonstrated by a study by Králová et al. (2021)[1], two Antarctic species of *F. flabelliforme* and *F. geliluteum*, derived from the isolated environment of the James Ross Island, were phenotypically MDR and, at the same time, contained multiple resistance genes to a broader spectrum of antimicrobial drugs. The varying antibiotic resistance profiles among flavobacterial strains of the same species in this study indicate the acquired nature of these traits, influenced by external factors. The influence of external factors related to environmental stress, bacterial epigenetics and horizontal gene transfer is

considered an important mechanism promoting the spread of antibiotic resistance also in polar regions[28,74]. The different antibiotic resistance in flavobacterial strains originating from two different environments, i.e. flowing and stagnant water, may be determined by the different dynamics of these waters. The current observations suggest that Antarctic bacteria from different ecosystems are genetically distinct due to the need to adapt to ecologically distinct habitats, which is also supported by studies conducted by other authors[75]. The narrower antibiotic resistance among flavobacterial strains derived from polar pond mats may be related to the greater stability of this environment and the availability of biogenic compounds and, consequently, to the lower competitiveness within the microbiocenosis. The community assembly may shape the diversity–functioning relationships in freshwater bacterial communities, which is an expression of the properties of the environment and the type of water[63].

Summary

This is the first study to demonstrate a relationship between antagonistic interactions with antibiotic resistance within flavobacteria, a component of polar-region microbial mats. The study revealed the complex nature of these interactions occurring at intra- and interspecies levels. It also demonstrated the distinctiveness of the relationships found among flavobacteria of the microbial mats of two types of water bodies, i.e. ponds and streams. The surprisingly high biodiversity of flavobacterial strains in the mat microbiocenosis is an example of the existing strong intra- and interspecies relationships that are key to maintaining structural and functional homeostasis. The results of the study demonstrate that there are individual patterns of antagonistic interactions and antibiotic resistance among the biotic components of mats. The nature of these patterns plays a crucial role in forming the structure and functions of microbial mats. In the mat microbiocenosis, strains of "superbacteria" are characterised by outstanding antagonistic potential and broad antibiotic resistance. As demonstrated by the study, environmental factors, mainly hydrological and trophic parameters of a pond, also play an important role in promoting specific patterns of intercellular interactions. The study also positively verified the hypothesis that antagonism at the species level in aggregated microbial mat systems is a common phenomenon and plays a significant role in shaping bacterial communities. Moreover, it was confirmed that antagonistic interactions are linked to the antibiotic resistance of strains, and the nature of the aquatic environment determines the intensity of these interactions. Although these findings on flavobacteria may not fully reflect all interactions within microbial mats, the obtained results can be used to develop a dynamic model of the formation of microbial mat communities.

Data availability

Sequence data of studied flavobacteria strains have been deposited in the GenBank NCBI database, under accession numbers OR739434–OR739483. The datasets generated and analysed during the current study are available from the corresponding author.

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Declarations

Competing interests
The authors declare no competing interests.

Additional information
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