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ORIGINAL PAPER

Metallo-Beta-Lactamase Producing *Pseudomonas aeruginosa* in a Healthcare Setting in Alexandria, Egypt

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Abstract

Pseudomonas aeruginosa has emerged as a major healthcare associated pathogen that creates a serious public health disaster in both developing and developed countries. In this work we aimed at studying the occurrence of metallo-beta-lactamase (MBL) producing *P. aeruginosa* in a healthcare setting in Alexandria, Egypt. This cross sectional study included 1583 clinical samples that were collected from patients admitted to Alexandria University Students' Hospital. *P. aeruginosa* isolates were identified using standard microbiological methods and were tested for their antimicrobial susceptibility patterns using single disc diffusion method according to the Clinical and Laboratory Standards Institute recommendations. Thirty *P. aeruginosa* isolates were randomly selected and tested for their MBL production by both phenotypic and genotypic methods. Diagnostic Epsilometer test was done to detect metallo-beta-lactamase enzyme producers and polymerase chain reaction test was done to detect imipenemase (IMP), Verona integron-encoded (VIM) and Sao Paulo metallo-beta-lactamase (IMP) encoding genes. Of the 1583 clinical samples, 175(11.3%) *P. aeruginosa* isolates were identified. All the 30 (100%) selected *P. aeruginosa* isolates that were tested for MBL production by Epsilometer test were found to be positive; where 19 (63.3%) revealed bla_{SPM} gene and 11(36.7%) had bla_{IMP} gene. bla_{VIM} gene was not detected in any of the tested isolates. Isolates of MBL producing *P. aeruginosa* were highly susceptible to polymyxin B 26 (86.7%) and highly resistant to amikacin 26 (86.7%). MBL producers were detected phenotypically by Epsilometer test in both carbapenem susceptible and resistant *P. aeruginosa* isolates. bla_{SPM} was the most commonly detected MBL gene in *P. aeruginosa* isolates.

Key words: *Pseudomonas aeruginosa*, Epsilometer test, metallo-beta-lactamases, MBL encoding genes

Introduction

Pseudomonas aeruginosa is considered one of the most leading causes of healthcare associated infections (HCAIs) worldwide (Varaiya *et al*., 2008). It is considered the fourth most commonly isolated nosocomial pathogen accounting for 10% of all HCAIs. *P. aeruginosa* infections can range from superficial skin infections to fulminant sepsis. Even colonization of such strains in critical systems can be fatal (Sivaraj *et al*., 2012).

World Health Organization (2015) has identified antimicrobial resistance as one of the three most important problems for human health. *P. aeruginosa* represents a phenomenon of resistance since all known mechanisms of antimicrobial resistance can be encountered; nevertheless enzyme production is the major mechanism of acquired resistance in these strains especially with β-lactam antibiotics, which are considerd a major line of treatment for *P. areuginosa*. Of these enzymes are the β-lactamases (Strateva and Yordanov, 2009). There are four classes of β-lactamases: A, C and D which act through a serine based mechanism and metallo-beta-lactamases (MBL); a class B type of β-lactamases that is the most worrisome and require bivalent metal ions, usually zinc as a cofactor for their activity (Bush and Jacopy, 2010). This group can be suppressed by bivalent ionic chelators as ethylene diamine tetra acetic acid (EDTA), but not inhibited by commercial β-lactamase inhibitors as clavulanic acid and tazobactam. They can hydrolyze β-lactams from all classes except the monobactams (Aoki *et al*., 2010).

MBL producing *P. aeruginosa* isolates were first reported in Japan in 1991, and since then there has been a substantial increase in the reporting of MBLs among carbapenem-resistant *P. aeruginosa* isolates worldwide (Pitout *et al*., 2005). These isolates increasingly have been responsible for several nosocomial outbreaks in tertiary centers in different parts of the world (Walsh, 2008). Also the association between infections caused by MBL producing *P. aeruginosa* and longer hospital stay with high mortality rates has been reported (Zavascki *et al*., 2006).

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The problem is aggravated by the fact that most of the MBL encoding genes reside on integrons and plasmids which in turn allows for widespread dissemination of these genetic elements, hence poses a threat for spread resistance patterns among the Gram-negative bacteria (Mohamed and Raafat, 2011).

As regards molecular structure: five MBL types have been widely recognized; imipenemase (IMP), Verona integron-encoded (VIM), Sao Paulo (SPM), German imipenemase (GIM) and Seoul imipenemase (SIM). Several types of MBL enzymes have been identified in *P. aeruginosa* among which the $_{\mathrm{VIM}}$-type enzymes appear to be the most prevalent. IMP is also considered one of the most important types of MBLs. Camagaro *et al.*, (2011) reported that after being restricted for more than ten years to Brazilian hospitals; SPM seems to become a global challenge, warning for the role of human traffic in spreading MBL genes (Salabi *et al.*, 2010).

It is well known that poor outcome occurs when patients with serious infections due to MBL producing organisms are treated with antibiotics to which the organism is completely resistant. Therefore early and proper detection of MBL producing Gram-negative bacilli especially *P. aeruginosa* is crucial; for optimal treatment of particularly critically ill and hospitalized patients and to permit rapid initiation of strict infection control procedures to prevent nosocomial spread and control the dissemination of resistance (Cuzon *et al.*, 2012). This work aimed at studying the occurrence of MBL producing *P. aeruginosa* in a healthcare setting in Alexandria, Egypt.

Experimental

Material and Methods

This cross sectional study was carried out during an 18-month period from January 2013 to June 2014. It included different clinical samples that were collected from patients admitted to the Alexandria University Students' Hospital (AUSH). Collected clinical samples were processed in AUSH laboratory and the Microbiology laboratory at the High Institute of Public Health (HIPH). The study was approved by the Ethics Committee of the HIPH. Informed consents of all enrolled patients were obtained before collection of samples and after explanation of the purpose and benefits of the research.

Sampling

Data collection. A questionnaire sheet including all the relevant information (name, age, sex, date of admission, medical history, diagnosis, antibiotic administration *etc.*) was filled in for every patient enrolled in the present study.

Samples collection and processing. A total of 1583 different clinical samples were collected during the study period from patients showing signs and symptoms suggestive of infection and were delivered to the laboratory. The samples were distributed as 660 respiratory samples (500 bronchoalveolar lavage (BAL) and 160 sputum samples), 446 urine samples, 209 blood samples, 142 pus and exudate samples, 58 peritoneal fluid samples, 35 ear discharge, and 33 conjunctival secretions. Collected samples were subjected to macroscopical and microscopical examination. The samples were cultured on blood (Oxoid 9191118 UK) and MacConkey's agar (Oxoid 567362 UK) plates. Plates were incubated aerobically at 37°C for 24 hours (Tille *et al.*, 2014).

Identification procedures of *P. aeruginosa*. After proper incubation of inoculated blood and MacConkey's agar plates, isolates that appeared as medium sized, grayish, opaque, large flat pigmented colonies, with feathered edges, producing a sweet or grape like odour either hemolytic or non hemolytic on blood agar plates, and were pale, non lactose fermenting on MacConkey's agar plates, and microscopically appeared as Gram-negative bacilli were further differentiated and identified according to standard microbiological methods (Tille *et al.*, 2014).

Antimicrobial susceptibility testing (AST). All 175 confirmed *P. aeruginosa* isolates were tested for their antibiotic susceptibility patterns using single disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (Patel *et al.*, 2014). The test was done on Mueller Hinton (MH) agar plates (Difco 00252-01), using the selected antibiotic discs. All antimicrobial discs used in this study were supplied by oxoid laboratories. After 24 hours aerobic incubation at 37°C, each plate was examined and inhibition zones were measured, recorded, and interpreted as susceptible (S), intermediate (I) or resistant (R) according to the interpretive criteria of CLSI (Patel *et al.*, 2014).

Isolates that were confirmed to be *P. aeruginosa* and tested for their antimicrobial susceptibility patterns were then subcultured on blood agar plates and incubated aerobically at 37°C for 24 hrs. Isolated colonies were inoculated on soft agar deeps and incubated aerobically at 37°C for 24 hrs.

Identification of metallo beta lactamase production by Epsilometer test (E-test). Thirty confirmed *P. aeruginosa* isolates were selected to be tested for their MBL enzyme production by E test. MBL diagnostic E-test strip consists of a double sided seven dilution range of imipenem (IP) (4 to 256 microgram/ml) and IP overlaid with EDTA (1 to 64 microgram/ml) (Pitout *et al.*, 2005). MBL E-test was performed according to the manufacturer's instructions (AB BioMerieux, Solna, Sweden). Isolates stored in agar deeps were subcultured on blood agar plates and were incubated aerobically at 37°C for 24 hrs. Individual colonies were picked from overnight agar plates and suspended in a 0.85% saline and were adjusted to a turbidity of 0.5 McFarland standard.

E-test MBL strips were applied to MH agar plates inoculated with adjusted suspensions. Seeded MH agar plates were incubated aerobically for 24 hrs at 37°C.The minimum inhibitory concentration (MIC) end points were read where the inhibition ellipses intersected the strip. A reduction of imipenem MIC in the presence of EDTA that is greater than or equal to eight-fold (IP/IPI > 8 mm) was interpreted as indicating MBL activity. The presence of a phantom zone or a deformation of the imipenem ellipse was also considered a positive result (Pitout *et al*., 2005).

Detection of bla_{IMP}, bla_{VIM}, and bla_{SPM} MBL genes by PCR. Thirty *P. aeruginosa* isolates that were confirmed as MBL producers by MBL diagnostic E-test, were tested for the presence of bla_{IMP}, bla_{VIM}, bla_{SPM} genes.

DNA extraction

Procedure. DNA for PCR was extracted by the boiling method. Two or three colonies were taken from fresh culture of the confirmed MBL *P. aeruginosa* isolates and suspended in 500 µl saline, then vortexed to get a uniform suspension. The cells were lysed by heating them at 100°C for 10 minutes, and then centrifuged at 12,000 rpm for 10 min. The supernatant was used directly as a template DNA in the PCR mixture.

DNA amplification. The extracted DNA was subjected to PCR amplification reaction using three pairs of primers specific for MBL genes (bla_{IMP}, bla_{VIM}, and bla_{SPM}). The DNA amplification was done using Dream Taq Green PCR Master mix (Thermo Scientific, Waltham, United States). The primers were purchased lyophilized; (Biosearch Tech, Petaluma, California, United States). They were reconstituted by the addition of sterile nuclease free water to a final concentration of 100 pico mol/µl, distributed in aliquots and stored at –20°C.

Primers sequence of MBL genes (Sader *et al*., 2005)

Primer	Sequence (5' to 3')
IMP 1	Sense: 5'dCTACCGCAGCAGAGTCTTTGC3' Antisense: 5'dGAACAACCAGTTTTGCCTTACC3'
VIM 2	Sense: 5'dATGTTCAAACTTTTGAGTAGTAAG3' Antisense: 5'dCTACTCAACGACTGAGCG3'
SPM 1	Sense: 5dCCTACAATCTAACGGCGACC3' Antisense: 5'dTCGCCGTGTCCAGGTATAAC3'

b-PCR amplification protocol. (I) Reaction mixtures were prepared using sterile nuclease free water. To each tube a total volume of 50 ml was reached by adding Master mix (25 µl), sense primer (1 µl), antisense primer (1 µl), DNA template (sample) (10 µl), nuclease free water (13 µl). A negative control was prepared by the addition of the same contents to the tube with 10 µl nuclease free water instead of the sample.

(II) The tubes were transferred to the thermal cycler (BioCycler TC-S, Boeco-Germany) for amplification. The thermocycler program conditions for bla_{IMP} and bla_{VIM} genes included: 30 cycles of amplification under the following conditions: denaturation at 95°C for 30 seconds, annealing for 1 minute at specific temperatures (bla_{IMP} at 45°C and bla_{VIM}–66°C), and extension at 72°C for 1 minutes/kb product (Khosravi *et al*., 2011). The cycling parameters of PCR to amplify bla_{SPM} gene were: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 minute and extension at 68°C for 1 minute. The cycle was followed by a final extension at 72°C for 10 minutes (Gaspareto *et al*., 2007).

DNA detection by gel electrophoresis. PCR products were loaded on 2% agarose in tris borate EDTA (TBE) containing 0.5 µl of ethidium bromide per ml. After electrophoresis, the gel was visualized under ultraviolet light.

The DNA bands were visualized on a 320 nm UV transilluminator and photographed. The gel was examined for specific bands; positive results of PCR were confirmed by detection of 432 bp band for bla_{IMP} gene, 500 bp band for bla_{VIM} gene and 650 bp band for bla_{SPM} gene as determined by the molecular weight markers run at the same time. (Khosravi *et al*., 2011; Gaspareto *et al*., 2007).

Statistical analysis of the data. Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (Kirkpatrick and Feeney, 2013). Qualitative data were described using number and percent. Quantitative data were described using Range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test, Fisher's exact test, Monte Carlo correction, independent t-test and Mann Whitney test. Significance of the obtained results was judged at the 5% level.

Results

The present study included 175 *P. aeruginosa* isolates that were recovered from a total of 1583 clinical samples. These samples were collected randomly from patients admitted to the AUSH (984 from ICUs and 599 from wards).

The highest percentage of *P. aeruginosa* isolates 75 (42.9%) were recovered from respiratory samples, followed by urine samples 57 (32.6%) and pus and exudate samples 33 (18.9%). From blood samples, four *P. aeruginosa* isolates were recovered (2.3%), while each of conjunctival secretion, ear discharge, and peritoneal fluid samples yielded two *P. aeruginosa* isolates (1.1% each).

In the current work, the highest percentage of resistance among 175 detected *P. aeruginosa* isolates was for aztreonam 150 (85.7%), followed by ceftazidime 140 (80%), cefipime 139 (79.4%), imipenem 137 (78.3%), ciprofloxacin 134 (76.6%), and meropenem 129 (73.7%).

On the other hand, the highest susceptibility percentages were recorded for polymyxin B 152 (86.9%), piperacillin tazobactam 52 (29.7%), 48 ofloxacin (27.4%), piperacillin 46 (26.3 %), gentamycin 39 (22.3%) and cefepime 28 (16%).

Of the 175 confirmed *P. aeruginosa* isolates, 30 isolates were randomly selected to be tested phenotypically for their MBL enzyme production by E test. Thirty selected *P. aeruginosa* isolates were as follows:

- Twenty *P. aeruginosa* isolates were resistant to three classes of β-lactams including: cephems, carbapenems and monobactams (aztreonam).
- Three *P. aeruginosa* isolates were resistant to three classes of β-lactams (penicillins, cephalosporins and carbapenems) and were susceptible to aztreonam.
- Seven *P. aeruginosa* isolates were resistant to three classes of antibiotics (cephems, aminoglycosides, and carbapenems), but were susceptible to imipenem, and 4 of them were also susceptible to meropenem.

This study showed that the highest percentage of MBL producing *P. aeruginosa* isolates were from respiratory samples 11/30 (36.7%), followed by urine, and pus and exudate samples 9/30 (30%) each. Only one isolate was recovered from an ear sample 1/30 (3.3%) (Fig. 1).

Thirty selected isolates were all positive for MBL production by MBL E test, and were tested for bla_{IMP}, bla_{VIM} and bla_{SPM} genes using conventional PCR (results as in Fig. 2). Of the 30 tested isolates; 19 (63.3%)

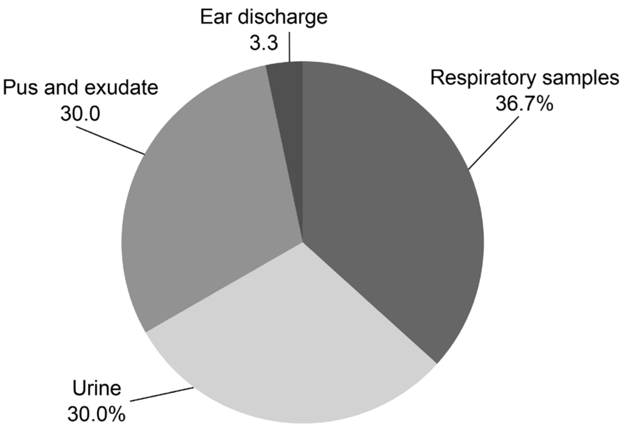

Fig. 1. Distribution of 30 MBL producing *P. aeruginosa* isolates according to type of samples.

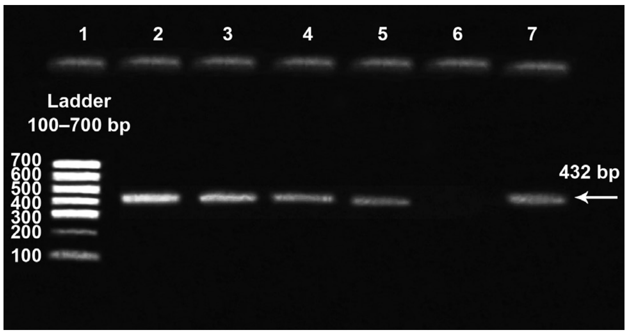

Fig. 2A bla_{IMP}

Lane 1: Marker 100 bp Ladder
Lane 2: Positive test strain for bla_{IMP} (432 bp)
Lane 3: Positive test strain for bla_{IMP} (432 bp)
Lane 4: Positive test strain for bla_{IMP} (432 bp)
Lane 5: Positive test strain for bla_{IMP} (432 bp)
Lane 6: Negative control
Lane 7: Positive test strain for bla_{IMP} (432 bp)

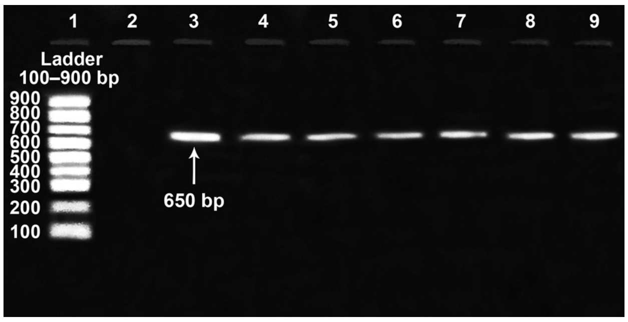

Fig. 2B bla_{SPM}

Lane 1: Marker 100 bp Ladder
Lane 2: Negative control
Lane 3: Positive test strain for bla_{SPM} (650 bp)
Lane 4: Positive test strain for bla_{SPM} (650 bp)
Lane 5: Positive test strain for bla_{SPM} (650 bp)
Lane 6: Positive test strain for bla_{SPM} (650 bp)
Lane 7: Positive test strain for bla_{SPM} (650 bp)
Lane 8: Positive test strain for bla_{SPM} (650 bp)
Lane 9: Positive test strain for bla_{SPM} (650 bp)

Fig. 2C bla_{VIM}

Lane 1: Marker 100 bp Ladder
Lane 2: Negative test strain for bla_{VIM} (500 bp)
Lane 3: Negative test strain for bla_{VIM} (500 bp)
Lane 4: Negative control
Lane 5: Negative test strain for bla_{VIM} (500 bp)
Lane 6: Negative test strain for bla_{VIM} (500 bp)
Lane 7: Negative test strain for bla_{VIM} (500 bp)
Lane 8: Negative test strain for bla_{VIM} (500 bp)

Fig. 2. A, B, C. Electrophoresis results for *P. aeruginosa* bla_{IMP}, bla_{SPM} and bla_{VIM} genes.

Table I
Distribution of the 30 patients with MBL producing *P. aeruginosa* isolates according to their gender and age.

Gender / Age in years	Male (n = 12)		Female (n = 18)		Total (n = 30)	
	No.	%	No.	%	No.	%
20– < 30	7	58.3	7	38.9	**14**	**46.7**
30–50	1	8.3	8	44.4	**9**	**30.0**
> 50	4	33.3	3	16.7	**7**	**23.3**
χ^2 (^{MC}p)	4.570 (0.104)					

^{2}: Chi square test; MC: Monte Carlo test

revealed *bla*$_{\text{SPM}}$ gene and 11(36.7%) had *bla*$_{\text{IMP}}$ gene, *bla*$_{\text{VIM}}$ gene was not detected in any of the tested isolates.

Of the 30 patients with MBL producing *P. aeruginosa* isolates, 14(46.7%) were of age group 20 ≤ 30 years, 9 (30.0%) belonged to the age group 30–50 years, and seven patients (23.3%) were above 50 years (Table I). Moreover, our data showed that the highest percentage of patients who revealed *P. aeruginosa* isolates with positive *bla*$_{\text{SPM}}$ gene were aged 20 ≤ 30 years old (47.4%), followed by those aged > 50 years old (31.6%).

In this study the majority of patients with MBL producing *P. aeruginosa* isolates 21 (70%) were admitted to the ICU and 9 (30%) were admitted in inpatient wards. In addition, the highest percentage (76.7%) was recovered from those who had duration of hospital stay of more than 7 days. Regarding antibiotic intake, the majority of patients 26/30 (86.7%) had taken antibiotics within 2 weeks from the study period, and this was found to be statistically significant (p ≤ 0.001). On the other hand, 23/30 (76.7%) patients were readmitted to the hospital within 3 months. This was statistically significant (p ≤ 0.006) (Table II).

Our work revealed that the 30 MBL producing *P. aeruginosa* isolates were highly susceptible to polymxin B 26 (86.7%), followed by piperacillin-tazobactam 11 (36.7%), then gentamycin 8 (26.7%). Seven (23.3%) isolates were susceptible to each of imipenem and ofloxacin. The highest percentage of isolates were resistant to Amikacin 26/30 (86.7%), followed by piperacillin and ciprofloxacin 24 /30(80% each) (Table III).

More than half of the patients who had *P. aeruginosa* isolates with *bla*$_{\text{IMP}}$ genes 6/11 (54.5%) were aged between 20 ≤ 30 years old, this was followed by those of 30–50 years old 3/11 (27.3%). The lowest percentage of *P. aeruginosa* isolates with *bla*$_{\text{IMP}}$ gene 2 (18.2%) was recovered from patients above 50 years. On the other hand, the highest percentage of patients who revealed positive *bla*$_{\text{SPM}}$ gene were aged 20 ≤ 30 years old 9/19 (47.4%), followed by those aged > 50 years old 6/19 (31.6%).

Table II
Distribution of 30 patients with positive MBL producing *P. aeruginosa* isolates according to risk factors.

Frequency of isolation / Risk factors	Patients with MBL producing *P. aeruginosa* isolates (n = 30)		Test of sig.	P
	No.	%		
Site of admission				
ICU (n = 126)	21	70.0	$\chi^2 = 0.072$	0.789
Ward (n = 49)	9	30.0		
Length of hospital stay (days)				
≤ 7 (n = 54)	7	23.3	$\chi^2 = 0.961$	0.327
> 7 (n = 121)	23	76.7		
Min. – Max.	3.0–39.0		Z = 0.519	0.604
Mean ±SD.	14.97 ± 10.12			
Median	12.50			
Associated diseases				
DM	22	73.3	$\chi^2 = 18.114^*$	< 0.001*
Cancer	18	60.0	$\chi^2 = 9.052^*$	0.003*
Related devices				
Mechanical ventilator	7	23.3	$\chi^2 = 0.784$	0.376
Urinary catheter	7	23.3	$\chi^2 = 8.823^*$	$^{FE}p = 0.024^*$
Readmission (n = 94)	23	76.7	$\chi^2 = 7.672^*$	0.006*
Antibiotic intake (n = 97)	26	86.7	$\chi^2 = 14.301^*$	< 0.001*

χ^2: Chi square test; Z: Z for Mann Whitney test; *: Statistically significant at p ≤ 0.05

Table III
Antimicrobial susceptibility patterns of 30 MBL producing *P. aeruginosa* isolates.

Susceptibility pattern / Antimicrobial agent	Susceptible S		Intermediate I		Resistant R	
	No	%	No	%	No	%
Penicillins						
– Piperacillin	4	13.30	2	6.7	24	80
β-lactam/β-lactamase inhibitor combinations						
– Piperacillin – tazobactam	11	36.7	8	26.7	11	70
Cephems						
– Ceftazidime	2	6.7	10	33.3	18	60
– Cefepime	5	16.7	4	13.3	21	70
Carbapenems						
– Imipenem	7	23.3	1	2.7	22	73.3
– Meropenem	4	13.3	6	20	20	66.7
Monobactams						
– Aztreonam	3	10	5	16.7	22	73.3
Aminoglycosides						
– Gentamycin	8	26.7	2	6.7	20	66.7
– Amikacin	1	3.3	3	10	26	86.7
Fluoroquinolones						
– Ciprofloxacin	5	16.7	1	33.3	24	80
– Ofloxacin	7	23.3	2	6.7	21	70
Lipopeptides						
– Polymyxin B	26	86.7	0	0.0	4	13.3

Table IV
Relation between results of blaIMP gene among 30 patients with MBL producing *P. aeruginosa* isolates and their risk factors.

Risk factors	*bla*$_{IMP}$ gene				Test of Sig.	P
	Negative (n = 19)		Positive (n = 11)			
	No.	%	No.	%		
Site of admission						
ICU	13	68.4	8	72.7	$\chi^2 = 0.062$	$^{FE}p = 1.000$
Ward	6	31.6	3	27.3		
Length of hospital stay (days)						
≤ 7	5	26.3	2	18.2	$\chi^2 = 0.258$	$^{FE}p = 0.686$
> 7	14	73.7	9	81.8		
Min. – Max.	4.0 – 37.0		3.0 – 39.0		Z = 0.129	0.897
Mean ± SD.	14.68 ± 9.84		15.45 ± 11.05			
Median	12.0		14.0			
Associated diseases						
DM	15	78.9	7	63.6	0.835	0.417
Cancer	11	57.9	7	63.6	0.096	1.000
Related devices						
Mechanical ventilator	4	21.1	3	27.3	0.151	1.000
Urinary catheter	4	21.1	3	27.3	0.151	1.000
Hospital readmission	7	36.7	9	81.8	5.662*	0.017*
Antibiotic intake	10	52.6	8	72.7	1.172	0.442

χ^2: value for Chi square; MC: Monte Carlo test
FE: Fisher Exact test Z: Z for Mann Whitney test *: Statistically significant at $p \leq 0.05$

Table V
Relation between results of blaSPM gene among 30 patients with MBL producing *P. aeruginosa* isolates and their risk factors.

Risk factors	bla_{SPM} gene				Test of Sig.	P
	Negative (n = 11)		Positive (n = 19)			
	No.	%	No.	%		
Site of admission						
ICU	7	63.6	14	73.7	$\chi^2 = 0.335$	$^{FE}p = 0.687$
Ward	4	36.4	5	26.3		
Length of hospital Stay(days)						
≤7	2	18.2	5	26.3	$\chi^2 = 0.258$	$^{FE}p = 0.686$
>7	9	81.8	14	73.7		
Min. – Max.	3.0 – 37.0		4.0 – 39.0		Z = 0.539	0.590
Mean ± SD.	14.18 ± 11.03		15.42 ± 9.84			
Median	11.0		14.0			
Associated diseases						
DM	1	9.0	5	26.3	3.135	$^{FE}p = 0.104$
Cancer	0	0.0	7	36.8	1.408	0.235
Related devices						
Mechanical ventilator	0	0.0	7	36.8	5.286*	$^{FE}p = 0.029$*
Urinary catheters	2	18.2	5	26.3	0.740	$^{FE}p = 0.658$
Hospital readmission	4	36.4	15	78.9	5.440*	$^{FE}p = 0.047$*
Antibiotic intake	3	27.3	13	68.4	4.739*	0.029*

χ^2: value for Chi square; MC: Monte Carlo test
FE: Fisher Exact test Z: Z for Mann Whitney test *: Statistically significant at $p \leq 0.05$

P. aeruginosa with positive bla_{IMP} gene were isolated from 7 patients with cancer and 7 with diabetes mellitus (DM) (63.6% each). In addition, 3 patients were on mechanical ventilators (27.3%) and 3 had urinary catheters (Table IV). On the other hand, *P. aeruginosa* isolates with bla_{SPM} gene were recovered from 5 (26.3.2%) patients, who had DM and 7 (36.8%) who suffered from cancer (Table V).

Discussion

P. aeruginosa has emerged as a major HCA pathogen in both developing and developed countries. This organism creates a serious public health disaster resulting in an enormous burden of morbidity, mortality and high health care cost, especially among high risk patients in ICUs (Morales *et al.*, 2012).

In the present study, 175 (11.1%) *P. aeruginosa* isolates were recovered from a total of 1583 clinical samples that were collected from patients admitted to the AUSH (984 were admitted to ICUs and 599 wards). A slightly higher percentage was reported by Divyashanthi *et al.* (2015), where 15.2% *P. aeruginosa* isolates were recovered from 788 tested clinical samples. In Nepal, Khan *et al.* (2014), reported that 194 (21.1%) *P. aeruginosa* isolates were identified from 917 collected clinical samples from patients with suspected *P. aeruginosa* infections. A much higher isolation rate (41.5%) was recorded by Sedighi *et al.* (2012), in Iran, where 106 *P. aeruginosa* isolates were detected from 255 gathered clinical samples. On the other hand, lower percentages of isolation were reported in a 3-year study that was conducted by Mohanasoundaram (2011) to determine the distribution rate and antimicrobial resistance pattern in *P. aeruginosa* among clinical samples (5%, 6.8% and 5% in 2008, 2009 and 2010, respectively).

One of the main concerns about *P. aeruginosa* is its remarkable ability to rapidly develop antibiotic resistance. The wide array of antimicrobial resistance mechanisms that have been described for *P. aeruginosa* is impressive and rivals those of other non-fermentative Gram-negative pathogens and illustrates the potential of this organism to respond swiftly to changes in selective environmental pressure. In recent years, Egypt has been considered among the countries that reported high rates of antimicrobial resistance (Zafer *et al.*, 2014)

In the current study, the susceptibility patterns of *P. aeruginosa* isolates were tested by a panel of antimicrobial agents according to CLSI recommendations. It was found that *P. aeruginosa* isolates showed high levels of resistance to many antibiotics. One of the alarming results is the high resistance against carbapenems; where 78.3% of isolates were resistant to imipenem and 73.7% were resistant to meropenem. In agreement with our results, Diab *et al.* (2013) revealed a high rate (72%) of imipenem resistance among *P. aeruginosa* isolates. In the Middle East the occurrence of imipenem resistant *P. aeruginosa* was also recognized. In Saudi Arabia, of a total of 350 *P. aeruginosa* isolates, 135 (38.57%) were found to be resistant to imipenem (Mohamed *et al.*, 2011). Hashemi *et al.*, (2016) reported that all their MBL-producing *P. aeruginosa* isolates were resistant to meropenem and imipenem.

Among 33 European countries participating in the European Antimicrobial Resistance Surveillance System (EARSS) in 2007, six countries reported carbapenem resistance rates of more than 25% among *P. aeruginosa* isolates (Souli *et al.*, 2008); the highest rate was reported from Greece (51%). Reasons for increased antimicrobial resistance in Greece are numerous as explained by Miyakis *et al.* (2011). As Greece has the highest antibiotic consumption rate in Europe, both in total and in out-patients, population mobility can introduce resistant strains and infrastructure and resources for infection control are insufficient. This, along with reduced awareness for detection, increases the likelihood of in-hospital spread of multi drug resistant organisms (MDROs). Many of these reasons exist in our country and can explain the high antimicrobial resistance encountered among *P. aeruginosa* isolates in the current study.

During the last decade, emergence and dissemination of the most prevalent MBL genes such as; bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, and the newly identified bla_{AIM} and bla_{NDM} have been extensively documented around the world. Because of the efficient carbapenemase activity of the MBL enzymes, they account for up to 40% of worldwide imipenem resistant *P. aeruginosa*. Xavier *et al.* (2010) stated that MBL enzymes increase antimicrobial MICs more effectively than does either efflux pump over-expression or porin down-regulation alone. Unfortunately, screening only carbapenem-resistant organisms for these enzymes, as most often performed, is suboptimal. With increasing reports of MBLs in carbapenem-susceptible isolates, it becomes crucial to improve laboratory methods used for detection of what is called: hidden MBLs (Ntokou *et al.*, 2008).

In the present study determination of MBL frequency in both carbapenem resistant and susceptible *P. aeruginosa* isolates was done by phenotypic and genotypic methods. Accordingly, 30 identified *P. aeruginosa* isolates were selected to cover: the commonly known definition of MBL producing *P. aeruginosa* isolates *i.e.* those that are resistant to all beta lactams but sensitive to monobactams (aztreonam), and isolates that showed resistance to carbapenems and aztreonam, together with isolates that were carbapenem (imipenem) sensitive to exclude hidden MBLs.

The most widely accepted standardized MBL screening test is the MBL E-test. However, due to the high cost and relative unavailability of E-test strips, many clinical microbiology laboratories use alternative screening methods, such as double-disk synergy test (DDST) and combined disk test (CDT). Although the DDST and the CDT assays are simple to perform and cheaper than the MBL E-test, they have shown discordant results, depending on the employed methodology, β-lactam substrates, MBL inhibitors, and bacterial genus tested (Picão *et al.*, 2008; Ranjan *et al.*, 2015). In this piece of work, all of the 30 (100%) selected *P. aeruginosa* isolates were found to be MBL producing strains using E-test. Similarly Bashir *et al.* (2011) reported that all their thirty isolates were positive for MBL producing *P. aeruginosa* by E-test.

In the current work, of the 30 identified MBL isolates; the highest percentage was recovered from respiratory samples 11 (36.7%), followed by 9 (30%) from each of urine and pus and exudate samples, and only one isolate (3.3%) was from ear discharge. This is nearly in agreement with Zavascki *et al.* (2006), who found that the lung was the most frequent site of nosocomial infection (50.3%), followed by urinary tract (20.5%), and skin and soft tissue (15.8%).

Many factors may contribute to the acquisition of MBL resistant enzymes. Patients in critical care units are likely to have higher probability for these isolates. It was found from this study that the highest percentage of MBL producing *P. aeruginosa* isolates was among ICU patients (70%). This was in line with Zavascki *et al.* (2006) and Lucena *et al.* (2014) who found that ICUs admission increased the risk for MBL producing *P. aeruginosa* infections.

One of the most important parameters for MBL acquisition is associated diseases. Of the 30 patients, who revealed MBL *P. aeruginosa* isolates, 22 (73.3%) had DM and 18 (60%) suffered from cancer. These results were statistically significant ($p < 0.001$ and $p = 0.003$, respectively). In concordance, Varaiya *et al.* (2008) reported that out of 33 (14.3%) MBL producing isolates, 24 (72.7%) were diabetic patients, and 6 (18.1%) were cancer patients. They attributed their findings to the associated immune deficiency among diabetic patients, and recurrent foot infections being good poly-microbial media for a high incidence of multidrug-resistant *P. aeruginosa*. In addition, Vaishali *et al.* (2013) reported that the presence of underlying diseases as DM is a significant risk factor in acquisition of MBL *P. aeruginosa* infection.

Other risk factors that were significantly associated with the presence of MBL producing *P. aeruginosa* infection in this study were the use of medical devices as mechanical ventilators and urinary catheters (23.3%) each, readmission to hospitals (76.7%), and history of previous antibiotic intake (86.7%). On the other hand, the length of hospital stay for more than 7 days represented 76.7%, but was not found to be statistically significant. Kumar *et al.* (2011), found that all MBL-positive patients were exposed to different risk factors as prolonged hospital stay for more than 8 days, catheterization, previous antibiotic use, and mechanical ventilation. In the study done by Vaishali *et al.* (2013) the duration of hospital stay of more than 10 days had 1.7 times risk of acquisition of MBL *P. aeruginosa* infection more than duration of hospital stay of less than or equal to10 days.

The occurrence MBL production is not restricted to carbapenem resistant strains, but some reports have argued about their presence in carbapenem susceptible organisms. They might be unrecognized as the MBL detection has not been routinely performed in most clinical microbiology laboratories (Picão *et al.*, 2012). Such organisms often carry hidden MBL genes. As a consequence, these isolates will be able to participate in horizontal MBL gene transfer with other Gram-negative pathogens and may contribute significantly to MBL related outbreaks. In the present study, of the 30 identified MBL producing *P. aeruginosa* isolates, 7 (23.3%) were imipenem sensitive and 4 (13.3%) were sensitive to meropenem. Diab *et al.* (2013) documented that among the studied imipenem susceptible isolates, 58.3% of which were proved to be MBL producers. A much lower percentage was published by Anoar *et al.* (2014) in Iraq, where of the 46 detected MBL producing isolates, 7 (3.95%) were meropenem sensitive. They explained their findings by the possibility that there might be hidden MBL genes among isolated strains which cannot be diagnosed by phenotypic tests, leading to the dissemination of these genes in the hospital silently among patients even within normal HCWs who can act as carriers for MBL genes in future.

In this piece of work, multi drug resistance was noticed. MBL producing *P. aeruginosa* had the highest percentage of resistance against amikacin 86.7%, followed by piperacillin and ciprofloxacin 80% each. In addition, carbapenems showed high percentages of resistance; imipenem and meropenem (73.3%, and 66.7%, respectively). Bhongle *et al.* (2012) found only one isolate sensitive to imipenem, but it was found to be positive for MBL, thus indicating that MBL producers could show susceptibility to imipenem. These isolates can appear to be susceptible to carbapenems though they carry carbapenemases, such organisms thus carry hidden MBL genes, whereby the microbiologists may remain unaware of their presence.

MBL producers are commonly known as those organisms that potently hydrolyze all beta-lactam antibiotics except aztreonam. In the current study, high resistance to monobactams (aztreonam) was detected (73.3%), and only 3 (10%) of the isolates were susceptible to aztreonam and conformed to such definition. A lower percentage of aztreonam resistance (45.1%) was reported by Zafer *et al.* (2014). In this study, 86.7% of MBL producing *P. aeruginosa* isolates were susceptible to polymyxin B. This supports the evidence that polymyxin B has increasingly become the last viable therapeutic option for MDR *Pseudomonas* infections. In accordance with the current results Bashir *et al.* (2011) recorded that MBL producers showed very high resistance to all antimicrobials except polymyxin B. However, resistance to amikacin (73%) and ciprofloxacin (55%) was lower when compared to the results in the present study (86.7% and 80%, respectively). Piperacillin tazobactam was the second most effective antibiotic after polymyxin B, where 36.7% of our isolates were sensitive to it.

Although phenotypic methods are considered to be useful and reliable in detecting MBL producers, results should be validated and confirmed by genotypic methods. In the present study, all the 30 (100%) identified MBL producing *P. aeruginosa* isolates by E test were further screened for the presence of MBL encoding genes (bla_{IMP}, bla_{SPM}, and bla_{VIM}) using conventional PCR. bla_{IMP} gene was the most prevalent MBL among the isolates accounting for 63.3%, while 36.7% of the isolates revealed bla_{IMP} gene and none of the isolates had bla_{VIM} genes.

In agreement with the results of this study, Gaspareto *et al.* (2007) stated that bla_{SPM} gene was the most common gene among MBL *P. aeruginosa* isolates (73%), and that no bla_{VIM} gene was detected. Camargo *et al.* (2011) reported that bla_{SPM} gene accounted for 71% of positive MBL strains, while bla_{IMP} was detected in 29%.

On the contrary, bla_{VIM} gene was detected in many Egyptian studies. Zafer *et al.* (2014) demonstrated that 58.3% were positive for bla_{VIM} gene, with bla_{IMP} gene detected in 2.1% and no bla_{SPM} gene was identified. Another study by Diab *et al.* (2013) revealed that 70% were positive for bla_{VIM}, while bla_{IMP} gene was not detected.

In the current work, all the 30 *P. aeruginosa* isolates that were identified as MBL producers by the phenotypic method E-test, were found to be MBL producers by the molecular method PCR; where 19 isolates had MBL_{SPM} gene and 11 had bla_{IMP} gene. A lower percentage was reported by Doosti *et al.* (2013); where 36/41 (87.8%) of isolates were phenotypically MBL positive, but PCR results confirmed presence of MBL genes in only 33/41 (80%) of isolates. bla_{IMP} producers have been detected worldwide: in Europe (Docquier *et al.*, 2003) and in the U.S. (Hanson *et al.*, 2006). In the current study, positive MBL IMP gene producers were more commonly isolated from respiratory samples (36.4%), followed by urine and pus and exudate samples (27.3% each). It was detected in only one ear discharge sample (9.1%).

In the current work, hospital readmission was found to be a significant risk factor for acquiring *bla*$_{\mathrm{IMP}}$ gene, where 81.8% of patients were readmitted to the hospital within 3 months of the study ($p = 0.017$).

Among MBL genes, *bla*$_{\mathrm{SPM}}$ has been widely detected in Brazil and Switzerland, the dissemination of this gene in various regions seems to be caused by a single epidemic *P. aeruginosa* clone (Salabi *et al.*, 2010) In the present study, *bla*$_{\mathrm{SPM}}$ gene producers were mostly recovered from respiratory samples (47.4%), followed by urine (26.3%) and pus and exudate samples (21.1%). In line to our data, Zavascki *et al.* (2006) in southern Brazil, described the first nosocomial outbreak of *P. aeruginosa* producing MBL SPM gene, and noted that $_{\mathrm{IMP}}$ isolates were highly revealed from respiratory samples (43.0%), followed by urine and surgical wound samples (33.3% and 5.9%, respectively). While Matos *et al.* (2016) documented that their study was the first report describing the detection of the bla$_{\mathrm{SPM}}$-1-like gene in northern Brazil.

In the present study, the majority of patients with positive *bla*$_{\mathrm{SPM}}$ gene *P. aeruginosa* isolates were admitted to the ICU (73.7%) and 14 (73.7%) had a hospital stay duration of more than 7 days. In addition 5 (26.3%) had DM and 7 (36.8%) suffered from cancer. It was also found that the use of mechanical ventilators was significantly associated with positive *bla*$_{\mathrm{SPM}}$ genes ($p = 0.029$). Other risk factors that were significantly associated with higher rates of positive *bla*$_{\mathrm{SPM}}$ gene were history of hospital readmission and antibiotic intake (78.9% and 68.4%, respectively). In agreement with the current findings, a study to evaluate risk factors for colonization or infection due to MDR *P. aeruginosa* carrying *bla*$_{\mathrm{SPM}}$ gene recorded that 50% of patients with positive *bla*$_{\mathrm{SPM}}$ gene had Foley's catheters, 21% were on mechanical ventilation, 78% had been previously hospitalized within the preceding year and all patients (100%) had taken antibiotics which was the main significant risk factor detected (Nouer *et al.*, 2005). Matos *et al.* (2016) reported that 20% (4/20) of their *P. aeruginosa* isolates were positive for the bla$_{\mathrm{SPM}}$-1-like gene, and that MDR occurred most frequently among isolates from adults who had been hospitalized for an average of 87.1 days, where the use of mechanical ventilation and urinary catheters were risk factors for infection.

In contrast with other studies (Zafer *et al.*, 2014; Mohd *et al.*, 2015) which suggested successful global dissemination of *bla*$_{\mathrm{VIM}}$ resistant gene and considered it to be of great concern, no *bla*$_{\mathrm{VIM}}$ genes were detected in the present work. Zafer *et al.*, in 2014 reported that *bla*$_{\mathrm{VIM}}$ gene was detected in 85% of MBL producing *P. aeruginosa* isolates, while Gonçalves *et al.*, (2017) reported that among the 157 analyzed *P. aeruginosa* strains, 5.3% were positive for *bla*$_{\mathrm{VIM}}$ gene.

Ignorance of rational antibiotics prescribing principles and prolonged clinical use of carbapenems for the treatment of MDR *P. aeruginosa* infections have been recognized as the main reasons behind MBL carbapenamase producing strains. Determination of MBL genes in MDR *P. aeruginosa* gives useful data about their epidemiology and risk factors associated with this group. Hence, early recognition of MBL producers is indispensable and necessitates rigorous infection control measures (Cantas *et al.*, 2013).

Conclusions

- MBL producing *P. aeruginosa* isolates were more prevalent among patients admitted to the ICUs.
- Polymyxin B was the most effective antimicrobial agent against MBL producing *P. aeruginosa* isolates, while amikacin was the least effective one.
- Monobactam (aztreonam) susceptible *P. aeruginosa* isolates were also found to be MBL producers as aztreonam resistant ones.
- MBL producers were detected phenotypically by E test in both carbapenem susceptible and resistant *P. aeruginosa* isolates.
- *bla*$_{\mathrm{SPM}}$ was the most commonly detected MBL gene in *P. aeruginosa* isolates.
- *bla*$_{\mathrm{SPM}}$ and *bla*$_{\mathrm{IMP}}$ MBL encoding genes were detected in both carbapenem susceptible and resistant *P. aeruginosa* isolates.
- Associated diseases (DM, cancer), indwelling urinary catheters, hospital readmission, and antibiotic intake were considered as significant risk factors for MBL producing *P. aeruginosa* infections.

Recommendations

1. Accurate laboratory methods including culture and antimicrobial susceptibility testing with routine screening for MBL production should be the base for proper diagnosis and management of *P. aeruginosa* infections.
2. Early and reliable detection of MBL production in *P. aeruginosa* isolates including monobactam and carbapenem susceptible and resistant strains; to permit timely institution of effective antimicrobial therapy and control dissemination of resistance in hospitals.

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ORIGINAL PAPER

Polish Physicians' Attitudes Towards Antibiotic Prescription and Antimicrobial Resistance

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Abstract

Antimicrobial resistance has been one of the biggest global current issues in medicine and public health. Overuse and imprudent use of antimicrobial agents are recognized as one of the leading causes of antibiotic resistance. The aim of this study was to analyze the attitudes of Polish physicians practicing at the community level towards antibiotics and antimicrobial resistance. The majority of physicians taking part in the survey believed that Polish people overuse antibiotics (98%). Most physicians (91%) considered that antimicrobial resistance is a major problem at present. The majority of physicians indicated the reasons for prescribing the antibiotic are related to health factors, such as optimal recovery (best effectiveness, least side effects) (80%), latest therapeutic guidelines (70%) and microbiological/epidemiological factors (63%). Knowledge of the *National Recommendations for the management of Community-Acquired Respiratory Tract Infections 2010* (NR-CA-RTI) developed within National Programme for Protection of Antibiotics was declared by 84% of respondents. Among those who are aware of the NR-CA-RTI, the majority follow them in their daily practice (91%). Among physicians, 62% are not familiar with the Centor/McIsaac scores used to differentiate bacterial and viral infections in patients presenting with a sore throat. Among physicians familiar with the scores, 90% use them in their daily practice. Rapid microbiological detection methods for Group A beta-hemolytic streptococcal pharyngitis are used only by 20% of respondents. Almost all of physicians declared readiness to use these tests. Main sources of information on antibiotics prescribing originate from Polish medical journals, scientific conferences organized by medical societies, pharmaceutical companies.

Key words: physicians attitudes, antibiotics, antimicrobial resistance, respiratory tract infections

Introduction

Antibiotic resistance has become one of the most important challenges for medicine and public health. The recent World Health Organization (WHO) report makes a clear case that resistance of common bacteria has reached alarming levels in many parts of the world (WHO, 2014). It was shown in many studies that antibiotic resistance is higher in countries with high consumption of this group of drugs (Bronzwaer *et al*., 2002). Poland is one of the few European Union countries where consumption of antibiotics has an increasing trend (ECDC, ESAC-Net, 2016).

In December of 2009 the first National Recommendations for the Management of Community-Acquired Respiratory Tract Infections – NR-CA-RTI (Hryniewicz *et al*., 2009) were published. They were based on Polish epidemiological data and susceptibility profile of the most common etiological agents and international literature reviewed by a panel of various specialists and broadly consulted by medical community in Poland. They were publicized and made available free of charge on the website of the National Programme on Antibiotic Protection (www.antybiotyki.edu.pl). They were updated in 2016.

In Poland more than 95% of antibiotics are prescribed by physicians practicing in the outpatient sector especially for respiratory tract infection which in majority of cases are of viral etiology (www.antybiotyki.edu.pl). This is why the aim of this study was to analyze the attitudes of Polish physicians practicing at the community level towards antibiotics and antimicrobial resistance. In addition the compliance of physicians antibiotic prescription with the National Recommendations on the NR-CA-RTI was evaluated.

Experimental

Materials and Methods

The questionnaire. The research instrument used in the study was a self-designed questionnaire to be completed by the respondents. The questionnaire differed in a section on the use of antibiotic therapy in

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particular indications, based on the medical specialty of the respondent. The questionnaire consisted of closed questions, semi-open questions, additional multiple choice single and multiple answer questions, and demographic items.

The areas covered in the questionnaire included:

1. Attitudes towards antibiotics.
2. Familiarity with the European Antibiotic Awareness Day campaign (EAAD).
3. Indications for antibiotic therapy.
4. Familiarity with and complying with current National Recommendations of the Management of Community-Acquired Respiratory Tract Infections.
5. Compliance of antibiotic prescriptions with the NR-CA-RTI
6. Source of knowledge on antibiotics and antimicrobial resistance.
7. Demographic and education data (medical practice setting, year of graduation and name of medical school).

Study design. The research was carried out between March 2011 and March 2012, during the medical conferences for 3 groups of physicians:

- paediatricians, 8th conference "Akademia po Dyplomie – Pediatria" [Postgraduate Academy – Paediatrics] in Warszawa on March 11–12, 2011;
- paediatric ENT, 18th Symposium "Paediatric Otolaryngology Days" in Mikołajki, on June 9–11, 2011;
- general practitioners (GPs), Top Medical Trends congress in Poznań on March 23–25, 2012.

The questionnaire was handed to be completed during above mentioned events to 3450 physicians (1500 paediatricians, 450 paediatric ENT physicians and 1500 GPs). The completed survey was returned by 18% of paediatricians, 32% of paediatric ENT physicians and 10.5% of GPs.

The participation was voluntary and anonymous. The organisers of the three conferences mentioned above consented to the questionnaire-based research to be carried out during the events.

Ethics. The study was approved by the Ethics Committee of Warsaw Medical University (Registration number: AKBE/45/13).

Data analysis. All analyses were carried out using the IBM SPSS Statistics for Windows 19.0 software package. Collected data was expressed as frequencies and percentages.

Some variables were recoded to chosen categories including binary coding to be used in logistic regression. To identify the sociodemographic factors related to attitudes and knowledge about the effectiveness and use of antibiotics and diagnostics tools multiple logistic regression was used. The reference groups were set as a medical specialties, medical practice, year of graduation and knowledge of the NR-CA-RTI. Odds ratios (ORs) with corresponding 95% confidence intervals (CIs) were calculated. For all test p-values of 0.05 or less were considered to be statistically significant.

Results

Study group characteristics. The study group consisted of 579 physicians representing three medical specialties: paediatrics (276), paediatric ENT (145) and GP (158). Five hundred sixty five responding physicians disclosed the year of graduation. In this cohort, 121 (21.4%) graduated from medical school before 1979, 213 (37.7%) graduated in 1980–1989, 231 (40.9%) graduated in 1990 and later. The majority of the respondents reported medical practice in an outpatient setting (N = 357; 61,7%), 222 respondents (38.3%) reported mixed setting inpatient-outpatient practice. The highest proportion of GPs and paediatricians reported outpatient practice only: 72.8% and 63.4%, respectively. The characteristics of the study group are summarized in Table I.

Attitudes towards antibiotics. The vast majority of respondents (98%) stated that antibiotics are overused in the Polish population. The opinions of physicians on the knowledge of antibiotics in the general public and the medical community differed significantly by medical specialty and practice setting (Table II). Most (67.9%) respondents claimed the physicians do not have sufficient knowledge regarding the use of antibiotics.

Ninety percent of respondents consider antimicrobial resistance to be a significant problem, as compared

Table I
Socio-demographic and professional characteristics of the study participants.

		Study participants n (%)	GPs n (%)	Paediatrics n (%)	Paediatric ENT physicians n (%)
Medical practice	outpatient only	357 (61.7)	115 (72.8)	175 (63.4)	67 (46.2)
	both outpatient and inpatient	222 (38.3)	43 (27.2)	101 (36.6)	78 (53.8)
Year of graduation	before 1979	121 (21.4)	30 (19.6)	70 (25.5)	21 (15.3)
	1980–1989	213 (37.7)	48 (31.4)	107 (38.9)	58 (42.3)
	1990 and later	231 (40.9)	75 (49.0)	98 (35.6)	58 (42.3)

Table II
Physician opinions regarding the knowledge of antibiotics by the public and the medical community.

There is a commonly held belief of inadequate knowledge of antibiotic use. It is legitimate for and applicable to:						
	n	The entire community without medical background (%)	A part of the community without medical background (%)	A part of the medical community (%)	Definitely NOT the medical community (%)	There is another group it is legitimate for and applicable to (%)
Total	579	24.0	61.3.	67.9	3.1	1.2
Medical specialties						
paediatrics	276	23.6	63.8	70.7	4.7	1.4
GPs	158	30.4	57.6	62.0	0.6	1.9
paediatric ENT physicians	145	17.9	60.7	69.0	2.8	0.0
sig.*		**0.039**	0.439	0.171	0.06	0.281
Medical practice						
outpatient only	357	25.2	61.1	65.5	4.5	1.1
both outpatient and inpatient	222	22.1	61.7	71.6	0.9	1.4
sig.*		0.390	0.876	0.128	**0.016**	0.805

*sig. – statistically significant (in bold), significance obtained using Chi^2-test

to 0.7% not considering it to be a problem and 8.7% stating it will become a problem in the future. Significant differences were seen in the expressed opinions on antimicrobial resistance and medical specialty represented (p = 0.025). The opinion that antimicrobial resistance was a significant problem nowadays was expressed by 94.9% of the GPs cohort, 90.4% of the paediatricians and 86.1% of the paediatric ENT cohort. Paediatric ENT physicians tended to express an opinion that antimicrobial resistance would be a problem of the future more often (12.5%) than paediatricians (9.6%) or the GPs (3.8%). The opinion that antimicrobial resistance was not a significant problem nowadays was expressed by 1.3% of the GPs cohort, 1.4% of the paediatric ENT cohort and none of the paediatricians.

Physician familiarity with the European Antibiotic Awareness Day campaign. Overall, 38% of physicians confirmed recent familiarity of the EAAD information campaign. The paediatricians (44.9%) tended to be more familiar with the EAAD as compared to the GPs (35.0%) and the paediatric ENT physicians (31.0%) p = 0.011.

Among the respondents who had recently heard of or participated in the EAAD campaign, 64.7% considered it to be necessary, 32.4% considered it to be beneficial, whereas 2.0%, considered it to be irrelevant for the knowledge of general public. Among 246 respondents, who admitted using EAAD promotional materials, 46.3% declared multiple use of the resources, 18.7% declared one time use whereas 35.0% were unable to state how many times they used them.

Indications for antibiotic therapy. The vast majority of the respondents pointed to health-related factors, that is optimum treatment of infection, ensuring highest efficacy and minimum adverse effects (79.6%), as the key to prescribing antibiotics, followed by the most current therapeutic guidelines (69.9%) and microbial/ epidemiologic factors (62.9%). Almost 9% of respondents take patient expectations into account when prescribing antibiotic therapy. Differences in including various factors when prescribing antibiotic therapy were observed depending on medical specialty and the year of graduation (Table III).

The GPs tend to consider economic factors when prescribing antibiotics more often (29.7%) than the two other specialties, whereas paediatricians tend to consider current therapeutic guidelines the most often (75.0%). Patient expectations were considered when prescribing antibiotics by 11.4% of GPs, 9.1% of paediatricians and 4.8% of paediatric ENT physicians.

More graduates from 1980–1989 or 1990 and later declared considering microbial/ epidemiologic factors and therapeutic guidelines when prescribing antibiotics, as compared to the pre 1979 graduates (Table III).

Familiarity with and complying with the National Recommendations on the Management of Community-Acquired Respiratory Tract Infections '2010. Overall, 569 respondents (84.4%) declared familiarity with the NR-CA-RTI. Significant differences were observed in the declared familiarity with the Recommendations by different medical specialties and medical practice settings (Table IV). This was definitely the highest among paediatricians (97.0%), followed by paediatric ENT physicians (81.8%) and GPs (64.7%). Almost 90.0% of physicians reporting mixed setting

Table III
Factors considered when prescribing antibiotics.

	N	Health-related (%)	Economic (%)	Epidemiologic/ microbiological (%)	Therapeutic guidelines (%)	Patient expectations (%)
Total	579	79.6	18.1	62.9	69.9	8.6
Medical specialties						
paediatrics	276	77.9	13.4	67.0	75.0	9.1
GPs	158	80.4	29.7	65.2	65.8	11.4
paediatric ENT physicians	145	82.1	14.5	52.4	64.8	4.8
sig.*		0.578	**< 0.001**	**0.010**	**0.040**	0.119
Medical practice						
outpatient only	357	79.0	19.3	61.6	66.1	9.5
both outpatient and inpatient	222	80.6	16.2	64.9	76.1	7.2
sig.*		0.634	0.345	0.433	**0.011**	0.335
Year of graduation						
before 1979	121	79.3	19.0	55.4	56.2	5.8
1980–1989	213	83.1	15.5	64.3	68.5	7.0
1990 and later	231	76.6	19.5	66.7	78.4	11.7
sig.*		0.238	0.513	0.105	**< 0.001**	0.098
Familiar with the National Recommendations on the Management of Community – Acquired Respiratory Tract Infections						
Yes	480	80.2	17.7	65.2	72.9	9.2
No	89	77.5	21.3	50.6	57.3	5.6
sig.*		0.563	0.414	**0.009**	**0.003**	0.273

* sig. – statistically significant (in bold), significance obtained using Chi^2-test

Table IV
Familiarity with the NR-CA-RTI.

	n	I know the National Recommendations on the NR-CA-RTI YES (%)	Adjusted ORs (95% CI)	sig.*	n	I know and follow the National Recommendations on the NR-CA-RTI YES (%)	Adjusted ORs (95% CI)	sig.*
Total	569	84.4	–	–	479	91.0	–	–
Medical specialties								
GPs	156	64.7	1	–	101	94.1	1	–
paediatrics	270	97.0	17.02 (7.67–37.76)	**< 0.001**	262	91.9	0.53 (0.19–1.48)	0.227
paediatric ENT physicians	143	81.8	2.16 (1.19–3.92)	**0.011**	116	87.9	0.39 (0.13–1.16)	0.092
Medical practice								
outpatient only	348	80.2	1	–	279	92.1	1	–
both outpatient and inpatient	221	91.0	2.88 (1.55–5.36)	**0.001**	200	89.5	0.80 (0.41–1.56)	0.511
Year of graduation								
1990 and later	231	81.0	1	–	186	90.3	1	
before 1979	118	85.6	1.24 (0.63–2.47)	0.531	101	89.1	0.83 (0.37–1.87)	0.652
1980–1989	206	88.8	1.85 (1.02–3.37)	**0.044**	183	92.9	1.37 (0.64–2.91)	0.416

* sig. – statistically significant (in bold)

practice declared familiarity with Recommendations, as compared to 80.2% of those reporting outpatient practice only (OR = 2.88, 95% CI = 1.55–5.36).

Among respondents declaring familiarity with the recommendations (n = 479), the vast majority (91.0%) follow them in their everyday practice: 94.1% of GPs, 91.9% of paediatricians and 87.9% of paediatric ENT physicians. At the same time 12.1 %, 8.8% and 5.9% of paediatric ENT physicians, paediatricians and GPs, respectively, declared they were familiar with yet did not follow the NR-CA-RTI. In the latter group (N = 43), the most frequently reported reasons for this attitude included following other recommendations (48.8%) and other reasons (44.2%), such as the need for further diagnostic testing, lack of access to the literature and own clinical experience.

Familiarity with and use of Centor/ McIsaac score. Overall, 38.1% of respondents stated familiarity with the Centor/McIsaac score used for differentiation between bacterial and viral pharyngitis (Table V). This was declared by 40.5% of GPs, 37.2% of paediatricians and 37.1% of paediatric ENT physicians.

Among those familiar with the Centor/McIsaac score, the majority graduated from medical school in 1990 or later (45.4%), followed by the 1980–1989 graduates (34.3%, OR = 0.61, 95% CI = 0.41–0.92) and the pre-1979 graduates (31.0%, OR = 0.56, 95% CI = 0.35–0.92). Familiarity with the Centor/McIsaac score was declared by 39.8% of physicians reporting familiarity the NR-CA-RTI and 29.8% of those unfamiliar with these recommendations (OR = 1.97, 95% CI = 1.11–3.51). Almost 90% of physicians reporting familiarity with the Centor/ McIsaac score use it in their everyday practice (Table V). Great majority (91.1%) of physicians using the Centor/McIsaac score declared familiarity with the NR-CA-RTI.

Using rapid diagnostic tests to detect Group A streptococcal (GAS) pharyngitis. Twenty percent of 569 respondents reported using rapid diagnostic tests to detect Group A streptococcal (GAS) pharyngitis. Differences in using it were observed between the three medical specialties (Table V). The rapid tests were used by the GPs more often (26.4%) than by other two specialties. The main reported barriers to use it were their inaccessibility and lack of reimbursement. At the same time, the majority of the respondents (95.3%) declared their willingness to use the test if it is reimbursed by the National Health Fund.

Compliance of antibiotic prescriptions with the NR-CA-RTI. Figure 1 shows the physician views (N = 434) on prescribing antibiotics in the following clinical cases:

- acute bronchiolitis in a 12-month infant with no additional risk factors,
- common cold in a 3-year-old,
- flu and flu-like symptoms in a 5-year-old,
- otitis media in a 2.5-year old, within the first 2 days since symptoms onset,
- rhinosinusitis without fever, facial pain or sore throat,
- I don't prescribe antibiotics in any of the above cases

Differences in prescribing decisions were observed depending on medical specialty and year of graduation. Almost 60% of GPs as compared to 46.7% of paediatricians stated they do not prescribe an antibiotic for

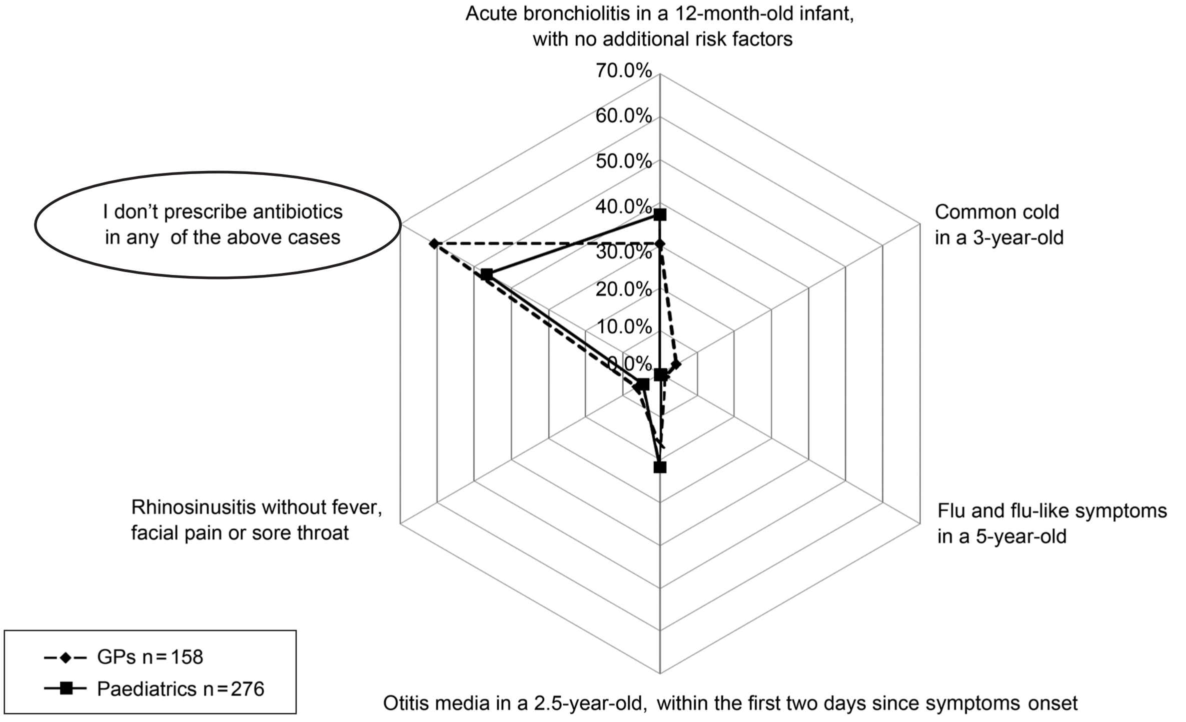

Fig. 1. Paediatricians and GPs views on prescribing antibiotics for certain indications. Choice of therapeutic option in line with the NR-CA-RTI was circled in black. Base: GPs and paediatricians (N = 434).

Table V
Familiarity of surveyed physicians with the Centor/McIsaac score and use of rapid diagnostic tests to detect Group A Streptococcal (GAS) pharyngitis.

	n	I know the Centor/ McIsaac score YES (%)	Adjusted ORs (95% CI)	sign.*	n	I use the Centor/ McIsaac score – physicians reporting familiarity with the score YES (%)	Adjusted ORs (95% CI)	sign.*	n	I use rapid diagnostic tests to detect Group A Streptococcal (GAS) pharyngitis (caused by *S. pyogenes*) (%) YES	Adjusted ORs (95% CI)	sign.*
Total	557	38.1	–	–	205	89.3	–	–	569	20.0	–	–
Medical specialties												
GPs	148	40.5	1	–	59	88.1	1	–	148	26.4	1	–
paediatrics	266	37.2	0.74 (0.46–1.18)	0.205	96	93.8	1.17 (0.32–4.25)	0.813	276	21.4	0.60 (0.35–1.03)	0.062
paediatric ENT physicians	143	37.1	0.76 (0.45–1.27)	0.294	50	82.0	0.43 (0.13–1.41)	0.162	145	11.0	0.28 (0.14–0.56)	**< 0.001**
Medical practice												
outpatient only	338	36.7	1	–	121	90.1	1	–	347	17.9	1	–
both outpatient and inpatient	219	40.2	1.08 (0.74–1.57)	0.698	84	88.1	0.92 (0.35–2.42)	0.862	222	23.4	1.56 (0.99–2.45)	0.054
Year of graduation												
1990 and later	227	45.4	1	–	100	86.0	1	–	230	18.7	1	–
before 1979	113	31.0	0.56 (0.35–0.92)	**0.021**	35	94.3	2.49 (0.51–12.16)	0.261	118	21.2	1.22 (0.68–2.17)	0.502
1980–1989	204	34.3	0.61 (0.41–0.92)	**0.017**	66	90.9	1.36 (0.46–3.95)	0.577	208	21.2	1.27 (0.77–2.08)	0.347
Familiar with the National Recommendations on the Management of Community-Acquired Respiratory Tract Infections												
I know the National Recommendations MCA-RTI	465	39.8	1	–	169	91.1	1	–	474	20.9	1	–
I don't know the National Recommendations MCA-RTI	84	29.8	1.97 (1.11–3.51)	**0.020**	10	76.0	3.28 (0.99–10.90)	0.052	85	14.1	1.87 (0.89–3.92)	0.098

* sig. – statistically significant (in bold)

any of the above indications (p = 0.033, OR = 0.60, 95% CI = 0.37–0.96), which is in line with the NR-CA-RTI.

Those who graduated in 1980–1989 (54.8%) and in 1990 or later (54.3%) more frequently stated they do not prescribe an antibiotic for any of the above indications, as compared to 43.0% of pre-1979 graduates. Then this follow in everyday practice (51.8%) National Recommendations for the Management of Community-Acquired Respiratory Tract Infections more frequently gave the correct answer, than this who do not use the Recommendations (27.6%, p = 0.012).

Figure 2 shows the first choice empirical antibiotic therapy declared by 143 GPs for the community-acquired pneumonia in a 40-year-old. More than 31% of GPs chose 1,000 mg of amoxicillin every 8 h for 7 days, which is in line with the NR-CA-RTI. Almost 40% of respondents suggested 1,000 mg of amoxicillin with clavulanic acid every 12 h for 7 days, 18.9% – 1,000 mg of amoxicillin every 12 h for 7 days, 6.3% – 500 mg of azithromycin once daily for 3 days and 2.8% – 625 mg of amoxicillin + clavulanic acid every 8 h for 7 days.

Overall, 81.5% of respondents declared the immediate use of an antibiotic for otitis media in infants below 6 months of age, which is in line with the NR-CA-RTI and which was confirmed by 91.7% of paediatricians, 85.5% of paediatric ENT and 60.1% of GPs (p < 0.001).

Sources of knowledge of antibiotics and antimicrobial resistance. The main sources of knowledge of antibiotics mentioned by our respondents included national medical journals (88.4%), conferences organised by medical societies (76.3%), conferences organised by pharmaceutical companies (54.2%) and the Internet (36.6%). International medical journals (15.2%) were less accessed sources of information on antibiotics (Table VI).

Differences were observed in utilizing different sources of knowledge, depending on medical specialty and year of graduation of respondents. National medical journals were identified as the main source of knowledge by 95.3% of paediatricians, 84.2% of GPs and 80.0% of paediatric ENT physicians (p < 0.001).

Conferences organised by medical societies were identified as the main source of knowledge on antibiotics by 87.6% of paediatric ENT physicians, 81.9% of paediatricians and 56.3% of GPs (p < 0.001). In all three specialties, more physicians pointed to conferences organised by medical societies rather than to ones organised by pharmaceutical companies as their main source of knowledge. Internet was mostly mentioned as the source of knowledge of antibiotics by those who graduated in 1990 or later (41.4%), followed by the 1980–1989 graduates (37.6%) and only by 27.3% of pre-1979 graduates (p = 0.036).

The conferences provided by pharmaceutical sector were selected by the majority of the 1980–1989 graduates (59.6%), followed by the pre-1979 graduates (58.7%), and to a significantly lower extent by those who graduated in 1990 or later (48.5%) p = 0.040.

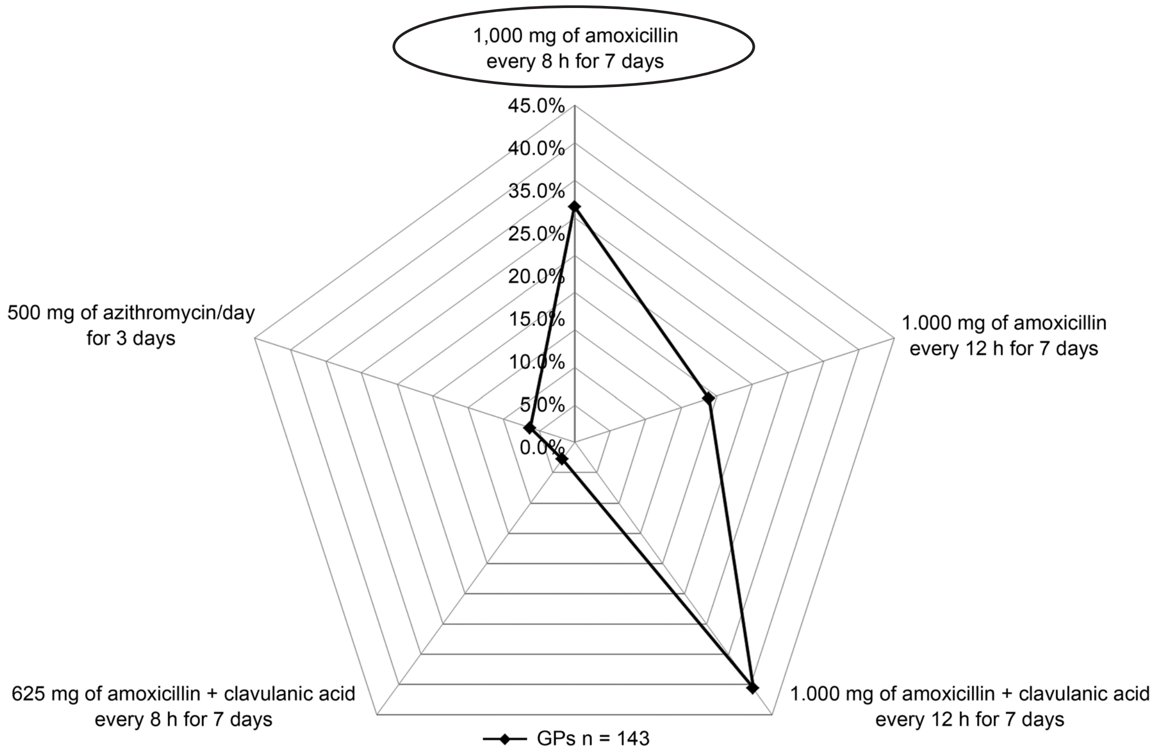

Fig. 2. Therapeutic decisions of GPs in the first episode of community-acquired pneumonia in a 40-year-old. Choice of therapeutic option in line with the NR-CA-RTI was circled in black. Base: GPs (N = 143).

Table VI
Sources of current knowledge of antibiotics.

	n	National medical journals (%)	International medical journals (%)	Conferences organised by medical societies (%)	Conferences organised by pharmaceutical sector (%)	Internet (%)
Total	579	88.4	15.2	76.3	54.2	36.6
Medical specialties						
paediatrics	276	95.3	14.1	81.9	58.3	34.1
GPs	158	84.2	14.6	56.3	52.4	38.0
paediatric ENT physicians	145	80.0	17.9	87.6	59.3	40.0
sig.*		**<0.001**	0.567	**<0.001**	**0.002**	0.445
Medical practice						
outpatient only	357	87.1	9.5	75.4	55.2	31.4
both outpatient and inpatient	222	90.5	24.3	77.9	52.7	45.0
sig.*		0.210	**<0.001**	0.478	0.560	**0.001**
Year of graduation						
before 1979	121	86.8	15.7	77.7	58.7	27.3
1980–1989	213	88.7	14.1	76.5	59.6	37.6
1990 and later	231	89.6	15.6	76.2	48.5	41.4
sig.*		0.728	0.884	0.950	**0.040**	**0.036**

* sig. – statistically significant (in bold), significance obtained using Chi^2-test

The vast majority of the respondents ($N = 578$; 97.9%) indicated a willingness to increase their knowledge of antibiotic use. Among our respondents, 3.3% of paediatricians stated no reason to broaden their knowledge of antibiotic use. The majority of those willing to increase their understanding of antibiotics graduated in 1990 or later (99.6%) and in 1980–1989 (98.1%), followed by slightly lower percentage of pre-1979 graduates (94.2%) $p = 0.004$. The highest proportion of respondents who stated no reason to broaden their knowledge of antibiotics consisted of pre-1979 graduates (5.8%).

The most popular future training topics indicated were: antimicrobial resistance of pathogens causing respiratory tract infections (72.7%), general principles of rational antibiotic use (69.8%) as well as microbiological diagnostics (68.6%).

Differences in participation in training on antibiotics therapy between the represented medical specialties were observed ($p < 0.001$). Pediatricians (45.3%) and paediatric ENT physicians (38.9%) reported participating in training on antibiotics therapy twice a year, whereas GPs (33.5%) reported receiving such training once a year. At the same time 21.3% of all respondents participate in training on antibiotics less often than once every year, with 7.0% stating that they have never participated in such training.

The main motivation for participating in training on antibiotic therapy was the need to update knowledge (80.8%) and broaden it (67.4%), followed by the need to get educational points (12.6%), wishing to meet one's friends (8.6%) and having uncertainty concerning prescribing antibiotics/treatment (6.4%).

The need to update knowledge was mentioned as a motivation to participate in training on antibiotics by 87.0% of paediatricians, 80.7% of paediatric ENT physicians and less often by GPs (70.3%) $p < 0.001$. Depending on practice setting, this need was expressed by 81.5% of outpatient practitioners, and 79.7% of mixed setting practitioners. The need to broaden the knowledge was definitely reported the most frequently among GPs (75.9%), followed by paediatricians (68.5%) and paediatric ENT physicians (55.9%) $p = 0.001$. Ten percent of GPs, 6.2% of paediatricians and only 2.8% of paediatric ENT physicians reported having uncertainty concerning prescribing antibiotics/treatment $p = 0.032$.

Discussion

Many reports from all over the world, as well as the results of the study described in this paper, suggest that physicians play the most important role in providing the public with information about the proper use of antibiotics (Napolitano *et al.*, 2013; TNS Opinion and Social, 2016). They decide whether to prescribe antibiotics or not. Thus physicians have a significant impact not only on antibiotic consumption, but also on their rational use. Family physicians play an especially important role in this process, with up to 90% of all antibiotics prescribed by primary care doctors (particularly by family physicians).

The data presented in this paper have revealed the attitude of Polish physicians towards antibiotic use and antimicrobial resistance. They indicated their high awareness of the threat of antimicrobial resistance. A similar attitude among physicians was shown in several studies from Europe and the USA as well as among medical students from various European countries (Bjorkman *et al*., 2013; Dyar *et al*., 2013; 2014). Data from England revealed that the awareness of antimicrobial resistance threat is higher among physicians practicing in hospitals and long term care than in an outpatient setting (Simpson *et al*., 2007). Our study, which tackled Polish physicians practicing in the ambulatory setting, indicated that they encounter the problem of antimicrobial resistance on a daily basis while doctors from the USA, France and Scotland regard this problem on a global level but not in their everyday practice (Wester *et al*., 2002; Giblin *et al*., 2004; Pulcini *et al*., 2011). Several studies which presented the opinions of both physicians and medical students noted that the main reason for the emergence and rapid spread of resistant bacteria is the overuse and improper use of antibiotics (Dyar *et al*., 2014).

When prescribing an antibiotic, several factors should be considered to help with the choice of a specific drug. Our study demonstrated that these factors included general health of a patient, therapeutic recommendations, and epidemiological situation. Similar answers were given by physicians from Iceland, Great Britain and Sweden who underlined the value of therapeutic recommendations and information on local epidemiology with regard to resistance (Petursson, 2005; Milos *et al*., 2014). Other studies indicate additional factors to be taken into consideration by physicians when prescribing an antibiotic (Stranberg *et al*., 2013; Ashworth *et al*., 2016; Strumiło *et al*., 2016). They can be divided into two groups. First, from a physician point of view, such as uncertainty of diagnosis, fear of patient's health deterioration and resulting claim for poor management, previous negative experience of post infectious complications, need to keep good relationship with patients in order not to loose them, and physician's personality. The second group of factors included patients' perspective and satisfaction (Shapiro, 2002; Ong *et al*., 2007). Interestingly, in our study patients' expectations accounted only for 10% of decision making.

It has been shown in many studies that additional diagnostic tools available at the physican's office may facilitate differentiation between virus and bacterial etiology and limit unnecessary antibiotic prescription (Fine *et al*., 2012; Palla *et al*., 2012). The best illustration is acute pharyngitis, in which an antibiotic prescription by family physician is the most common. The rapid test detecting antigen of *Streptococcus pyogenes*, a leading bacterial etiology of acute pharyngitis, allows in many cases to avoid giving antibiotic for viral infections. The test is strongly recommended by various scientific societies including IDSA.

The most spectacular promotion of rapid streptococcal test at the physician office started in 2002 in France. The National Insurance Company advised its use in patients above 3 years of life suspected of bacterial pharyngitis and was made available free of charge to every physician practicing in an outpatient setting. In 2012 more than 60% of French physicians declared to use the test in every day practice in children between 3 and 16 years of age. An antibiotic is only given when the *S. pyogenes* test is positive (Michel-Lepage *et al*., 2014).

The Polish NR-CA-RTI advice both usage of Centor/McIsaac scale and rapid Strep-test (Hryniewicz *et al*., 2009) and almost 80% of physicians participating in the study are acquainted with the NR-CA-RTI although only one fourth of Polish physicians regularly has access to the test in their office. However, all of them would be happy to use the test in every day work provided it is reimbursed by health service payer as is the case in other countries. Additional help can be obtained in differentiation between viral and bacterial pharyngitis by using the so-called Centor/McIsaac score, which is based on clinical signs and symptoms. Good correlation was shown between positive streptococcal test, culture and high score (Stefaniuk *et al*., 2017). Fewer than half of the Polish physicians participating in our study were not acquainted with this score however they claimed to know the Recommendation.

As it was already mentioned almost 80% of questioned Polish physicians know the NR-CA-RTI. However, only about 50% complies with them in regard to antibiotic prescription for acute bronchiolitis, common cold, flu, otitis media, facial pain and sore throat, and community-acquired pneumonia. The best compliance with the NR-CA-RTI was noticed in the case of acute otitis media in children younger than 6 months, whom immediate antibiotic usage is indicated. The high doses of amoxicillin recommended in NR-CA-RTI are justified because Polish study on community respiratory isolates indicate high percentage of *S. pneumoniae* non-susceptible to penicillin (www.koroun.edu.pl).

Several strategies were described limiting antibiotic usage. One of them is the so-called delayed prescription practiced in many countries. However, it is rarely practiced in Poland again because of difficulties in access to a physician. It is mostly used in the case of acute otitis media in children and called a watchful waiting strategy, which is also included in the Polish National Recommendations but unfortunately rarely practiced because of difficulties with accesses to physician for additional visit.

Many international studies targeting physicians and medical students have shown that the most efficacious way to improve antibiotic prescription is direct education (Figueiras *et al*., 2001; McGettigan *et al*., 2001). They also demonstrated their great willingness to broaden knowledge on antibiotic therapy and antibiotic resistance, which should be included in the medical curriculum and continuous education programs (Minen *et al*., 2010; Dyar *et al*., 2014). The same was observed in our study in which the great majority of participating physicians declared interest in updating knowledge on antibiotics and their use in therapy in the time of growing resistance.

The ease of internet access and consultation with "dr Google" may impact physician-patient interactions. Wrongly interpreted medical information by a not medically educated patient can lead to unreasonable health concerns and influence expectations from a physician concerning therapeutic decisions.

Several studies pointed at the role of pharmaceutical companies in increased antibiotic consumption due to often aggressive promotion and additional benefits for prescribers. This view was supported by the results of a study in Spain performed among family physicians and the conclusions presented stated that there is a need to limit the contribution/participation of companies in educational events if a drop in needless prescription is to be expected (Caamano *et al*., 2002). Physicians should have access to objective evidence-based information in order to achieve proper antibiotic prescription. Our own data indicated that half of Polish physicians get updates on antibiotics and their proper use during meetings organized by pharmaceutical companies. This mostly refers to those who completed their medical studies before 1989. They also more often use not current therapeutic recommendations. On the other hand, some papers underline the value of educational initiatives undertaken by pharmaceutical companies not only in countries with low resources. The majority of British general practitioners declared in 2001 that pharma people are their major source of current therapeutic recommendations (McGettigan *et al*., 2001). The great popularity of pharma sponsored educational meetings is also due to the fact of free access to them.

The threat created by rapidly growing resistance has promoted several educational campaigns on the European level organized by ECDC and global level managed by WHO (Huttner *et al*., 2010; Earnshaw *et al*., 2014; Chaintarli *et al*., 2016). Poland joined the European initiative immediately when the European Antibiotics Awareness Day was established in 2008. Our data revealed that almost 40% of Polish physicians participating in this study come across EAAD and more than 60% declared that it is beneficial for building awareness to antibiotic resistance.

The results of this study revealed several gaps in knowledge of physicians concerning proper use of antibiotics. Further more focused educational activities of medical community need to be performed under umbrella of the National Programme for Protection of Antibiotics and according to the results of our study.

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ORIGINAL PAPER

Bioconversion of 16-dehydropregnenolone Acetate to Exclusively 4-androstene-3,17-dione by *Delftia acidovorans* MTCC 3363

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Abstract

Delftia acidovorans MTCC 3363 was found to convert 16-dehydropregnenolone acetate (16-DPA) exclusively to 4-androstene-3, 17-dione (AD). Addition of 9α-hydroxylase inhibitors was not required for preventing the accumulation of byproducts. The effect of pH, temperature, substrate concentration, surfactants and carrier solvents on this bioconversion has been studied. 16-DPA was maximally converted in buffered medium at pH 7.0, at temperature 30°C and 0.5 mg ml^{-1} substrate concentration. Detergent addition and temperature above 35°C had deleterious effect on bioconversion. Dioxan was found to be the best carrier solvent for biotransformation of 16-DPA to AD.

Key words: *Delftia acidovorans* MTCC 3363, 4-androstene-3,17-dione, 16-dehydropregnenolone acetate and steroid bioconversion

Introduction

Most of the steroid drugs are derivatives of 4-androstene-3,17-dione (AD) and 1,4 androsta-diene-3,17-dione (ADD) (Kieslich, 1985; Sedlaczek, 1988; Perez *et al.*, 2006). Production of AD and related steroids is largely dependent on microbial transformation of natural sterols (Galan *et al.*, 2016; Liu and Lo, 1997; Rodriguez-Garcia *et al.*, 2016). Soy sterol (from soy oil industry) and wood sterols (from paper pulp industry) are two main sources of natural plant sterols used as raw material in steroid bioconversion (Donova and Egorova, 2012; Perez *et al.*, 2006; Yao *et al.*, 2013). Owing to the structural similarity, 16-dehydropregnenolone acetate, also known as 3β-(acetyloxy) pregna-5,16-dien-20-one, is used for chemical synthesis of steroid hormones. It can be a potential raw material for steroid bioconversion (Zhang and Guo, 2011). 16-DPA is produced by chemical degradation of solasodine and diosgenin obtained from plant sources (Asolkar and Chadha, 1979; Goswami and kotoky, 2003). Banerjee *et al.* (2003) reported the microbial conversion of 16-DPA to 17-ketosteroids, by mixed culture of *Pseudomonas diminuta* MTCC 3361 and *Commamonas acidovorans* MTCC 3362. Conversion of 16-DPA could be a potential alternative route for production of AD, which is currently dependent mainly on soy sterol bioconversion. Solasodine can be directly converted to AD (Shukla *et al.*, 1992) but lower bioconversion yield is a concern. Moreover, availability of solasodine is limited. Microbial conversions offer a single step route to important steroidal intermediates under mild conditions of temperature and pressure.

A soil isolate was found to convert 16-DPA exclusively to AD (Fig. 1). This strain was identified as *Delftia acidovorans* and assigned accession number MTCC 3363.

Optimization of various parameters is required to utilize biotransformation capabilities of *D. acidovorans* MTCC 3363. In the current study, effect of pH, temperature, substrate concentration, some detergents and carriers on bioconversion of 16-DPA to AD by *Delftia acidovorans* MTCC 3363 has been studied.

Experimental

Materials and Methods

Microbial strain. *D. acidovorans* MTCC 3363 was obtained from MTCC, Chandigarh, India. The strain was grown and maintained on nutrient agar slants. For storage the slants were kept at 4–8°C in a refrigerator.

Culture media. Medium used for growth and bioconversion consisted of ($g l^{-1}$) peptone, 5; yeast extract, 2; beef extract, 1; sodium chloride, 5. To study the

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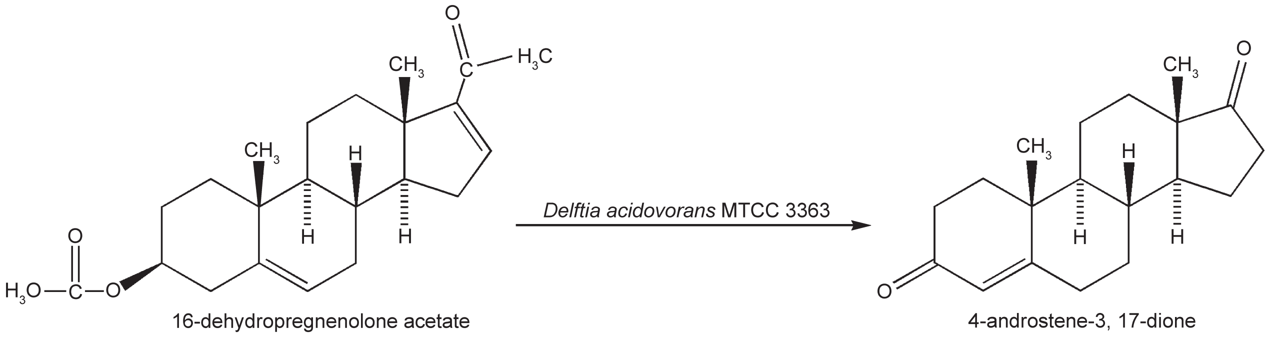

Fig. 1. Biotransformation of 16-dehydropregnenolone acetate (16-DPA) to 4-androstene-3,17-dione (AD) by *D. acidovorans* MTCC 3363.

effect of pH, medium was supplemented with 1%, 1 M sodium phosphate buffer of suitable pH in place of sodium chloride.

Sterols and steroids. 16-DPA, AD and ADD were obtained from Sigma Aldrich, USA.

Other chemicals. Peptone, yeast extract, beef extract and sodium chloride, were obtained from Himedia laboratories, India. Tween 40, Tween 80 and Triton X-100 was purchased from SD Fine-chem Ltd., India. DMF, dioxan and glycerol were procured from Merck specialties Pvt. Ltd. India. Refined soy oil was obtained from local market. The chemicals used were of laboratory reagent grade.

Biotransformation. All bioconversion experiments were conducted in triplicates in 100 ml Erlenmeyer flasks containing 20 ml bioconversion medium. The media was supplemented with 0.01 mg ml^{-1} 16-DPA as an inducer. After adjusting to desired pH, the medium was dispensed in flasks and autoclaved at 15 psi for 20 min. The medium was inoculated by 2 ml of exponentially growing seed culture of *D. acidovorans* MTCC 3363. Seed was grown in nutrient broth medium for 24 hours at 30°C at 200 rpm in gyratory shaker. Inoculated flasks were incubated on a gyratory shaker (200 rpm, 1.5 cm eccentric throw) at desired temperature. After 12 h of incubation, 16-DPA (0.5 mg ml^{-1}) dissolved in dimethylformamide/other substrate carriers (1% of culture volume) was aseptically added to the culture medium. Mol% conversion was calculated as per the formula:

$$\text{Mol\% conversion} = \frac{(\text{concentration of AD in broth})}{(0.804 \times \text{substrate concentration})} \times 100$$

The factor 0.8 in denominator of the formula represents the ratio of molecular weights of AD and 16-DPA.

Analysis of products. At regular intervals, 1 ml sample of bioconversion medium was aseptically drawn from bioconversion flasks. The sample was extracted twice by 2 ml ethyl acetate. The solvent layers were separated and pooled in a fresh tube. The residual water was removed by addition of anhydrous sodium sulfate. Aliquot (0.2 ml) of ethyl acetate extract was taken for quantitative analysis of bioconversion product and rest of the extract was concentrated under vacuum for qualitative analysis by thin layer chromatography. Qualitative analysis of the bioconversion products and residual substrate was carried by thin layer chromatography as described by Shah *et al.* (1980). Identification was done by colour of the spot and R*f* of co-chromatographed authentic samples. AD was estimated by modified Zimmermann reaction as described by Ahmad and Johri (1991). Briefly; The samples (as such or diluted as required) were dried over vacuum at room temperature. The residue was re-dissolved in 0.1 ml of 10% m-dinitrobenzene in pyridine. To each tube 0.05 ml 2.5 N aqueous KOH was added. The tubes were incubated at 45°C for 30 min in a water bath. After completion of reaction, the reaction mixture was cooled to room temperature and diluted with freshly prepared mixture of ethyl acetate and pyridine (1:1). The absorbance was recorded immediately at 572.5 nm on Shimadzu 2401-PC spectrophotometer. Concentration of AD was calculated using a calibration graph.

For preparation of calibration graph, 5 mg AD dissolved in 100 ml ethyl acetate was used as stock solution. 0.1 ml to 1.0 ml aliquots form stock solution was pipette out into separate test tubes and estimated as described above.

The data obtained for replicates was averaged and standard deviation determined. The inter sample variance was analysed by one-way ANOVA and t-test.

Results

The present work brings to foreground the suitability of *D. acidovorans* MTCC 3363 for the bioconversion of 16-DPA to AD. A single intermediate was accumulated during this bioconversion, which was utilized completely as the fermentation progressed. AD is the most important steroidal derivative as it can be transformed to multiple products including ADD by different microorganisms (Zhang *et al.*, 2013).

Effect of temperature. Incubation temperature of 30°C was found suitable for bioconversion of 16-DPA to AD. At this temperature, maximum ~ 71.8 mol% conversion was recorded after 120 h incubation (Table I). AD was the only bioconversion product at 30°C. Mol% yield of AD was reduced at 35°C, yielding 55.6 mol%. Appreciable quantities of ADD (as evidenced by TLC) were recorded at 35°C. No accumulation of AD was found when the flasks were incubated at 40°C.

Table I
Effect of temperature on bioconversion of 16-DPA to AD by *D. acidovorans* MTCC 3363.

Temperature (°C)	Mol% conversion after incubation period (h)			
	48	72	96	120
30	62.25 ± 3.18	59.59 ± 2.24	65.86 ± 1.59	71.85 ± 1.48
35	53.60* ± 3.03	54.21* ± 2.08	55.60* ± 4.28	53.03* ± 4.18
40	ND	ND	ND	ND

Data are mean of three replicates ± standard deviation.
ND = not detected.
* significance at $\alpha = 0.05$; $^{\#}$ represents significance at $\alpha = 0.1$

Effect of pH. The effect of pH was studied using buffered biotransformation medium at 30°C. The effect of pH on bioconversion of 16-DPA to AD by *D. acidovorans* MTCC 3363 in buffered media is presented in Table II. A maximum of 82.88 mole% bioconversion was recorded at neutral pH. Slightly acidic or alkaline pH resulted in less AD accumulation. However, AD accumulation was reduced more in acidic pH (6.5), as compared with alkaline pH (7.5) resulting in ~ 69 and 77 Mol% bioconversion respectively.

Table II
Effect of pH on bioconversion of 16-DPA to AD by *D. acidovorans* MTCC 3363.

pH	Mol% conversion after incubation period (h)			
	48	72	96	120
6.5	59.25 ± 4.91	64.63 ± 1.69	69.15 ± 4.16	63.15 ± 2.08
7.0	73.36* ± 1.91	76.53* ± 3.77	82.88* ± 1.35	74.73* ± 1.48
7.5	69.32* ± 3.73	76.40* ± 0.47	77.26$^{\#}$ ± 3.83	73.27* ± 1.56

Data are mean of three replicates ± standard deviation.
* significance at $\alpha = 0.05$; $^{\#}$ represents significance at $\alpha = 0.1$

Table III
Effect of substrate concentration on bioconversion of 16-DPA to AD by *D. acidovorans* MTCC 3363.

16-DPA (mg/ml)	AD (mg/ml) accumulated at incubation period (h)			
	48	72	96	120
0.5	0.20 ± 0.027	0.23 ± 0.038	0.25 ± 0.022	0.21 ± 0.037
1^{a}	0.31* ± 0.046	0.32* ± 0.024	0.34* ± 0.047	0.26$^{\#}$ ± 0.012
1^{b}	0.33* ± 0.022	0.35* ± 0.024	0.31$^{\#}$ ± 0.047	0.28* ± 0.009
1.5	0.47* ± 0.038	0.50* ± 0.043	0.50* ± 0.009	0.41* ± 0.032

Data are means of three replicates ± standard deviation;
^{a} substrate added as a single dose; ^{b} substrate added in two split doses at 12 h (I) and 24 h (II).
* significance at $\alpha = 0.05$; $^{\#}$ represents significance at $\alpha = 0.1$

Effect of substrate concentration. The amount of AD accumulated at different substrate loadings is presented in Table III. At 0.5, 1.0 and 1.5 mg ml^{-1} substrate, highest mol% conversion was recorded at 96 hours. A maximum of 61.63, 42.81 and 41.42 mol% bioconversion was recorded at 0.5, 1.0 and 1.5 mg ml^{-1} 16-DPA loading respectively. The Mol% conversion reduced correspondingly with the increase in substrate concentration. No significant difference in AD accumulation was observed between the two modes of substrate addition. However, split dosing favored achieving highest mol% conversion (43.50 mol%) within 72 hours. TLC analysis revealed differences in 16-DPA utilization pattern by *D. acidovorans* MTCC 3363 at different substrate loadings. The substrate was completely utilized within 72 hours at 0.5 mg ml^{-1} of 16-DPA, whereas, it persisted for 96 and 120 h at 1and 1.5 mg ml^{-1} 16-DPA loading, respectively.

Effect of surfactants. Steroidal precursors like 16-DPA are hydrophobic in nature and form aggregates, limiting the mass transfer. Surfactants improve dispersion of hydrophobic aggregates. Effect of some nonionic surfactants (Tween 40, Tween 80, and triton X-100) was tested at 0.1 and 0.5% concentrations on biotransformation of 16-DPA (0.5 mg ml^{-1}) to AD by *D. acidovorans* MTCC 3363 (Fig. 2). In this study, all the concentrations of tested surfactants negatively influenced AD accumulation.

Effect of carrier solvents. Dimethyformamide (DMF), dioxan, soy oil and glycerol were tested as carrier solvents for 16-DPA biotransformation at 0.5 mg ml^{-1} (Table IV). All the carrier solvents were used at 0.1% concentration (with respect to the medium). Dioxan was found best amongst tested carriers, 77.46 mol%

Table IV
Effect of carrier solvents on bioconversion of 16-DPA to AD by *D. acidovorans* MTCC 3363.

Carrier	Mol% conversion after incubation period (h)			
	48	72	96	120
DMF	50.33 ± 5.80	70.78 ± 5.48	70.94 ± 4.83	40.33 ± 5.86
Dioxan	61.82* ± 5.85	74.73 ± 0.97	77.46 ± 5.22	53.84* ± 4.88
Soy oil	ND	ND	ND	ND
Glycerol	48.09 ± 2.67	40.16* ± 0.79	31.80* ± 0.78	30.72$^{\#}$ ± 3.94

Data are mean of three triplicates ± standard deviation.
Volume of carrier solvents used was 1% of incubation medium.
ND = not detected.
* significance at $\alpha = 0.05$; $^{\#}$ represents significance at $\alpha = 0.1$

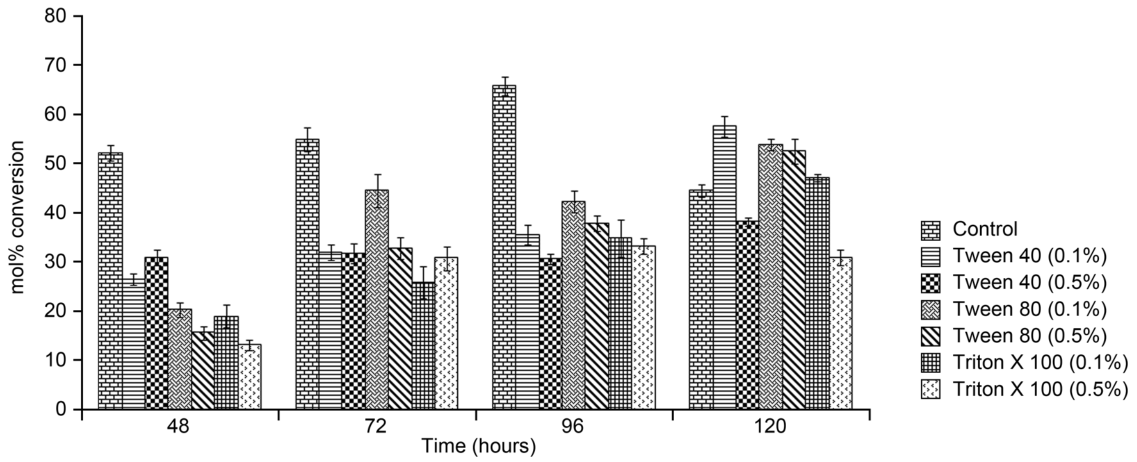

Fig. 2. Effect of some nonionic surfactants on bioconversion of 16-DPA to AD by *D. acidovorans* MTCC 3363.

biotransformation was recorded (as maximum) at 96 h. DMF yielded similar trend of AD accumulation as dioxan with slightly lower AD accumulation. Interestingly, with soy oil as carrier solvent, no biotransformation was recorded. Using glycerol as a carrier solvent resulted in peak conversion within 48 hours (48.09 mol%) which reduced on further incubation.

The effect of various concentrations of dioxan (1, 1.5 and 2%) was studied for accumulation of AD on biotransformation of 16-DPA by *Delftia acidovorans* MTCC 3363 (Fig. 3). Maximum biotransformation was recorded with 1% dioxan. Increasing the dioxan quantity resulted in decrease in AD accumulation.

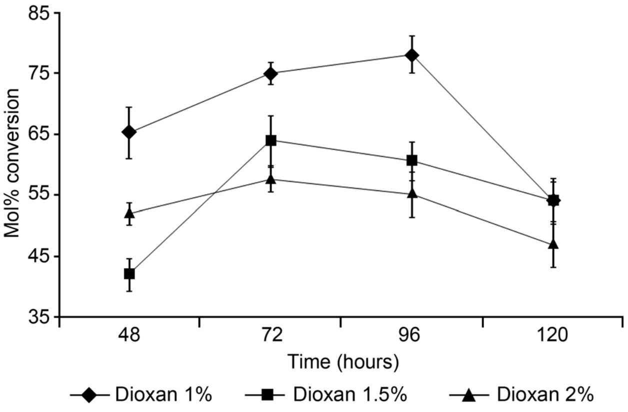

Fig. 3. Effect of concentration of dioxan on bioconversion of 16-DPA to AD by *D. acidovorans* MTCC 3363.

Identification of bioconversion product. The isolated and purified bioconversion product was identified by UV, IR spectra and co-TLC with authentic sample. UV λ_{max} 241 nm, IR (KBr) cm^{-1}, 1736 (17 – C = O), 1661.1 and 1614.8 (Δ^4 – 3 – C = O); co-TLC with authentic sample, R*f* = 0.54 ± 0.03, colour with 2% ceric ammonium sulphate in 60% sulphuric acid – Greenish blue confirmed the product to be AD (Fig. 4).

Discussion

Active hydrogen ion concentration *i.e.* pH is considered as an important factor for biological reactions. Biotransformation of 16-DPA into AD by *D. acidovorans* MTCC 3363 requires neutral pH for optimal bioconversion while slightly acidic or alkaline pH resulted in reduced biotransformation. Slightly acidic pH proved more detrimental as compared to alkaline pH as less AD was accumulated on a comparative basis. Previous reports suggest that the optimum biotransformation requires neutral to slightly alkaline pH for bacteria mediated biotransformation of steroid substrates. Shiwei and Youhua (1982) observed the maximum bioconversion of 16-dehydro-allopregnane-3-acetoxy-

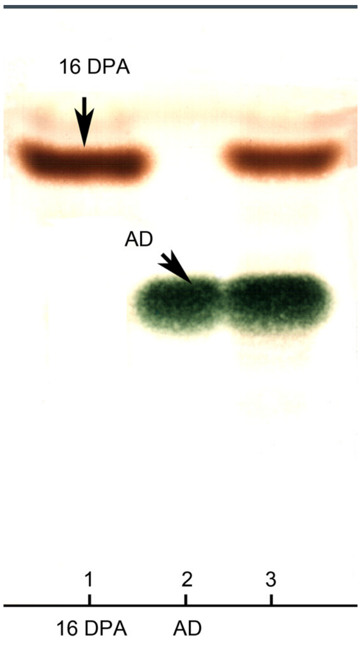

Fig. 4. Thin layer chromatography (TLC) showing authentic 16-DPA (Lane-1), AD (Lane-2) and bioconversion of 16-DPA to AD by *D. acidovorans* MTCC 3363 (Lane-3).

20-one in *p*H range of 7.0 to 8.0. Nagasawa *et al.* (1970) found that 16-dehydroprogesterone was converted only 17.5 mole% up to 48 h at pH 7.0. Viola *et al.* (1983) reported 20% conversion of pregnenolone acetate to AD along with minor quantities of testosterone, testosterone acetate and testolactone at pH 7.5.

Alongside to the pH, temperature affects the physiology of steroid biotransformation. Changes in temperature can enhance/suppress the activity of enzymes. In the present study accumulation of ADD along with AD at 35°C was recorded while at 30°C only AD was a major product. The accumulation of ADD along with AD indicates appreciable steroid 1(2)-dehydrogenase activity at 35°C. The results are in agreement with Shukla (1994), who obtained maximum accumulation of ADD at 35°C. The absence of biotransformation products at 40°C is suggestive of inactivation of steroid degrading enzymes responsible for AD and ADD bioconversion. Roy *et al.* (1991) reported rapid loss of activity of *Mycobacterium* cells on elevating the temperature from 28 to 35°C and total cessation of activity at 41°C during sitosterol side chain cleavage.

Reduced bioconversion of steroids (in terms of mol%) at high substrate concentrations is a frequently reported phenomenon attributed to the substrate toxicity (Sallam *et al.*, 1977; Roy *et al.*, 1991; Shukla 1994). In the present study, splitting the steroid substrate addition in two equal doses did not reveal higher bioconversion of 16-DPA. However, addition of substrate in two equal doses shortened the time of maximum product accumulation. When substrate is added into two equal doses, maximum product accumulation was achieved within 72 hours, while adding substrate in single bolus, has taken 96 hours for maximum accumulation of the AD. No significant change in accumulation of ADD was recorded. Increasing the substrate loading resulted in increased product accumulation but it does not reflected in linear increase in mol% conversion. The effect can possibly be attributed to increased influx of substrate (higher diffusion pressure) leading to a higher conversion, while causing the toxicity to restrict the mol% conversion to a lower value.

Zhang and Guo (2011) reported, 16-DPA being insoluble in water, hence is not highly amenable to microbial bioconversion. Although the carrier solvents may exert toxic effects on the organisms, they are routinely employed to dissolve the steroid substrate to improve its mass transfer (Fernandes *et al.*, 2003). The use of natural carrier solvents is assumed to be advantageous in terms of viability (by acting as reservoirs of substrate, restricting the exposure of cells to the toxic effects) and thus biotransformation. In this study, soy oil and glycerol were included as less toxic carrier solvents. The soy oil inhibited accumulation of AD from 16-DPA. On the other hand using glycerol as a carrier solvent resulted in less AD accumulation which further degraded. The inhibition of 16-DPA conversion in case of soy oil could be attributed to a preference of substrate utilization *D. acidovorans* MTCC 3363. However, the degradation of product with glycerol indicates towards a co-metabolism of product with the carrier.

Maximum amount of AD was accumulated with dioxan as a carrier solvent. However, at higher concentrations, dioxan adversely affected the biotransformation. The reduction in biotransformation can be attributed to toxic effects of the solvent. On the comparative scale, DMF performed better than soy oil and glycerol.

Surfactant reduces the aggregation of hydrophobic molecules and facilitates better dispersion of the substrate in the medium (Wang *et al.*, 2004; Malaviya and Gomes, 2008). In the present study reduction/delay in the biotransformation in presence of non ionic surfactants was recorded. The reduction in AD accumulation can be contributed to the toxic effects of the surfactants, primarily exerted by cell wall lipid dissolution (Wang *et al.*, 2005).

Greater amounts of detergents are needed in order to completely dissolve the sterol substrate, although it is shown by Atrat *et al.* (1992) that there is no direct correlation between detergent mediated dissociation of steroidal aggregates and the transformation activity of the bacterial cells.

The present study clearly demonstrates the unique potential of single product accumulation (AD) by *D. acidovorans* MTCC 3363. Moreover, the applicability of 16-DPA as an alternate steroid substrate to soy sterols is shown. Under optimized conditions, fairly high conversion of 16-DPA to AD have been obtained with this strain in shake flasks. Further scale up at laboratory and pilot scale fermentation levels is necessary to use the potential of the organism for commercial production of AD from 16-DPA. Moreover, faster growth rate of the organism as compared to the routinely employed *Mycobacterium* sp. for the conversion of steroids to AD might prove advantageous at industrial scale.

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ORIGINAL PAPER

Changes in the Concentration of Carbonyl Compounds during the Alcoholic Fermentation Process Carried out with *Saccharomyces cerevisiae* Yeast

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Abstract

The aim of the study was to determine the influence of the source material and the applied *S. cerevisiae* strain on the concentrations of carbonyl fractions in raw spirits. Acetaldehyde was the most common aldehyde found, as it accounted for 88–92% of the total amount of aldehydes. The concentration of acetaldehyde in maize, rye and amaranth mashes was highly correlated with fermentation productivity at a given phase of the process, and reached its highest value of 193.5 mg/l EtOH in the first hours of the fermentation, regardless of the yeast strain applied. The acetaldehyde concentration decreased over the time with the decreasing productivity, reaching its lowest value at the 72nd hour of the process. The final concentration of acetaldehyde depended on the raw material used (*ca* 28.0 mg/l EtOH for maize mashes, 40.3 mg/l EtOH for rye mashes, and 74.4 mg/l EtOH for amaranth mashes). The effect of the used yeast strain was negligible. The overall concentration of the analyzed aldehydes was only slightly higher: *ca* 30.3 mg/l EtOH for maize mashes, 47.8 mg/l EtOH for rye mashes, and 83.1 mg/l EtOH for amaranth mashes.

Key words: *Saccharomyces cerevisiae*, alcoholic beverages, fermentation technology, yeast fermentation

Introduction

Controlling the concentration of carbonyl compounds in raw spirits and other fermented alcoholic beverages is very important, because these compounds affect organoleptic features of the products and can be harmful to human health. The application of different raw materials and yeast strains not only influences the course and yield of the fermentation process, but it also affects the composition of volatile by-products. These compounds determine the sensory quality of alcoholic beverages. The accepted levels of volatile fractions in spirits and alcoholic beverages are regulated by law in many countries. Therefore the identification of all factors that can modify the composition of this group of compounds is of utmost importance. Carbonyl compounds, *i.e.* aldehydes and ketones, constitute one of the fractions that affect the sensory quality of spirits and alcoholic beverages (Biernacka and Wardencki, 2012; Cachot *et al.*, 1991; Longo *et al.*, 1992; Plutowska *et al.*, 2010). Carbonyl compounds have various fruity or floral flavours that resemble the scent of apples, lemons or nuts. They have different detection thresholds and differ in the relative influence on the organoleptic features of alcoholic beverages (Moreno-Arribas and Polo, 2009; Ribéreau-Gayon *et al.*, 2006a). Acetaldehyde can account for up to 90% of the total amount of carbonyl fraction in the spirits. Short-chain and branched aldehydes are produced by the yeast during the alcoholic fermentation process from sugars, fatty acids and amino acids (Lambrechts and Pretorius, 2000; Longo *et al.*, 1992; Moreno-Arribas and Polo, 2009).

A disturbance in the reaction of decarboxylation or reduction, caused either by a decreased availability of thiamine pyrophosphate, magnesium or zinc ions, or by the redox potential that favors rather oxidation than reduction processes, can result in an elevated concentration of acetaldehyde in the fermentation medium (Cheraiti *et al.*, 2010; Moreno-Arribas and Polo, 2009). An increased concentration of acetaldehyde can also result from an alteration in fermentation procedure parameters such as oxygenation of the medium, pH, the concentration of fermenting sugars, yeast strain, inoculum size, temperature at which the process is conducted,

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and the distillate storing conditions (Cachot *et al.*, 1991; Cheraiti *et al.*, 2010; Li and Orduña, 2011; Liu and Pilone, 2000). It has to be stressed that the presence of inhibitors of enzymatic reactions (especially those catalyzed by dehydrogenases) can result in the production of small amounts of higher aldehydes that are produced along metabolic pathways leading also to higher alcohols (Moreno-Arribas and Polo, 2009). The inhibition effect is brought about by many heavy metals and chemical compounds, including mycotoxins (Kłosowski and Mikulski, 2010; Ueno and Matsumoto, 1975).

The presence of aldehydes in the spirits and alcoholic beverages produced by the fermentation industry is determined by many of the aforementioned factors. The composition of the fermentation media, that depends on the raw material used, is crucial in this context. The production of aldehydes during the fermentation process, including acetaldehyde, is an individual feature of each yeast species and strain (Longo *et al.*, 1992; Li and Orduña, 2011; Liu and Pilone, 2000). Various species and strains of wine yeast can produce from 4 to 490 mg of aldehydes per one liter of must (Lambrechts and Pretorius, 2000; Liu and Pilone, 2000; Longo *et al.*, 1992). The application of different raw materials during the media preparation as well as changes in the concentration of fermenting sugars can increase the concentration of aldehydes in the medium (Biernacka and Wardencki, 2012; Cachot *et al.*, 1991; Li and Orduña, 2011). The elevated glucose concentration in the wine must, from 10 g/l to 240 g/l, increased the concentration of acetaldehyde by more than 100 mg/l (Li and Orduña, 2011). The application of high gravity (HG) mashes increased the acetaldehyde concentration by more than 480 mg/l of raw distillate (Mikulski *et al.*, 2014).

The aim of the study was to examine the differences in both the kinetics of carbonyl compounds production and the composition of carbonyl fraction in the distillates obtained by fermentation of various starchy materials, *i.e.* maize, rye and amaranth grain, with the use of two *S. cerevisiae* strains: D-2 and As-4. The authors also made an attempt at establishing the correlation between some fermentation parameters, such as the stage of the process and the application of various raw materials and yeast strains, and the concentration of selected aldehydes. The type of raw material used can influence the fermentation kinetics and the aldehyde concentration. Being aware of these relationships can be useful in the industrial practice, because the level of carbonyl compounds in liquors is an important quality parameter (subjected to law and industry regulations in many countries) that affects the sales price. Moreover, the assessment of the concentration of aldehyde contaminants, including acetaldehyde, in the spirits and alcoholic beverages is of utmost importance, because aldehyde contaminants are listed by the World Health Organization (WHO) among potentially carcinogenic compounds (Nascimento *et al.*, 1997).

Experimental

Materials and Methods

Raw materials. The fermentation media were prepared with the use of maize grain (Anna variety), rye grain (Danikowskie Złote variety) and amaranth seeds (*Amaranthus cruentus* L.). Rye and maize grain were purchased from Rolnas Ltd, Kotomierz, Poland. Amaranth grain was delivered by APC Kuma Ltd., Bydgoszcz, Poland. The starch concentration in the samples of maize, rye and amaranth grain, determined by Evers' polarimetric method (BS EN ISO 10520:1998), was 69.1%, 50.3% and 52.5%, respectively.

Microorganisms. Fermentation media were obtained by mixing ground grain with water (1:3.7; real extract value: 16.5° Brix) and inoculated with two distillers yeast strains: *S. cerevisiae* D-2 or As-4. These strains, originating from a collection of pure cultures of the Institute of Agricultural and Food Biotechnology, (Warsaw, Poland) are commonly used in the Polish distilling industry. Dry yeast were rehydrated by stirring in of distilled water and then added to the mashes in the amount of 1 ml/l of mash ($1.05 \pm 0.07 \times 10^9$ CFU/ml), according to the vendor recommendations.

Enzymes preparations. The enzymatic hydrolysis of starch and non-starch polysaccharides was carried out with Novozymes® (Bagsvaerd, Denmark) preparations. All mashes were prepared with the same set of enzymes applied at doses recommended by the producer. Starch liquefaction was carried out with thermostable α-amylase (Termamyl 120L), the dose was 150 ml/ton of starch. For starch saccharification an *Aspergillus* preparation SAN Super 240 l was used (1000 ml/ ton of starch). For hydrolysis of non-starch polysaccharides in the mashes, Viscozyme 120 l preparation was applied. The preparation exhibits the activity of arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. The recommended dosage was 200 ml per 1 ton of the raw material. All enzymatic preparations were applied at doses recommended by the producer.

Mash preparation. All fermentation media were prepared under laboratory conditions with the application of pressureless liberation of starch (PLS) technology. After the hydrolysis of polysaccharides, the mashes were cooled to 30°C, then inoculated with yeast cream and subjected to fermentation for 72 h at 37°C. The total amount of starch was identical in each variant of fermentation (12.9 g 100 ml of mash). The initial concentrations of starch hydrolysis products and fructose in maize, rye and amaranth mashes are presented in Fig. 1.

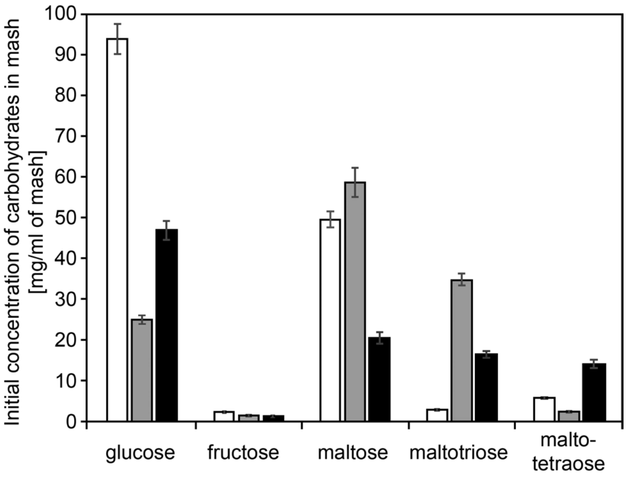

Fig. 1. The initial concentration of sugars in the fermentation media (white bars – maize mashes, grey bars – rye mashes, black bars – amaranth mashes).

Analytical methods

Analysis of the alcoholic fermentation process. Fermentation factors were defined as follows (Kłosowski *et al.*, 2010). Fermentation productivity: the amount of absolute ethanol produced in one liter of mash within 1 h (ml EtOH/l/h); fermentation yield: the amount of absolute ethanol obtained from 100 kg of starch (l/EtOH 100 kg of starch). The ethanol concentration was measured with a chromatograph by Agilent Technologies, model 1260, equipped with a refractometric detector. Chromatographic separation was carried out using a Hi-Plex H column (Agilent technologies) operating at 60°C under isocratic conditions with 5 mM H_2SO_4 as the eluent, with the flow rate of 0.6 ml/min. The ethanol concentration was determined with the use of external standards (ESTD) and an appropriate calibration curve. Integration and quantification were performed with Chem-Station LC by Agilent Technologies. The separation parameters were in agreement with the manufacturer's recommendations for examining ethanol concentration in fermentation media.

Determination of the alcoholic fermentation volatile by-products. Analysis of the aldehyde fraction was performed after 16, 24, 36, 48, 65 and 72 hours of the process. Distillate samples were obtained with a glass distillation apparatus equipped with 25 bubble-cap plates. The ethanol concentration in such obtained raw spirits was 91.0 ± 0.5% v/v. The content of aldehydes in the distillates was determined by capillary gas chromatography method using the Agilent Technologies 7890 chromatograph with FID detector on the 50-m long Agilent Technologies CP WAX 57 CB column with the internal diameter of 0.32 mm. Chromatographic separation conditions were described in Kłosowski and Mikulski (2010).

Analysis of the products of enzymatic degradation of polysaccharides. The products of enzymatic hydrolysis of polysaccharides were analyzed by high-performance liquid chromatography (HPLC) directly after enzymatic hydrolysis. Before the analysis of sugars, the fermentation media samples were diluted and filtered through a 0.44 µm filter. Chromatographic separation was carried out using a chromatograph by Agilent Technologies, model 1260, equipped with a refractometric detector, under conditions described in 2.5.1. The concentration of sugars was determined with the use of external standards (ESTD) and appropriate calibration curves. Integration and quantification were performed with Chem-Station LC by Agilent Technologies. The separation parameters followed the manufacturer's recommendations for examining the concentration of carbohydrates in fermentation media.

Statistical analysis. Statistical analysis (analysis of variance, determination of SD), was carried out using Statistica software, version 10. ANOVA and RIR Tukey's tests were applied at the significance level of $\alpha < 0.05$. The data were from three independent experiments.

Results and Discussion

Concentration of selected aldehydes in the distillates at subsequent hours of the fermentation process. The concentration of acetaldehyde, isobutyraldehyde, valeraldehyde, isovaleraldehyde, propionaldehyde in the obtained distillates at the subsequent hours of the fermentation of maize, rye and amaranth mashes was analyzed (Fig. 2, Fig. 3). Acetaldehyde was the main component of the aldehyde fraction, both at the

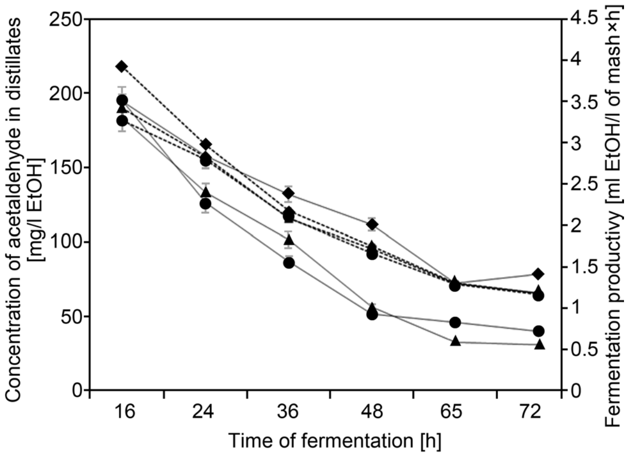

Fig. 2. Fermentation productivity (dotted line) and the concentration of acetaldehyde (solid line) in the mashes *vs* fermentation time (closed triangles – maize mashes with D-2 strain, open triangles – maize mashes with As-4 strain, closed circles – rye mashes with D-2 strain, open circles – rye mashes with As-4 strain, closed diamonds – amaranth mashes with D-2 strain, open diamonds – amaranth mashes with As-4 strain).

beginning of the process and after 72 hours of fermentation. At the 16th hour of the process, the compound accounted for almost 88% of the total amount of aldehydes. Its concentration at the first hour of the process was *ca* 193.5 mg/l EtOH in all distillates, with no statistically significant differences across the analyzed samples (Fig. 2). At the next hours of the process a decrease in the acetaldehyde concentration was observed, due to the activity of alcohol dehydrogenase (ADH) that reduced the aldehyde to ethanol. Similar changes in the concentration of acetaldehyde were also observed by other researchers (Cachot *et al.*, 1991; Li and Orduña, 2011), who indicated that the acetaldehyde reduction at the initial phase of the fermentation process is relatively less efficient in comparison to the pyruvate decarboxylation process. At the subsequent hours of the process, the accumulated acetaldehyde, a hydrogen acceptor, is gradually reduced to ethanol which results in a decrease of its concentration in the fermentation medium.

During the first 24 hours of the fermentation, the reduction of the accumulated acetaldehyde was the fastest in maize and rye media, irrespective of the yeast strain used (D-2 or As-4). After 24 hours, the acetaldehyde concentration in these media decreased by as much as 63 mg/L EtOH as compared to the concentration of 192 mg/l EtOH recorded at the 16th hour of the process (Fig. 2). This downward trend lasted till the end of the fermentation. It was also observed that the concentration of acetaldehyde depended on the yeast strain used. The differences found were statistically significant. The lowest final acetaldehyde concentration, *ca* 26.1 ± 0.4 mg/l EtOH, was reported for maize spirits obtained with As-4 yeast strain (Fig. 2). The final acetaldehyde concentration in rye spirits reached the level of *ca* 40.3 mg/l EtOH, with no statistically significant differences across the rye mashes analyzed. The highest final acetaldehyde concentration, more than 70 mg/l, was in amaranth spirits, irrespective of the yeast strain used (Fig. 2). The results suggest that the yeast metabolism, especially the kinetics of acetaldehyde concentration, can be modified by the available medium components. Such relationships for rye distillates were shown by other authors. It was demonstrated that the concentration of particular volatile by-products can be significantly changed even by the rye variety used (Pietruszka and Szopa, 2014).

Similar changes in the acetaldehyde concentration were reported by Cachot *et al.* (1991), who observed the highest acetaldehyde concentration (exceeding 340 mg/l EtOH) during the first 8 hours of alcoholic fermentation of cane molasses. The concentration decreased to *ca* 20 mg/l EtOH at the 24th hour of the process. Li and Orduña (2011) also observed a downward trend in the acetaldehyde concentration during the subsequent hours of the alcoholic fermentation. These authors also reported significant differences in the acetaldehyde concentration in the media fermented with various *S. erevisiae* strains. The ongoing acetaldehyde reduction was also observed during the alcoholic fermentation in the wine making procedure, but because of a lower temperature of the process, the reduction reaction was slower and could take more than ten days (Pan *et al.*, 2011).

The analysis of distillates at subsequent hours of the fermentation process revealed that the concentration of isobutyraldehyde, propionaldehyde, valeraldehyde and isovaleraldehyde in the spirits was decreasing over time. This phenomenon can be explained by the ongoing reduction of the aldehydes to their alcohol counterparts, *e.g.* propionaldehyde to propanol, isobutyraldehyde to isobutanol and isovaleraldehyde to isoamyl alcohol (Nykänen and Suomalainen, 1983). In the examined samples of spirits obtained from maize media the concentration of isobutyraldehyde decreased from *ca* 8 mg/l EtOH at the 16th hour of the fermentation to 1 mg/l EtOH at the 72nd hour of the process (Fig. 3A), regardless of the yeast strain applied. In spirits obtained from rye mashes, the isobutyraldehyde concentration was *ca* 4 mg/l EtOH and did not change much during the whole fermentation process, regardless of the yeast strain used (D-2 or As-4). In the distillates obtained from the amaranth media, isobutyraldehyde concentration decreased during the fermentation by *ca* 2.5 mg/l EtOH in comparison with that measured at the16th hour of the process, but then it remained at a higher level of *ca* 5 mg/l EtOH (Fig. 3A). The analysis of isovaleraldehyde concentrations at the 16th hour of fermentation revealed significant differences between distillates from media prepared with different raw materials. An elevated concentration of the aldehyde was reported for maize and rye spirits. The highest concentration of isovaleraldehyde, *ca* 15 mg/l EtOH, was observed at the 16th hour of the fermentation in the maize spirits, slightly lower, *ca* 12.5 mg/l EtOH, was reported for rye spirits (Fig. 3B), irrespective of the yeast strain applied. However, in both maize and rye spirits the isovaleraldehyde concentration decreased during the subsequent hours, and at the 72nd hour of the process the aldehyde was fully reduced (Fig. 3B). In amaranth distillates neither valeraldehyde nor isovaleraldehyde was detected (Fig. 3B, C). The presence of both aldehydes in maize and rye distillates is associated with the metabolism of leucine that is present in the fermentation media. This amino acid is deaminated *via* Ehrlich pathway to a corresponding α-keto acid, then decarboxylated to isovaleraldeyde, and reduced to 3-methyl-1-butanol (Ribéreau-Gayon *et al.*, 2006b).

Spirits obtained from rye mashes had the highest initial valeraldehyde concentration, *ca* 19 mg/l EtOH, irrespective of the yeast strain used (Fig. 3C). Similarly

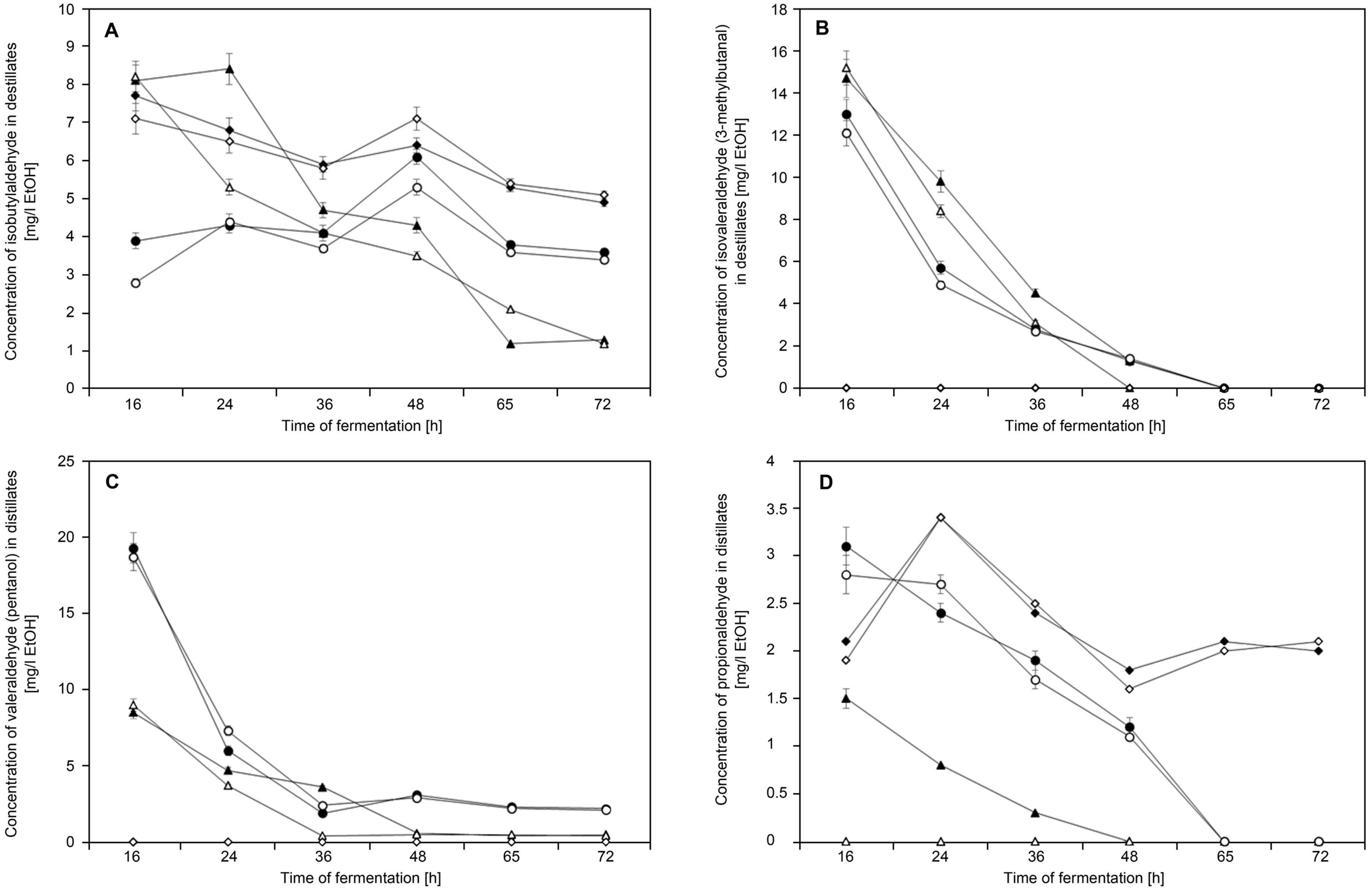

Fig. 3. The concentration of selected aldehydes in the obtained distillates at the subsequent hour of the alcoholic fermentation: **A** – isobutyraldehyde, **B** – isovaleraldehyde, **C** – valeraldehyde, **D** – propionaldehyde (closed triangles – maize mashes with D-2 strain, open triangles – maize mashes with As-4 strain, closed circles – rye mashes with D-2 strain, open circles – rye mashes with As-4 strain, closed diamonds – amaranth mashes with D-2 strain, open diamonds – amaranth mashes with As-4 strain).

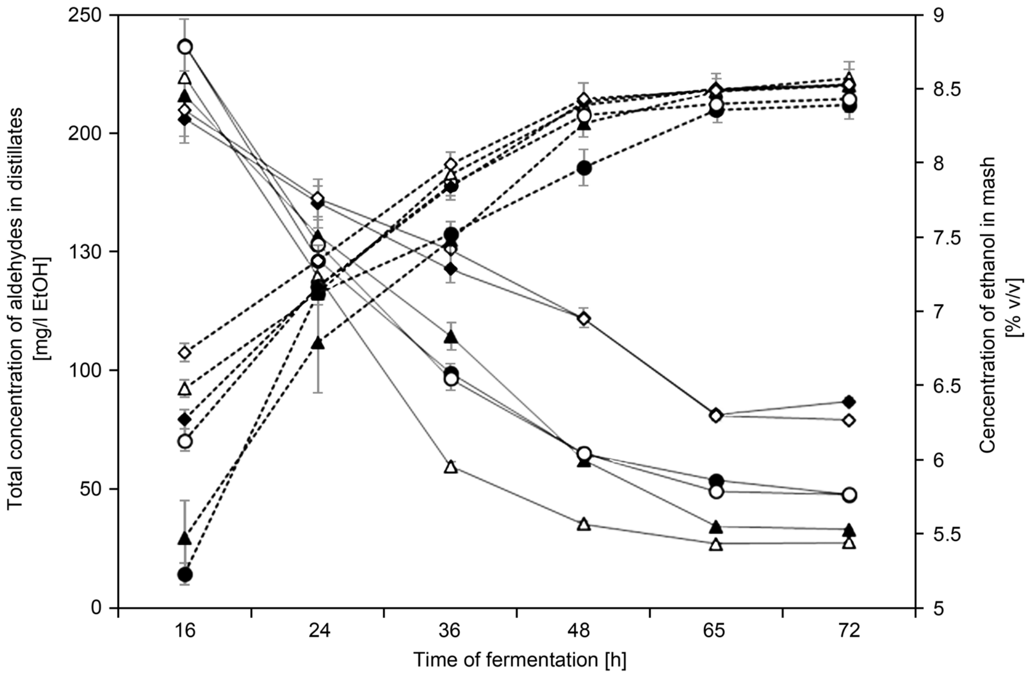

Fig. 4. The overall concentration of aldehydes in the spirits (solid line) and the ethanol concentration in the mashes (dotted line) during the alcoholic fermentation process (closed triangles – maize mashes with D-2 strain, open triangles – maize mashes with As-4 strain, closed circles – rye mashes with D-2 strain, open circles – rye mashes with As-4 strain, closed diamonds – amaranth mashes with D-2 strain, open diamonds – amaranth mashes with As-4 strain).

to the isovaleraldehyde, the concentration of valeraldehyde in rye and maize spirits was decreasing over the fermentation time, but its concentration at the 72nd hour of the process was 2 mg/l EtOH, irrespective of the yeast strain applied, so the aldehyde was not fully reduced at the end of the process (Fig. 3C).

All analyzed spirit samples had a low concentration of propionaldehyde (Fig. 3D). In the distillate obtained from a maize mash, propionaldehyde was not found at all (in samples inoculated with As-2 strain), or it was detected only at the 16th hour of the fermentation process at the concentration of 1.5 mg/l EtOH (in samples inoculated with D-2 strain) (Fig. 3D). The concentration of propionaldehyde in rye distillates decreased from 3 mg/l EtOH at 16th hour of the fermentation to zero at 72nd hour, regardless of the yeast strain applied (Fig. 3D). In amaranth distillates the propionaldehyde concentration was *ca* 2 mg/l EtOH and constant over the time, regardless of the yeast strain used.

The effect of alcohol fermentation kinetics on the total concentration of aldehydes in the obtained distillates. A correlation between the fermentation productivity and the overall concentration of aldehydes was observed, including acetaldehyde as the main component of the aldehyde fraction. The highest ethanol concentration, fermentation productivity and yield at the initial phase of the process were associated with the elevated concentration of aldehydes, especially acetaldehyde. It must be stressed, however, that statistically significant differences in productivity between different yeast strains were observed at the beginning of the fermentation process and were not accompanied by significant differences in the acetaldehyde concentration. At this fermentation stage the productivity of As-4 yeast strain was statistically higher than that of D-2 strain, regardless of the raw material used, but no significant differences in the acetaldehyde concentration were found (Fig. 2, Fig. 4, Table I). At the 16th hour of the fermentation, in maize and rye mashes inoculated with As-4 the yield was higher by ca. 7.4 l EtOH/100 kg of starch in comparison to that of the mashes inoculated with D-2 strain (Table I). In the following hours of the fermentation process the fermentation activity of D-2 was rising so that at the 65th hour of the process no statistically significant differences in the yield between the two strains were observed. At this stage of the process no differences in the yield between different raw materials were found (Fig. 4, Table I). At the 72nd hour of the alcoholic fermentation the ethanol concentration, the fermentation yield and the ratio of the actual yield to the theoretical one were similar in all media analyzed, reaching 8.49% v/v, 65.8 l EtOH/100 kg of starch, and 91.6%, respectively (Fig. 4, Fig. 5, Table I).

During the subsequent hours of the process, the concentration of the analyzed aldehydes decreased with decreasing fermentation yield, as an effect of the ongoing reduction of aldehydes to their corresponding alcohols (Nykänen and Suomalainen, 1983). Although no statistically significant differences in the parameters of the fermentation process were observed at

Table I
Fermentation yield at subsequent hours of the alcoholic fermentation process.

Raw material	Yeast strain	Fermentation yield [l EtOH/100kg of starch] at subsequent hours of the alcoholic fermentation process					
		16	24	36	48	65	72
Maize grain	D-2	42.35a ± 1.94	52.57ac ± 2.63	57.91a ± 0.62	64.03abc ± 0.70	65.66a ± 0.62	65.96a ± 0.85
	As-4	50.17bd ± 0.46	55.13abc ± 0.62	61.32b ± 0.85	64.96b ± 1.01	65.73a ± 0.62	66.35a ± 0.85
Rye grain	D-2	40.50a ± 0.54	52.03c ± 0.62	58.23a ± 0.62	61.71c ± 0.93	64.73a ± 0.70	64.96a ± 0.77
	As-4	47.46c ± 0.54	55.52ad ± 0.77	60.78b ± 0.54	64.42ab ± 0.77	65.04a ± 0.85	65.27a ± 0.70
Amaranth grain	D-2	48.56bc ± 0.46	55.45ae ± 0.46	60.72b ± 0.70	65.13ab ± 1.01	65.83a ± 0.77	65.99a ± 0.70
	As-4	52.04d ± 0.46	56.85bde ± 0.77	61.88b ± 0.62	65.29ab ± 0.77	65.75a ± 0.70	66.06a ± 0.70

The mean values given in columns with different letter index are significantly different ($\alpha < 0.05$).

the 72nd hour of the process, the concentration of all analyzed aldehydes differed significantly across the source materials used (Fig. 4). The lowest final concentration of aldehydes was found in the distillates obtained from maize media, regardless of the yeast strain used (D-2 or As-4). The aldehyde concentration in the maize media was *ca* 30.3 mg/l EtOH and it was lower than that observed in rye and amaranth distillates by *ca* 17.5 mg/l EtOH and 52.8 mg/l EtOH, respectively. The results justify the conclusion that the application of different yeast strains does not considerably influence the final acetaldehyde concentration. The most pronounced differences in the acetaldehyde concentration was observed between samples taken at different process stages. The final acetaldehyde concentration was associated with the type of source material used. This effect can be caused by different availability of minerals that are enzyme cofactors, such as zinc and magnesium, in the source material. The active site of alcohol dehydrogenase contains a bound zinc atom. Magnesium and thiamine pyrophosphate are cofactors of pyruvate decarboxylase. Different concentrations of Zn and Mg cations in the maize, rye and amaranth grain can affect the activity of ADH and pyruvate decarboxylase and thus influence the acetaldehyde concentration in the distillates (Lorenz and Wright, 1984; Mikulski and Kłosowski, 2015; Moreno-Arribas and Polo, 2009; Ribéreau-Gayon *et al.*, 2006b).

Conclusions

A general relationship was observed: there was a correlation between the ethanol productivity and the acetaldehyde concentration at a given fermentation stage. A high productivity at the initial phase of the process is accompanied with an elevated acetaldehyde concentration. The drop in the productivity at the final phase of the fermentation is correlated with a decrease in the concentration of acetaldehyde. Therefore, should any disturbing factors prematurely terminate or

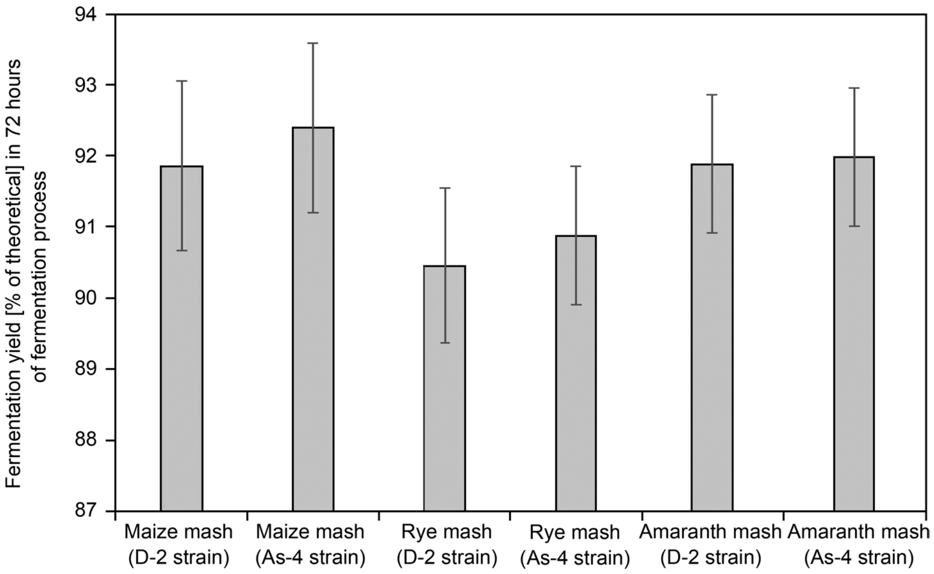

Fig. 5. The ratio of the actual yield of the alcoholic fermentation to the theoretical one for all mashes analyzed at the 72nd hour of the process.

considerably shorten the technological process of the alcoholic fermentation, an elevated concentration of carbonyl compounds would have to be expected. The final concentration of acetaldehyde depends significantly on the type of source material used, without any noticeable influence of the applied yeast strain.

Similar relationships for the other aldehydes (propionaldehyde, valeraldehyde, isovaleraldehyde) are much more difficult to find. These compounds are also products of yeast metabolism, but they are not directly associated with the main alcoholic fermentation pathway. However, a certain trend can be observed: the concentration of these aldehydes in the distillate samples taken at subsequent fermentation stages gradually decreases over the time.

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ORIGINAL PAPER

The Ability of a Novel Strain *Scheffersomyces* (Syn. *Candida*) *shehatae* Isolated from Rotten Wood to Produce Arabitol

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Abstract

Arabitol is a polyalcohol which has about 70% of the sweetness of sucrose and an energy density of 0.2 kcal/g. Similarly to xylitol, it can be used in the food and pharmaceutical industries as a natural sweetener, a texturing agent, a dental caries reducer, and a humectant. Biotechnological production of arabitol from sugars represents an interesting alternative to chemical production. The yeast *Scheffersomyces shehatae* strain 20BM-3 isolated from rotten wood was screened for its ability to produce arabitol from L-arabinose, glucose, and xylose. This isolate, cultured at 28°C and 150 rpm, secreted 4.03 ± 0.00 to 7.97 ± 0.67 g/l of arabitol from 17–30 g/l of L-arabinose assimilated from a medium containing 20–80 g/l of this pentose with yields of 0.24 ± 0.00 to 0.36 ± 0.02 g/g. An optimization study demonstrated that pH 4.0, 32°C, and a shaking frequency of 150 rpm were the optimum conditions for arabitol production by the investigated strain. Under these conditions, strain 20BM-3 produced 6.2 ± 0.17 g/l of arabitol from 17.5 g/l of arabinose after 4 days with a yield of 0.35 ± 0.01 g/g. This strain also produced arabitol from glucose, giving much lower yields, but did not produce it from xylose. The new strain can be successfully used for arabitol production from abundantly available sugars found in plant biomass.

Key words: *Scheffersomyces shehatae*, arabitol production, biotechnological process optimization, biotransformation, yeast identification

Introduction

The pentitol L-arabitol, similarly to its enantiomer xylitol, has been included in the list of the top twelve biomass-derivable chemicals designated for further biotechnological research (Erickson *et al.*, 2012). Just like xylitol, arabitol can be used as a natural sweetener that offers a number of health benefits, including its ability to reduce dental caries and prevent the formation of adipose tissue and accumulation of fat in the digestive tract (Mingguo *et al.*, 2011). Because arabitol is a low-calorie product (only 0.2 kcal/g) with low glycemic and low-insulinemic indices and anticariogenic and prebiotic effects (Koganti *et al.*, 2011), it is an excellent sugar substitute for diabetic patients. This polyol can also be used as a texturing agent, a humectant, a softener, and a colour stabilizer in the production of foods and pharmaceuticals (Kumdam *et al.*, 2013). Biotechnological production of arabitol from monosaccharides, such as L-arabinose and glucose, or from waste substrates (*e.g.* glycerol) by yeasts such as *Candida* spp., *Pichia* spp., *Debaryomyces* spp., *Wickerhamomyces* spp., and *Saccharomycopsis* spp. represents an efficient and cost-effective alternative to chemical production (Saha and Bothast, 1996; Koganti *et al.*, 2011).

Among the yeasts screened for their ability to produce arabitol from L-arabinose, some of the most frequently reported are those from the genus *Candida* (McMillan and Boynton, 1994; Saha and Bothast, 1996; Kordowska-Wiater *et al.*, 2008). McMillan and Boynton (1994), for example, showed that L-arabinose was metabolized to arabitol, among others, by the yeasts *Candida shehatae* and *Candida tropicalis*, with the latter being the best producer of arabitol, giving a yield of 1.02 g/g during cultivation in a medium containing yeast nitrogen base and arabinose. In a screening study of 49 L-arabinose-utilizing yeast strains, Saha and Bothast (1996) demonstrated that *Candida entomaea* NRRL Y-7785 was a superior secretor of L-arabitol (a yield of about 0.7 g/g). Kordowska-Wiater *et al.*

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(2008) reported that *Candida parapsilosis* DSM 70125 and *C. shehatae* ATCC 22984 produced arabitol at yields of 0.28–0.78 g/g and 0.25–0.5 g/g, respectively, depending on the shaking speed (100–200 rpm), in a medium containing 20 g/l of L-arabinose and yeast- and malt extracts. Watanabe *et al.* (2010) selected from over 1600 yeast strains, a single strain, closely related to *Candida subhashii*, designated NY7122, which was able to produce L-arabitol and ethanol from L-arabinose as the sole carbon source (Watanabe *et al.*, 2010).

C. shehatae (syn. *Scheffersomyces shehatae*) is mainly known as a yeast able to assimilate the pentose D-xylose and metabolise it to ethanol or xylitol, especially in processes that use hemicellulosic hydrolysates (Girio *et al.*, 1989; Jeffries and Kurtzman, 1994; Tanimura *et al.*, 2012; Antunes *et al.*, 2014). Kurtzman and Suzuki (2010) have proposed the new genus *Scheffersomyces* to accommodate some *Pichia* and *Candida* species (among others *C. shehatae*) connected with woody habitats on the basis of phylogenetic relationships among these species. *S. shehatae* is an environmental species isolated from wood and soil. Apart from D-xylose, it also ferments glucose, galactose and trehalose (Kurtzman *et al.*, 2011). Its fermentative effectiveness depends on oxygen availability, with optimum levels of ethanol and xylitol production being achieved under oxygen-limited and anaerobic conditions. The ability of *S. shehatae* to produce arabitol from pentoses and hexoses has been much less investigated.

In the present study, eleven yeast strains isolated from rotten wood were screened for their ability to assimilate L-arabinose. A single strain which was found to be able to produce arabitol from L-arabinose and glucose was subjected to molecular identification and morphological and biochemical characterization, and tested for arabitol production efficiency.

Experimental

Material and Methods

Isolation, preliminary identification and maintenance of the yeast isolates. Yeast strains were isolated from samples of rotten wood (as a source of pentose sugars) collected from Polish forests. Five grams of wood sample was shaken with 45 ml of physiological saline in Erlenmeyer flasks on a reciprocal shaker for 10 min. The suspensions were diluted decimally and plated onto Petri dishes containing YGC agar (yeast extract 5 g/l, glucose 20 g/l, chloramphenicol 0.1 g/l, agar 15 g/l) (BTL, Łódź, Poland). The Petri dishes were incubated at 28°C for 5 days, and the single colonies of yeasts obtained were transferred separately to new Petri dishes with YGC agar and incubated. The purification procedure was repeated twice. The microorganisms forming colonies were identified microscopically, then inoculated onto YPG agar slants (yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/l, agar 20 g/l) (BTL, Łódź, Poland) and, after incubation at 28°C for 3 days, they were maintained at 4°C. They were deposited in the Culture Collection of the Department of Biotechnology, Human Nutrition and Science of Food Commodities of the University of Life Sciences in Lublin, Poland. *C. shehatae* ATCC 22984 was used as a reference strain.

Screening of arabinose-assimilating yeasts. Yeast isolates were screened using a modified method by Subtil and Boles (2011). Petri dishes with selective YNB-A agar (yeast nitrogen base 6.7 g/l, L-arabinose 20 g/l, agar 20 g/l) were inoculated with ten microlitres of three decimal dilutions of all the yeasts studied. The Petri dishes were incubated at 28°C for 5 days. Strains which showed growth on YNB-A were selected for the second stage of the screening procedure.

Optimization experiment of arabitol production from arabinose. The inoculation medium was composed of L-arabinose 20 g/l, yeast extract 3 g/l, malt extract 3 g/l, $(NH_4)_2SO_4$ 5 g/l, and KH_2PO_4 3 g/l, pH 5.5. The cultivation medium was composed of L-arabinose 20 g/l, 50.0 g/l, or 80.0 g/l, yeast extract 3 g/l, malt extract 3 g/l, $(NH_4)_2SO_4$ 5 g/l, and KH_2PO_4 3 g/l. The pH was adjusted to 5.5. In the study of the effect of pH on arabitol production, the pH of the medium was adjusted to 3.5, 4.0, 4.5, 5.0, or 6.0.

For each strain, a loopful of cells from a slant was transferred into a tube with the inoculation medium and incubated at 28°C for 24 h. Then, the cultivation medium was inoculated with 2% (v/v) of the inoculum and incubated in a rotary shaker (Infors HT Minitron, Infors AG, Switzerland) at 150 rpm and 28°C for 4 or 5 days. A study of the effect of rotational speed on arabitol production was conducted at shaker speeds of 100 and 200 rpm. The impact of temperature was investigated by incubating yeast cells at 24 and 32°C. On the basis of the results obtained from the optimization experiments, a verification experiment was carried out in inoculation and cultivation media containing 20 g/l of arabinose under the following conditions: pH 4.0, 32°C, and 150 rpm. Incubation was continued for four days The samples were collected to measure pH, biomass concentration, L-arabinose utilization, and production of L-arabitol, and ethanol (where applicable) every 24 h. The biotransformation experiment was performed in triplicate.

Cultivation experiment of arabitol production from glucose or xylose. The inoculation medium was composed of D-glucose 50 g/l, yeast extract 3 g/l, malt extract 3 g/l, $(NH_4)_2SO_4$ 2 g/l, and KH_2PO_4 3 g/l. The cultivation medium was composed of glucose 50 g/l or 100 g/l, yeast extract 3 g/l, malt extract 3 g/l, $(NH_4)_2SO_4$

2 g/l, and KH_2PO_4 3 g/l. The medium for D-xylose metabolism was composed of D-xylose 20 g/l, yeast extract 3 g/l, malt extract 3 g/l, $(NH_4)_2SO_4$ 5 g/l, and KH_2PO_4 3 g/l. The pH of all the media was adjusted to 5.5. The inoculum and productive cultures were prepared as above. The cells were cultivated for 5 days at 28°C and 150 rpm. The cultures were analysed for pH, biomass concentration, D-glucose or D-xylose utilization, and production of arabitol and other products (glycerol, ethanol, xylitol, and ribitol) every 24 h. The cultivation experiment was performed in triplicate.

Morphological characterization. For macroscopic morphology observations, the yeast strains were grown on YPG agar (BTL, Łódź, Poland) at 28°C for 2–4 days. Intravital microscopy imaging was performed at a magnification of 1000 × with a Delta Optical Evolution 300 optical microscope (Delta Optical, Poland) equipped with an HDCE-50B camera using ScopeImage Dynamic Pro (Delta Optical, Poland) software. Then, the colonies and the cells were characterized according to Yeasts of the World software (ETI-Biodiversity Center, Amsterdam, Netherlands) and Kurtzman *et al.* (2011).

Biochemical characterization and identification of the yeast strains by the Biolog System™. The Biolog System™ (Biolog YT MicroPlate™; Biolog Inc. Hayward, CA, USA) is designed for classification of yeasts on the basis of their carbon source utilisation profiles (assimilation of 59 substrates and oxidation of 35 substrates). The system used in this study was equipped with a multichannel pipette, a computer-linked absorbance and turbidity growth reader, and Biolog Microlog System 3 (5.2) software for data management. Before use, the selected yeast strain was cultured on Biolog Universal Yeast Agar (BUY™) and incubated at 25°C for two days. Next, cells were removed from agar surface with a sterile swab and suspended in sterile water at a specified density (47% T). One hundred microliters of the cell suspension was inoculated into each of the 96 wells of the Biolog YT MicroPlate, which was then incubated in the dark at 27°C for 72 h. The data were recorded with a plate reader at 590 nm every 24 h up to 72 h. For identification, the MicroPlate was read in the MicroStation™ reader and compared to the YT database. The percent of total carbon source utilisation response following oxidation (metabolism) tests (%) was calculated using optical density (OD 590 nm) data for each well, corrected by subtracting the values for the blank well (inoculated, but without a carbon source). The carbon substrates were divided into the following groups: amino acids, carbohydrates, carboxylic acids, polymers, polyalcohols, and miscellaneous. For each series, the corrected absorbance values of a particular group of substrates were summed up and expressed as percent of the average absorbance value of this substrate group for selected yeast strain.

For assimilation tests, turbidity was measured in wells containing carbon substrates assembled into the following groups: carbohydrates, carboxylic acids, polymers, polyalcohols, glycosides, miscellaneous, and others. The rate of assimilation of each substrate was measured by optical density (OD 590 nm), and positive, negative and partial responses were recorded as +, –, and +/– respectively.

Genetic identification. DNA was extracted from yeast cells (20–30 mg) which had been transferred to 2-ml Eppendorf tubes and disintegrated by grinding in liquid nitrogen to a fine powder. DNA was obtained using a Plant and Fungi DNA Purification Kit (EURx, Poland), according to the manufacturer's instructions. After spectrophotometric analysis of DNA purity and concentration (NanoDrop, ThermoScientific, USA), DNA samples were stored at –20°C. ITS1 (5-TCCG-TAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTC-CGCTTATTGATATGC-3') primers were used for the identification of the yeasts according to White *et al.* (1990). PCR reactions were run on a SensoQuest Labcycler (SensoQuest GmbH, Germany) in a 25 μl volume using 2 × PCR Master Mix (Thermo Scientific Fermentas, Lithuania) with 20 pmol of each primer and 20 ng of DNA. Thermal cycling conditions were as follows: an initial step at 95°C for 3 min followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and finally 72°C for 8 min. Then, the amplification product obtained after electrophoresis in a 1.5% (wt/v) agarose gel with ethidium bromide in 1 × TBE was visualised using the GelDoc 2000 gel documentation system (Bio-Rad, USA), and the size of the PCR product was determined using the GeneRuler 100 bp plus DNA Ladder (Thermo Scientific Fermentas, Lithuania).

The purified PCR product obtained using ITS1 and ITS4 primers was directly sequenced at Genomed, Poland, by the Big dye Terminator method using an ABI 3730xl Genetic Analyzer. The DNA sequence obtained was compared with the sequences found in the NCBI GenBank database and then submitted to NCBI GenBank under accession number KP783503.1.

Analysis of sugars and polyols. Samples of cultures were centrifuged at 9 000 × g for 15 min, and the supernatants were used for HPLC analysis. In each sample, after deproteinization by acetonitrile, the concentrations of L-arabinose, glucose, xylose, arabitol, glycerol, ribitol, and xylitol were determined using a chromatograph from Gilson Inc. (Middleton, WI, USA) equipped with a Bio-Rad Aminex Carbohydrate HPX 87H column (Bio-Rad Laboratories Inc., Hercules, CA, USA), and a refractive index detector (Knauer GmbH, Berlin, Germany). 0.05 M sulphuric acid was used as the mobile phase at a flow rate of 0.5 ml/min, and the temperature of separation was 42°C. Chromax 2007 software version 1.0a (Pol-lab, Poland) was used for the integration and analysis of chromatograms. Qualitative and quantitative analysis of the examined substrates and products was performed by comparison of peaks' areas and retention times with those obtained for pure chemicals.

Arabitol (glycerol, ribitol) yield was calculated as grams of product per grams of sugar consumed. Productivity was calculated as grams of product per grams of sugar consumed per hour.

Ethanol analysis. The deproteinised supernatants were analysed using a gas chromatograph/mass spectrometer (GC/MS) (Model GC2010, Shimadzu, Japan) coupled with an MS-EI apparatus (model QP 2010Plus, Shimadzu, Japan) and an auto injector (model AOC-20i, Shimadzu, Japan). The gas chromatograph was equipped with a 25 m × 0.32 mm (0.3 μm film thickness) CP-WAX 57 CB (Agilent J&W) column operated in the splitless mode with the valve closed for 0.3 min. The carrier gas was helium at a flow rate of 1.8 ml/min. The injector and detector temperature was 200°C. The column oven temperature was set at 50°C for 6 min, then increased at a rate of 3°C/min to 120°C and held for 3 min, and finally raised at a ramp rate of 15°C/min to 190°C, and held for 2 min. Data were acquired in the SCAN-mode (20–400 m/z). GC peaks were identified by comparing the MS fragmentation pattern and the relative retention time with those of the reference compounds. Quantitative determination of a constituent was performed on the basis of the calibration curve of the dose-peak area of a corresponding pure compound. Ethanol yield and productivity were calculated in the same way as arabitol yield and productivity.

Biomass and pH measurement. The biomass was determined by measuring the optical density (OD) at 600 nm using a BioRad Smart Spec Plus spectrophotometer (BioRad, USA). Then, the relationship between OD and dry cell weight was calculated on the basis of a previously prepared calibration curve. The biomass yield was calculated as grams of dry cell weight per grams of sugar consumed. pH of the cultures was monitored every day using an electronic pH-meter (Hanna Instruments, Poland).

Statistical analysis. Data on arabitol production by the investigated yeasts were expressed as mean ± standard deviation. Differences among mean arabitol concentrations, yields, and productivities were tested for statistical significance at $p < 0.05$ using the analysis of variance and Fisher's test for univariate groups (STATISTICA 8.0, StatSoft Inc., Tulsa, USA).

Results and Discussion

Identification and characterization of yeast strains. During the isolation procedure, eleven yeast strains was obtained. It was shown that only four isolates were able to assimilate L-arabinose on YNB-A agar, and only one strain, designated 20BM-3, produced arabitol from arabinose in liquid medium at a yield above 0.2 g/g. This strain was characterized morphologically and biochemically. After three days of incubation on YPG agar, strain 20BM-3 formed 3 mm-diameter colonies which were white-cream, convex, smooth, glistening and butyrous with a smooth edge (Fig. 1). The cells of this strain were round and oval (2–4 × 3.5–6 μm) with multilateral budding. They occurred singly or in pairs and started to form a pseudomycelium (Fig. 2).

In the genetic identification procedure, the region containing the 3' end of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and the 5' end of 26S rDNA was amplified by PCR using ITS1 and ITS4 primers. On the basis of the DNA

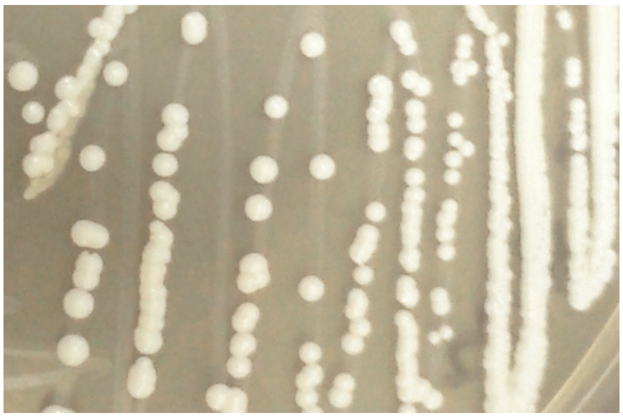

Fig. 1. Colonies of *S. shehatae* strain 20BM-3 on YPG agar.

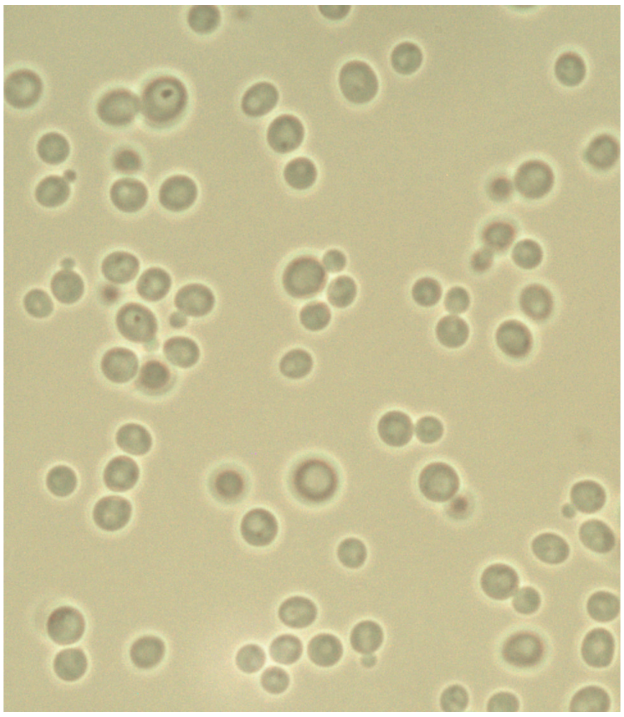

Fig. 2. Morphology of cells of the yeast *S. shehatae* strain 20BM-3, 1000×.

Table I
Ability of *S. shehatae* strain 20BM-3 to assimilate different substrates acc. the Biolog system after 48 h.

Group of substrates	Carbon substrate	Result	Group of substrates	Carbon substrate	Result
Carbohydrates	D-Cellobiose	(+)	Miscellaneous	Succinic Acid Methyl Ester + D-Xylose	(+/–)
	Gentiobiose	(+/–)		N-Acetyl-L-Glutamic Acid + D-Xylose	(+/–)
	Maltose	(+)		Quinic Acid + D-Xylose	(+/–)
	Maltotriose	(+)		D-Glucuronic Acid + D-Xylose	(–)
	D-Melezitose	(+/–)		Dextrin + D-Xylose	(+/–)
	D-Melibiose	(+/–)		α-D-Lactose + D-Xylose	(+/–)
	Palatinose	(+)		D-Melibiose + D-Xylose	(–)
	D-Raffinose	(+/–)		D-Galactose + D-Xylose	(+/–)
	Stachyose	(+/–)		m-Inositol + D-Xylose	(+/–)
	Sucrose	(+/–)		1,2 Propanediol + D-Xylose	(–)
	D-Trehalose	(+)		Acetoin + D-Xylose	(–)
	Turanose	(+/–)	Others	Succinic Acid Mono-Methyl Ester	(–)
	α-D-Glucose	(+)		N-Acetyl-D-Glucosamine	(+/–)
	D-Galactose	(+)		D-Glucosamine	(–)
	D-Psicose	(+/–)		Tween 80	(+)
	L-Rhamnose	(+/–)	Polyalcohols	Maltitol	(+/–)
	L-Sorbose	(+/–)		D-Mannitol	(+/–)
	L-Arabinose	(–)		D-Sorbitol	(+/–)
	D-Arabinose	(–)		Adonitol	(+/–)
	D-Ribose	(–)		D-Arabitol	(+/–)
	D-Xylose	(+/–)		Xylitol	(+/–)
Carboxylic acids	Fumaric Acid	(+/–)		i-Erythritol	(+/–)
	L-Malic Acid	(+/–)		Glycerol	(+/–)
	Bromo-Succinic Acid	(–)	Polymers	Dextrin	(+/–)
	L-Glutamic Acid	(+/–)		Inulin	(+)
	Amino-Butyric Acid	(+/–)	Glycosides	α-Methyl-D-Glucoside	(+/–)
	α-Keto-Glutaric Acid	(+/–)		β-D-Methyl-Glucoside	(+/–)
	2 Keto-D-Gluconic Acid	(+)		Amygdalin	(–)
	D-Gluconic Acid	(+/–)		Arbutin	(–)
				Salicin	(+/–)

sequence of the amplified product (656 basepairs), which was compared to DNA sequences in NCBI GenBank, the isolate 20BM-3 was identified as *Scheffersomyces shehatae* (syn. *C. shehatae*).

Identification performed by the Biolog system on the basis of the growth profile called the metabolic fingerprint confirmed that isolate 20BM-3 belonged to the genus *Candida*. The Biolog system was additionally used as a rapid and convenient tool for detailed biochemical characterization of the investigated yeast. Table I summarizes the results of the carbon source assimilation tests for the isolated strain, and Fig. 3 shows the results of the oxidation tests. Some scientists (Praphailong *et al.*, 1997; Foshino *et al.*, 2004; Wang *et al.*, 2008) have used the Biolog system for the identification of marine yeasts and yeast strains in food and beverages. It should be noted that there are several differences between the metabolic profile of *S. shehatae* obtained using the Biolog system and the biochemical characteristics of this yeast according to Kurtzman *et al.* (2011) and Yeasts of the World software. These differences, regarding the assimilation of pentoses, especially L-arabinose and D-xylose, are probably due to insufficient aeration of the Biolog plates. As the screening experiment demonstrated, the strain reported here was able to assimilate L-arabinose and D-xylose, but it needed oxygen and growth factors supplied in the cultivation medium. The key role of aeration in arabinose assimilation has been highlighted by Fonseca *et al.* (2007). On the other hand, there are some reports about strains of *S. shehatae* not able to assimilate arabinose (Antunes *et al.*, 2014).

Arabitol production from L-arabinose. The screening procedure using the medium containing 20 g/l of L-arabinose suggested that the isolate identified as

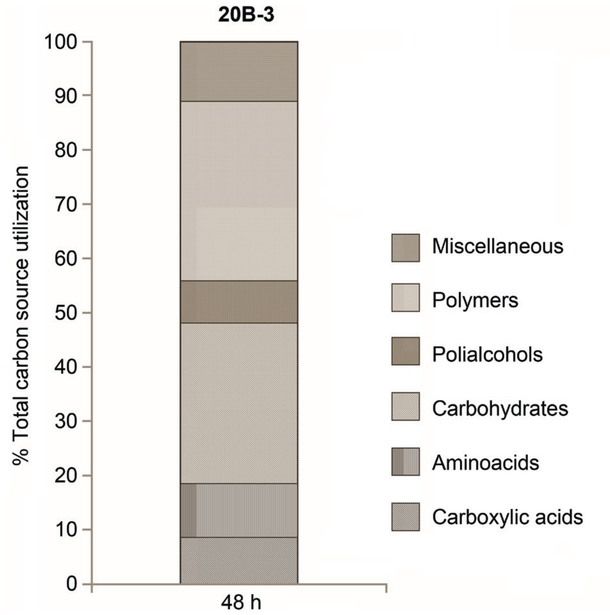

Fig. 3. Ability of *S. shehatae* strain 20BM-3 to oxidize different substrates after 48 h according to the Biolog system.

S. shehatae strain 20BM-3 was a promising arabitol producer and so, in further stages of the study, this strain was cultivated under different conditions using the "one variable at a time" optimization method. In this set of experiments, media containing different initial concentrations of L-arabinose as the carbon source (20.0, 50.0, and 80.0 g/l) and different initial pH values (3.5–6.0) were used. The yeast was incubated at different temperatures (24–32°C) and different rotational speeds (100–200 rpm), which had been selected on the basis of previous literature reports by different scientists (Kordowska-Wiater, 2015). The higher concentrations of the sugar (50.0 and 80.0 g/l) were chosen to check the ability of the yeast to produce arabitol in media of higher osmolarity. The results of the experiments are presented in Table II. The novel strain assimilated about 20.0 g/l of arabinose when cultured for 4 and 5 days, respectively, in media containing 20 and 50 g/l of the pentose, and was able to use about 30 g/l of this sugar in the medium containing 80 g/l of arabinose over 5 days.

Table II
Parameters of L-arabitol production by the novel *S. shehatae* strain 20BM-3 depending on biotransformation conditions.

Conditions (variables)	Production parameters							
	Cult. time (h)	Residual arabinose (g/l)*	Maximum $C_{A\text{-}ol}$ (g/l)*	$Y_{A\text{-}ol}$ (g/g)*	$p_{A\text{-}ol}$ (g/g × h)*	C_B (g/l)*	Y_B (g/g)*	Final pH
Initial C_A (g/l)[1]								
20	96	3.05 ± 0.41	4.03 ± 0.00^{b}	0.24 ± 0.03^{a}	0.002 ± 0.00^{a}	1.94 ± 0.01	0.11 ± 0.00	3.9
50	120	32.40 ± 0.06	6.42 ± 0.28^{a}	0.36 ± 0.02^{b}	0.003 ± 0.00^{a}	2.04 ± 0.04	0.12 ± 0.00	4.85
80	120	49.90 ± 0.07	7.97 ± 0.67^{a}	0.26 ± 0.02^{a}	0.002 ± 0.00^{a}	1.76 ± 0.02	0.06 ± 0.00	4.50
Initial pH[2]								
3.5	96	5.42 ± 0.46	4.82 ± 0.51^{bc}	0.33 ± 0.05^{ac}	0.003 ± 0.00^{b}	2.31 ± 0.06	0.16 ± 0.04	3.1
4.0	96	4.04 ± 0.21	5.88 ± 0.09^{de}	0.37 ± 0.00^{a}	0.004 ± 0.00^{a}	2.81 ± 0.09	0.17 ± 0.06	3.44
4.5	96	4.18 ± 0.58	5.28 ± 0.33^{cd}	0.33 ± 0.03^{ac}	0.003 ± 0.00^{b}	2.47 ± 0.14	0.16 ± 0.09	4.46
5.0	96	4.33 ± 0.63	4.35 ± 0.38^{ab}	0.28 ± 0.01^{bc}	0.003 ± 0.00^{b}	2.42 ± 0.23	0.15 ± 0.00	5.13
5.5	96	3.83 ± 0.06	4.04 ± 0.06^{a}	0.25 ± 0.00^{b}	0.003 ± 0.00^{b}	2.86 ± 0.31	0.18 ± 0.02	4.23
6.0	96	5.83 ± 0.23	3.13 ± 0.42^{f}	0.22 ± 0.03^{b}	0.002 ± 0.00^{b}	2.42 ± 0.14	0.17 ± 0.01	4.95
Shaker rot. speed (rpm)[3]								
100	96	12.94 ± 0.79	2.32 ± 0.06^{a}	0.33 ± 0.03^{a}	0.003 ± 0.00^{a}	1.49 ± 0.04	0.21 ± 0.00	5.8
200	96	6.98 ± 0.30	2.22 ± 0.26^{a}	0.17 ± 0.02^{b}	0.002 ± 0.00^{a}	1.66 ± 0.01	0.13 ± 0.00	5.5
Temp. (°C)[4]								
24	96	13.05 ± 0.55	1.72 ± 0.07^{b}	0.25 ± 0.01^{a}	0.003 ± 0.00^{a}	1.59 ± 0.02	0.23 ± 0.03	5.85
32	96	4.93 ± 0.86	4.65 ± 0.47^{a}	0.31 ± 0.01^{b}	0.003 ± 0.00^{a}	2.27 ± 0.06	0.15 ± 0.00	4.8
Verific. experim.^	96	2.48 ± 0.04	6.2 ± 0.17^{e}	0.35 ± 0.01^{a}	0.004 ± 0.00^{a}	2.59 ± 0.13	0.15 ± 0.01	3.6

* mean values ± standard deviations;
$C_{A\text{-}ol}$ – arabitol concentration; $Y_{A\text{-}ol}$ – arabitol yield; $p_{A\text{-}ol}$ – arabitol productivity;
C_A – arabinose concentration; C_B – biomass concentration; Y_B – biomass yield;
[1] stable conditions: pH 5.5; 28°C; 150 rpm;
[2] stable conditions: 20 g/l arabinose; 28°C; 150 rpm;
[3] stable conditions: 20 g/l arabinose; pH 5.5; 28°C;
[4] stable conditions: 20 g/l arabinose; pH 5.5; 150 rpm;
^ stable conditions: 20 g/l arabinose; pH 4.0; 32°C 150 rpm;
Values with the same superscript letters within a column and one variable are not significantly different ($p < 0.05$).

Production of arabitol was quite effective, with yields of 0.24–0.36 g/g. It is worth noting that biomass production was similar in all cultures within the whole incubation period. The pH of all cultures decreased to 3.9–4.85 during the incubation time. A study of the effect of the initial pH of the medium on arabitol secretion by the novel strain demonstrated that maximum product concentrations and yields were obtained at pH in the range of 3.5–4.5 (Table II), with an optimum observed at pH 4.0. This shows that the yeast preferred lower values of this variable for effective production of arabitol, and, accordingly, acidified the medium during the experiments. By contrast, the effect of pH on biomass production was rather negligible, with the maximum biomass concentration, obtained at pH 5.5, exceeding only slightly the concentration obtained at pH 4.0. The effects of rotational speed and temperature of cultivation on arabinose consumption and arabitol and biomass production by *S. shehatae* strain 20BM-3 were much more salient. A high concentration and a high yield of the polyol were obtained during incubation of the yeast at 32°C and 150 rpm, whereas rotational speeds of 100 and 200 rpm were unfavourable for this yeast (Table II). It is known that rotational speed strongly affects the availability of oxygen to the cells during arabinose catabolism. The initial metabolic pathway for L-arabinose degradation involves redox transformations: L-arabinose is reduced by an unspecific NADPH-linked aldose reductase to arabitol, which is then converted to L-xylulose by NAD-linked dehydrogenase before entry to the pentose phosphate pathway. Under low oxygen conditions, arabitol accumulates because of poor regeneration of NAD necessary for the conversion by arabitol dehydrogenase, and the further stages of the catabolic pathway are stopped or slowed down. Conversely, high oxygenation favours biomass production because the metabolic pathways are more energy-efficient (Fonseca *et al.*, 2007). The analysis of all the combinations of culture conditions for the novel strain *S. shehatae* 20BM-3 suggested that the optimal conditions for this strain were as follows: concentration of arabinose in the medium 20 g/l (because higher concentrations of arabinose were not assimilated completely), pH 4.0, incubation temperature 32°C, and rotational speed 150 rpm. Worth noting is the fact that these conditions were not only conducive to arabitol production, but also promoted yeast growth. For comparison, *C. shehatae* ATCC 22984 cultivated under the same conditions was, generally, a less effective arabitol producer, especially during incubation in the medium with 80 g/l of L-arabinose and at lower pH values of the media (3.5–4.5). Table III compares the effectiveness of the novel strain to that of the reference strain cultivated under the same conditions and over the same incubation time. In another study on *C. shehatae* ATCC 22984, Kordowska-Wiater *et al.* (2008) obtained similar arabitol yields from 20 g/l L-arabinose at 28°C and 100 rpm (0.3–0.35 g/g) or 200 rpm (0.2–0.25 g/g); lower yields were obtained at 32°C or 24°C and 150 rpm, and better results were observed at 28°C and 150 rpm (0.5 g/g), which may be explained by the different volumes of the cultures. Experimental verification of the results of the optimization study confirmed that pH 4.0, 32°C and 150 rpm exerted a positive effect on arabitol production by the new strain *S. shehatae* 20BM-3, which secreted 6.2 ± 0.17 g/l of the product after 4 days with a yield of 0.35 ± 0.01 g/g and a productivity of 0.004 g/g × h. Under these conditions, the yeast assimilated arabinose slightly faster than at 28°C, producing similarly low amounts of biomass (Table II). Taking into account the results obtained at 28°C and 32°C, it may be concluded that both temperatures are acceptable and which of them is used for industrial-scale production of arabitol will ultimately depend on economic considerations.

Arabitol production from xylose and glucose. The ability of the novel strain to produce arabitol from glucose and xylose was investigated in shaken cultures. The yeast assimilated both sugars. D-xylose was transformed to xylitol (0.2 g/l after 2 days) and ethanol (0.45 g/l after 3 days); arabitol was not detected. Bideaux *et al.* (2016), who constructed a metabolic network for

Table III
The comparison of selected parameters of arabitol production by strain 20BM-3 with parameters obtained for reference strain *C. shehatae* ATCC 22894 expressed as % of growth (+) or decrease (–) of parameter value.

	% of growth of production parameters		
	Maximum $C_{A\text{-ol}}$*	$Y_{A\text{-ol}}$*	$p_{A\text{-ol}}$*
Initial C_A (g/l)[1]			
20	9.21	10.23	0
50	42	28.57	50
80	4428	1300	1333
Initial pH[2]			
3.5	53.5	65	59.01
4.0	47.74	37	36.37
4.5	13.55	6.45	7.63
5.0	–21.76	–20	–25
5.5	6.88	1.21	13.33
6.0	–5.15	29.41	28.72
Shaker rotation speed (rpm)[3]			
100	–3.29	–5.71	–6.40
200	149.28	–10.52	–8.40
Temp. (°C)[4]			
24	–9.37	13.63	11.36
32	–12.89	6.89	6.82

* results calculated on the basis of mean values; other designations as in Table II.

Table IV
Parameters of L-arabitol, glycerol, ethanol and ribitol production from glucose by the novel *S. shehatae* strain 20BM-3.

Initial C_G (g/l)[1]	Production parameters				
	Cult. time (h)	Residual glucose (g/l)*	Maximum C_{A-ol} (g/l)*	Y_{A-ol} (g/g)*	p_{A-ol} (g/g × h)*
50	48	1.81 ± 0.01	0.77 ± 0.41	0.016 ± 0.00	0.0003 ± 0.00
100	120	41.28 ± 0.36	4.00 ± 0.36	0.07 ± 0.00	0.0006 ± 0.00
			Maximum C_{G-ol} (g/l)*	Y_{G-ol} (g/g)*	p_{G-ol} (g/g × h)*
50	24	10.43 ± 0.99	1.21 ± 0.35	0.03 ± 0.05	0.001 ± 0.00
100	96	50.24 ± 5.76	3.09 ± 0.33	0.06 ± 0.04	0.0006 ± 0.00
			Maximum C_{E-ol} (g/l)*	Y_{E-ol} (g/g)*	p_{E-ol} (g/g × h)*
50	72	1.81 ± 0.05	0.78 ± 0.03	0.016 ± 0.00	0.0002 ± 0.00
100	120	41.28 ± 0.36	1.15 ± 0.07	0.02 ± 0.01	0.0002 ± 0.00
			Maximum C_{R-ol} (g/l)*	Y_{R-ol} (g/g)*	p_{R-ol} (g/g × h)*
50	72	1.81 ± 0.05	0.12 ± 0.05	0.0025 ± 0.0	0.00003 ± 0.00
100	96	50.24 ± 5.76	2.44 ± 0.12	0.05 ± 0.01	0.0005 ± 0.00

* mean values ± standard deviations;
C_G – glucose concentration; C_{A-ol} – arabitol concentration; Y_{A-ol} – arabitol yield; p, $_{A-ol}$ – arabitol productivity;
C_{G-ol} – glycerol concentration; Y_{G-ol} – glycerol yield; p_{A-ol} – glycerol productivity; C_{E-ol} – ethanol concentration;
Y_{E-ol} – ethanol yield; p_{E-ol} – ethanol productivity; C_{R-ol} – ribitol concentration; Y_{R-ol} – ribitol yield; p_{R-ol} – ribitol productivity;
[1] stable conditions: pH 5.5; 28°C; 150 rpm.

xylose conversion in *C. shehatae* ATCC 22984 on the basis of metabolic fluxes, also found that arabitol was not secreted during xylose catabolism. In our experiment, we obtained a biomass concentration of 2.22 g/l, which was similar to that obtained in the yeast culture grown on arabinose. In this present study, *S. shehatae* 20BM-3 assimilated glucose at concentrations of 50 or 100 g/l, secreting arabitol into the broth, at concentrations of 0.77 ± 0.41 and 4.00 ± 0.36 g/l after 2 and 5 days, respectively. A second product was glycerol at concentrations of 1.21 ± 0.35 and 3.09 ± 0.33 g/l, respectively. This yeast strain also produced small quantities of ethanol and ribitol. The parameters of arabitol, glycerol, ribitol, and ethanol production from glucose are presented in Table IV. Biomass production was quite low, reaching, after 5 days, 3.2 and 1.83 g/l from 50 or 100 g/l of glucose, respectively, which confirms that strain 20BM-3 preferred lower sugar concentrations. To compare, *C. shehatae* ATCC 22984 produced 0.8 and about 3 g/l of arabitol from glucose within the same time and under identical conditions. The pathway of glucose catabolism for conversion of glucose to arabitol leads *via* the glucose phosphorylation stage and conversion to D-ribulose-5-phosphate (or D-xylulose-5-phosphate) followed by dephosphorylation and reduction of D-ribulose (or D-xylulose) to arabitol, which may either enter the pentose phosphate pathway or be secreted by the cells (Kordowska-Wiater, 2015). It is clear that the yields and productivities obtained by strain 20BM-3 are rather low, but there exist methods of improving the strain's production of arabitol from glucose, which is an abundant and cheap source of carbon. For example, Fromanger *et al.* (2010), who investigated *C. shehatae* ATCC 22984 in fed-batch cultures in media containing xylose or glucose in aerobic conditions, observed that this yeast strain produced large quantities of biomass and CO_2 and lower quantities of ethanol, glycerol, xylitol, and ribitol, but not arabitol, from xylose, whereas glucose was metabolized mainly to biomass and CO_2 with small amounts of glycerol, arabitol, ethanol, and ribitol, but not xylitol, which was similar to our findings. In oxygen-limited conditions, they detected the same products, but at different concentrations, with ethanol being the main metabolite of both xylose and glucose. The ability of *C. shehatae* FPL-Y-049 to produce traces of arabitol from glucose during ethanol production from wood hydrolyzate has been reported by Sreenath and Jeffries (2000).

Conclusion

The novel yeast strain isolated from rotten wood, designated 20BM-3, was found to be able to produce arabitol efficiently from arabinose and much less efficiently from glucose. This strain was genetically identified as *S. shehatae* and was characterized morphologically and biochemically using the Biolog system. It secreted arabitol in batch cultures at average concentrations of 4.03–7.97 g/l, depending on the initial concentration of arabinose in the medium, under moderate aeration conditions at 28°C and an initial pH 5.5, with average yields in the range of 0.24–0.36 g/g. It was observed that the yeast used about 20–30 g/l of L-arabinose in the medium. The initial pH of the medium (in the range of 3.5–6.0) was found to have an impact on arabitol production, with pH 4.0 being the preferred value. The analysis of all the combinations of culture conditions for the novel strain *S. shehatae* 20BM-3 demonstrated that optimal growth was obtained at arabinose concentration in the medium 20 g/l, initial pH 4.0, incubation temperature 32°C, and rotational speed 150 rpm. Further experiments showed that this novel strain was also able to produce arabitol from glucose, but only at low yields. The results obtained in this work encourage continuation of screening studies in search of novel yeast strains possessing the ability to produce arabitol from different sugars *e.g.* those abundant in plant biomass. They also point to the importance of optimization studies and genetic modification studies in looking for new ways of obtaining this polyol.

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ORIGINAL PAPER

Metagenomic Analysis of Soil Bacterial Community and Level of Genes Responsible for Biodegradation of Aromatic Hydrocarbons

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Abstract

The aim of the studies was to compare the composition of soil bacterial metabiomes originating from urbanized areas and areas contaminated with hydrocarbons with those from agricultural soil and forest soil obtained from a protected wild-life park area. It should be noted that hydrocarbons are everywhere therefore bacteria capable of their utilization are present in every soil type. In the hydrocarbon-contaminated soil and in the soil of anthropogenic origin, the bacteria belonging to Gammaproteobacteria were dominant (28.4–36.6%), whereas in the case of agricultural soil and protected wild-life park soil their ratios decreased (22.8–23.0%) and were similar to that of *Alphaproteobacteria*. No statistically significant changes were observed in terms of the Operational Taxonomic Unit identified in the studies soils, however, based on the determined alpha-diversity it can be established that contaminated soils were characterized by lower biodiversity indices compared to agricultural and forest soils. Furthermore, the dioxygenase level was also evaluated in the studied soils, which are genes encoding crucial enzymes for the decomposition of mono- and polycyclic aromatic hydrocarbons during the biodegradation of diesel oil (PAHRHDαGN, PAHRHDαGP, xylE, Cat 2,3, ndoB). It was concluded that both the population structure of the soil metabiome and the number of genes crucial for biodegradation processes differed significantly between the soils. The level of analysed genes showed a similar trend, as their highest number in relations to genes encoding 16S RNA was determined in urban and hydrocarbon-contaminated soil.

Key words: aromatic hydrocarbons biodegradation, presence of genes responsible for degradation, soil bacterial community, soil metabiome

Introduction

From the human perspective the value of soil is mainly defined by the role it plays in food production processes. However soil also has several other functions which are crucial to the life of humans, animals and plants, although its contribution to the functioning of ecosystems is often non-direct and remains unseen (Brady and Weil, 1999). Soil allows to limit the results of climate changes and acts as a regulator of water drainage, it decreases the temperature in urban areas during hot periods and, at the same time, increases the humidity of air, it also helps to preserve the biological diversity in urban areas, allowing the survival of several species of plants, animals and microorganisms (Lal, 2004; Seneviratne, 2010). One of the fundamental differences between urban soil and soil originating from rural areas is the fact that the first is subjected to strong changes due to anthropogenic activity (de Kimpe and Morel, 2000). The first negative phenomenon associated with the sealing of soil is increasing its density and agglomeration, which results in issues in terms *e.g.* of proper water intake. The second notable threat to soils in urban areas is associated with their local contamination. Degradation of soils in UE-28 proceeds at a rate of approx. 2.46 t/ha/year (Panagos *et al.*, 2015), and some researchers claim that the rate may be as high as 2.76 t/ha/year (Bosco *et al.*, 2015). In Europe, an average of 0.3–1.4 tons of soil per ha is created annually (Verheijen *et al.*, 2009). Due to this reason several scientists treat soil as a non-renewable resource. Aside from heavy metals and liquid ionic, hydrocarbons are one of the most dangerous types of contaminants. Pollution with hydrocarbons often occurs during accidents and leakage of diesel oil during its transport or storage (Piotrowska-Cyplik and Czarnecki, 2003; Sydow *et al.*, 2015; Ławniczak *et al.*, 2016; Sydow *et al.*, 2016). From human perspective, the environmental contamination

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with petroleum hydrocarbons is a notable environmental and health problem. These compounds, due to their hydrophobic nature, may penetrate through cell membranes and damage the cells, which are not adopted to their presence. Such compounds are often toxic, mutagenic and cancerogenic (Marecik *et al.*, 2012).

A detailed analysis of physicochemical parameters, physical phenomena and determination of the contamination rate allows to select a proper remediation technique. The UE Directive.

2004/35/CE indicates that natural bioremediation methods are a solution with limited human intervention in the processes of returning the environment to its primary state. The use of bioremediation aims to clean-up the polluted areas, it does not require the application of complex apparatuses and it can be used *in situ*. It employs biological systems to decrease the concentrations and (or) completely remove several chemical compounds, such as petroleum and its processing products, aliphatic and aromatic hydrocarbons (with the exception of polycyclic aromatic hydrocarbons and polychlorinated biphenyls), organic solvents and others (Lisiecki *et al.*, 2014). The increasing use of bioremediation for the biodegradation of hydrocarbons results from its ecological and economic merits, as it does not interfere with the natural clean-up processes and soil subjected to bioremediation may be reused (Marquez-Rocha *et al.*, 2001). It is general accepted that in order to achieve complete degradation of a mixture of petroleum hydrocarbons, the contribution of numerous microorganisms which cooperate with each other is necessary. Several authors emphasize the high potential of soil for conducting natural attenuation processes during a long time period, which is of particularly importance, since this approach guarantees that its structure remains intact and limits the costs of bioremediation (Szczepaniak *et al.*, 2016). However, in order to allow the natural attenuation processes to proceed, the bacterial microflora must be sufficiently rich in species capable of degrading petroleum hydrocarbons. Evaluation of the soils biodegradation potential has a notable practical implication, as it allows to indicate the need to introduce external microbial cultures with a high biodegradation potential, to establish the range of such operations and to monitor the biodegradation process not only based on the changes of pollutant concentrations, but also by determining the abundance of the introduced bacterial consortia and evaluation of their functioning by measuring the expression of genes crucial for the biodegradation process (Cébron *et al.*, 2008).

The aim of this study was to compare the composition of the bacterial metabiome and the presence of selected genes encoding crucial enzymes responsible for the decomposition of aromatic hydrocarbons in soils originating from urban areas and areas contaminated with hydrocarbons in relation to agricultural soil and soil originating from a protected wild-life area (forest soil). In order to fully identify all the microorganisms present in the soils and evaluate their genetic pool, the sequencing of the soil metabiome using Next Generation Sequencing with the use of MiSeq Illumina was assumed. Changes in the number of enzymes initiating the decomposition of polycyclic aromatic hydrocarbons (dioxygenases (PAHRHDαGN, PAHRHDαGP)), monoaromatic hydrocarbons (catechol 2,3-dioxygenase (xylE, Cat 2,3) and naphtalene 1,2-dioxygenase (ndoB) was analysed in the analysed soil samples with respect to the gene encoding 16S rNA using the RT-PCR method.

Experimental

Materials and Methods

The origin of soil samples. The soil used in the studies originated from the Wielkopolska region in Poland and was collected from four sites with different usage. Soil A originated from a forest in the area of the landscape park (Poland) (N 52.534032; E 17.047875); Soil B originated from an agricultural field used to grow wheat (N 52.551539, E 16.99974); Soil C originated from the roadside vicinity of a road near Poznan (Poland) (N 52.524980, E 16.976538); Soil D originated from a petrol oil station (N 52.560036, E 16.999887). The surface layer of soil from each area was collected from 10 different spots, each sample contained 100 g of soil. The samples were collected from a depth of 10–20 cm, then they were combined and mixed. The soil near the road was collected in the direct vicinity of the drive way. In the case of the remaining soils a regular method of sampling was applied, since a uniform distribution of contaminants was assumed. The total mass of the combined soil obtained upon combination of all individual samples was 1 kg.

Identification of microorganisms

DNA extraction. Total DNA was extracted from soil sample using Genomic Mini AX Soil kit (A&A Biotechnology) according to manufacturer's instruction. The extracted DNA was quantified using Quant-iT HS ds.-DNA assai kit (Invitrogen) on Qubit2 fluorometer (Ławniczak *et al.*, 2016).

PCR amplification. Region IV of bacterial 16S RNA gene was amplified using universal primers 515F and 806R: containing reverse complement of 3' Illumina adapter, golay barcode, reverse primer pad, reverse primer linker and reverse primer (Table I). Products

Table I
Characteristics of sequencing and real-time PCR primers.

Primers	Sequence (5’ to 3’)	Reference
PCR amplification		
Forward 515F Reverse 806R	AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA[1] CAAGCAGAAGACGGCATACGAGATXXXXXXAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT[2]	Caporaso *et al.*, 2012
RT-PCR		
Cat 2,3 Forward Reverse	AGGTGCTCGGTTTCTACCTGGCCG ACGGTCATGAATCGTTCGTTGAG	Laramee *et al.*, 2000
xylEb Forward Reverse	GTGCAGCTGCGTGTACTGGACATGAGCAAG GCCCAGCTGGTCGGTGGTCCAGGTCACCGG	Panicker *et al.*, 2010
ndoB Forward Reverse	CACTCATGATAGCCTGATTCCTGACCCCGGCG CCGTCCCACAACACACCCATGCCGCTGCCG	Panicker *et al.*, 2010
PAH-RHDα GN Forward Reverse	GAGATGCATACCACGTKGGTTGGA AGCTGTTGTTCGGGAAGAYWGTGCMGTT	Cébron *et al.*, 2008
PAH-RHDα GP Forward Reverse	CGGCGCCGACAAYTTYGTNGG GGGGAACACGGTGCCRTGDATRAA	
16 S rDN**A** 968 Forward 1401 Reverse	AACGCGAAGAACCTTAC CGGTGTGTACAAGACCC	

[1] contaning 5’ Illumina adapter, forward primer pad, forward primer linker and forward primer sequence
[2] containing reverse complement of 3’ Illumina adapter, golay barcode, reverse primer pad, reverse primer linker and reverse primer

were purified in Clean-Up columns (A&A Biotechnology) according to manufacturer’s protocol. The libraries were constructed from amplicons using NEBNext® DNA Library Prep Master Mix Set for Illumina (New England Biolabs UK). Then the libraries were polled at equimolar concentration. Sequencing was conducted on an Illumina MiSeq (Illumina, USA) using paired-end (2 × 250) MiSeq Reagent Kits v2 (Illumina, USA). Sequencing primers were based on Caporaso *et al.* 2012 (Table I). The sequencing reaction was performed with MiSeq Illumina instrument and MiSeq Reagent Kit v2 (2 × 250bp) (Ławniczak *et al.*, 2016).

Bioinformatic analysis. The sequencing data was processed using CLC Genomic Workbench 8.5 and CLC Microbial Genomics Module 1.2. (Qiagen, USA). Total number of reads ranged from 438 216 to 461123. After sequencing, the reads were demultiplexed to the probes and the overlapping paired-end reads were merged (70% of total reads) and trimmed to yield fragments of 289 nt. Just fragments which passed the merging were retained for downstream processing. Chimeric reads (from 27121 to 29785) were filtered and remaining sequences were assigned to operational taxonomic units (OTUs). Number of reads which passed merging and trimming ranged from 136576 to 154294. Reads were clustered against the SILVA v119 99% 16S rRNA gene database (Quast *et al.* 2013).

RT-PCR. Genes level was analysed using a Power SYBR Green PCR Master Mix (Life Technologies) on ABI 7500 SDS (Applied Biosystems). Primers used for real-time PCR are listed in Table I. Total bacterial RNA was quantitated by real-time PCR amplification of fragment of bacterial 16S ribosomal RNA with universal bacterial primers and TaqMan MGB probe using TaqMan Universal Master Mix II (Life Technologies) on ABI 7500 SDS (Applied Biosystems). Sequences of primers and probe used are listed in Table I. All analysis was done in triplicates. In order to compare the gene expression in each sample, the mean expression index was calculated according to formula: C_T target/C_T 16S using data from 3 analyses. This parameter reflects the expression level of a specific gene compared to the expression level of the universal gene (16S RNA) in the whole metabiome (Szczepaniak *et al.*, 2016).

Results and Discussion

Analysis of the soil metabiome is a challenging scientific problem due to the properties of soil, methods and efficiency of isolation of genetic material and the use of different molecular biology methods in order to identify the soil microbial species (Kozdrój, 2013; Szczepaniak *et al.*, 2015). The use of metagenomic 16S

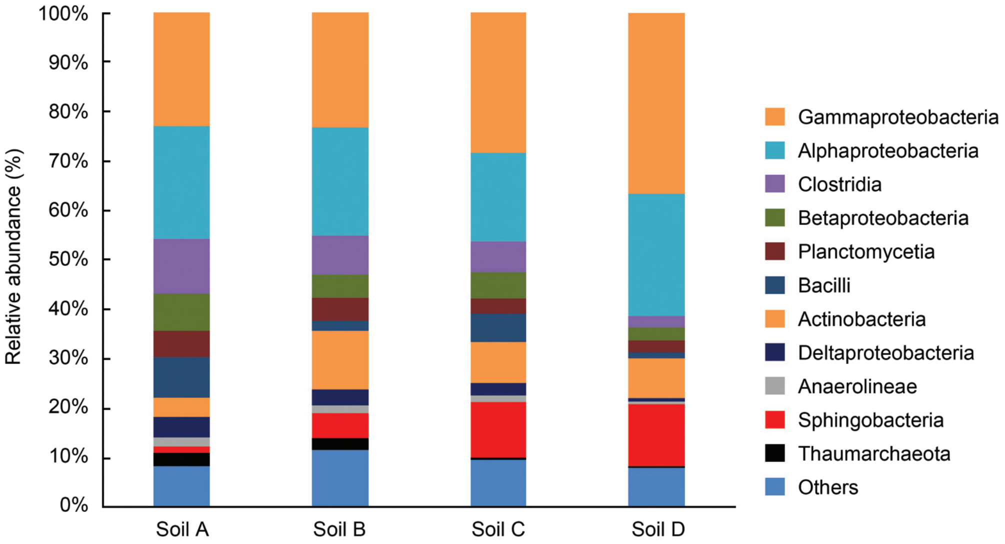

Fig. 1. Relative abundance of bacterial classes (A) and quantitative changes in RhlA, RhlC, PAHRHDαGN, PAHRHDαGP expression level genes (B) present in the soil after 1 and 3 months of biodegradation.

analysis method based on the MiSeq platform allows to notably expand the knowledge regarding the composition of the soil microbiome. This tool is particularly useful during comparison studies of genomes originating from different environments (Szulc *et al.*, 2014). In order to identify the bacterial consortium (M), a metagenomes analysis of the gene encoding 16S rRNA was conducted on the basis of V4 hypervariable region of the 16S rRNA gene (Fig. 1) (Caparoso *et al.*, 2012).

Metagenomic 16S analysis of the soil metabiome indicated that soil originating from the forest (A) and agricultural soil (B) were the most diverse in terms of microbial species. In the bacterial metabiome the identified species belonged to 64 classes, including the following dominant classes: Alphaproteobacteria (22.83%) and Gammaproteobacteria (22.88%) as well as Clostridia (11.11%), Bacilli (8.40), Betaproteobacteria (7.55%) and Planctomycetia (5.21%). The growth of crops and application of agicultural processes resulted in the dominance of Gammaproteobacteria in the agricultural soil (soil B), which accounted for 23.06% of the total population. The remaining dominant classes included Alphaproteobacteria (21.92%), Actinobacteria (11.94%) and Clostridia (8.05%). The ratio of the remaining groups of microorganisms did not exceed 5%.

As a result of applying different agricultural processes, including different fertilization methods and use of plant protection agents, the metabiome of agricultural soils was characterized by a lower population variety, which was reflected by a lower value of alpha-diversity indices. In the studies carried out by Newman *et al.* (2016) regarding the influence of glyphosate on the metabiome of rhizosphere soil in soybean and corn, it was established that three phyla dominated in control soil: Proteobacteria, Acidobacteria, and Actinobacteria. The use of glyphosate significantly increased the ratio of Proteobacteria (particularly Gammaproteobacteria) in the soil metabiome, whereas the ratio of Acidobacteria notably decreased. Furthermore, in the study of Zhou *et al.* (2015), which focused on the influence of long-term fertilization on the changes of the soil metabiome, a decrease of the total number of 16S rRNA gene and decrease of bacterial diversity was observed in the case of soils treated with mineral fertilizers. Dominant phyla, which increased their abundance as a result of fertilization, included: Proteobacteria, Acidobacteria, Actinobacteria, Actinobacteria and Proteobacteria. On the other hand, the ratio of Acidobacteria and Nitrospirae in the population was notably decreased. It should therefore be assumed that other factors may also lead to changes in the composition of soil bacteria populations. The presence of hydrocarbon contaminants in soil may be one of such factors.

Petroleum hydrocarbons are compounds which undergo decomposition in soil due to activity of several groups of microorganisms. Several different microbial species participate in the biodegradation of hydrocarbons, ranging from strictly aerobic to strict anaerobic bacteria. Several Gram-positive (Rhodococcus or Bacillus) as well as Gram-negative (Alcaligenes, Acinetobacter, Pseudomonas) species are also characterized by relatively broad substrate spectrum (Szczepaniak *et al.*, 2015).

The progress of hydrocarbon biodegradation processes carried out *in situ* depends on the conditions in a given area and the present microflora. During the early stages n-alkanes are biodegraded by the bacteria and when only persistent compounds are left (branched alkanes, mono- and polycyclic aromatic hydrocarbons), the biodegradation process is conducted by bacteria

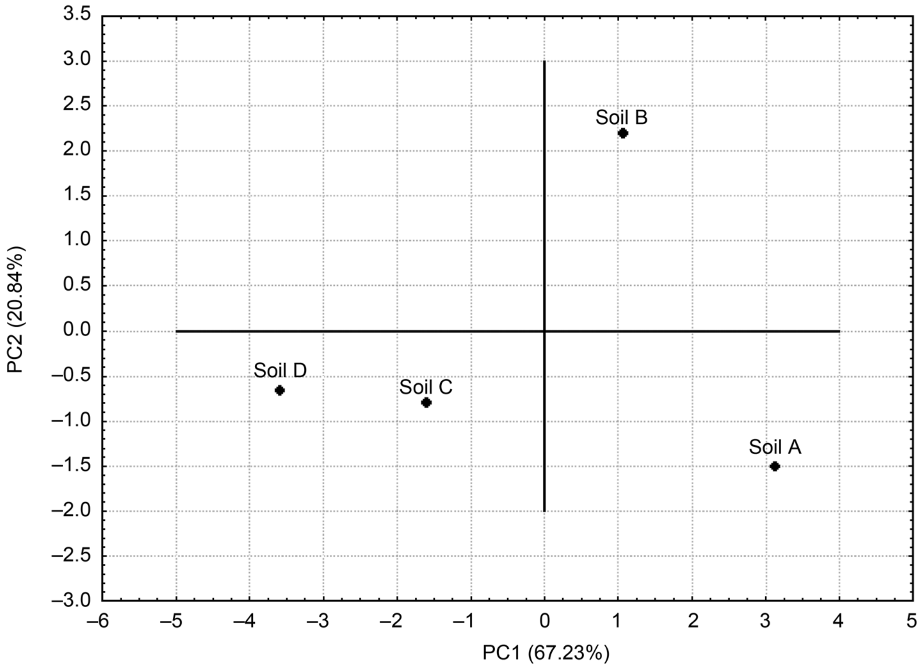

Fig. 2. Principal component analysis (PCA) for examining correlations between relative abundance of bacterial classes and genes: RhlA, RhlC, PAHRHDαGN, PAHRHDαGP expression during PAHs biodegradation.

characterized by a lower growth rate and unique metabolic abilities (Mrozik and Piotrowska-Seget, 2010).

In the soil samples collected from the roadside (soil C) and soil collected form a former fuel station (soil D), bacteria belonging to the Gammaproteobacteria class were clearly the dominant group. Their ratio in the soil samples collected from the roadside (soil C) was at 28.4%, whereas in the case of soil collected from a former fuel station (soil D) the value reached 36.63%. The ratio of Alphaproteobacteria was similar to the remaining soils and ranged from 17.86 to 24.78%. A notable ratio of bacteria belonging to the Sphingobacteria (11–12%) and Actinobacteria (8.0–8.4%) class was also observed. The ratio of the remaining identified classes of microorganisms did not exceed 6%.

The Principal Component Analysis (PCA) was used in order to evaluate the changes in the bacterial soil metabiome. Figure 2 shows how the analysed bacterial metabiomes were grouped. Two first main components are decisive and describe the variability of the primary data in 84.0%. The first main component carried approx. 67.23% of the data regarding microbial populations contained in the input variables. It includes the following positively correlated variables: Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria and Bacilli; as well as the negatively correlated: Clostridia and Flavobacterium. The second main component is mainly responsible for the presence of Actinobacteria, Planctomycelia and Others, and described the variability of the analysed data in 20.84%. The high variability of the soil metabiome depending on its place of origin was presented on Fig. 2. The presence of anthropogenic contaminants in soil (soil C and D) caused notable changes in the soil metabiome, which are visible in the lower-left part of the figure. The remaining metabiomes of soils A and B are present in separate quarters of the figure.

The determined alpha diversity (Table II) of soil originating from the forest area (soil A) and agricultural soil (soil B) were significantly higher compared to soil originating from urban and contaminated areas (soil C and D) ($p < 0.05$).

A rational approach to the bioremediation process should limit the activities which may change the

Table II
Alpha diversity measured using number of OTUs, Shannon's index, Chao 1 bias-corrected and phylogenetic diversity.

	Soil A	Soil B	Soil C	Soil D
OTU observed	1893 ± 51	1944 ± 71	1426 ± 29	1312 ± 44
Shannon's index	5.8 ± 0.2	5.7 ± 0.3	4.8 ± 0.2	4.1 ± 0.1
Chao 1 bias-corrected	1358 ± 187	1398 ± 154	1089 ± 122	1012 ± 115
Phylogenetic diversity	4.98 ± 0.31	4.86 ± 0.22	3.95 ± 0.34	3.68 ± 0.21

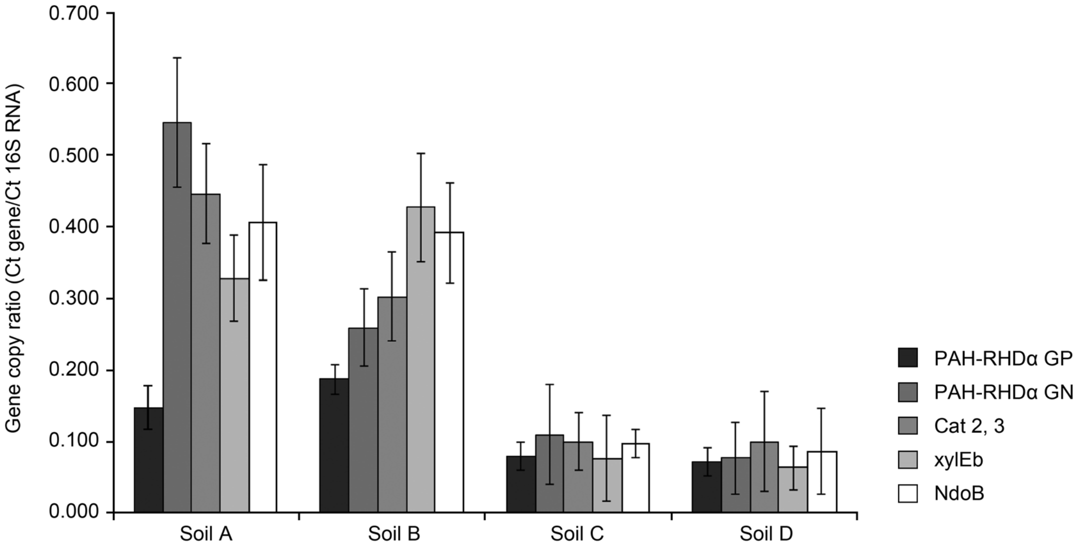

Fig. 3. Relative abundance of bacterial classes (A) and quantitative changes in RhlA, RhlC, PAHRHDαGN, PAHRHDαGP expression level genes (B) present in the soil after 1 and 3 months of biodegradation.

composition and structure of soil and therefore disrupt the biological equilibrium by introducing new groups of microorganisms. To date, the studies regarding the bioremediation of natural environments rarely include analyses of the environmental genetic pool, which would contribute to valuable data regarding its natural attenuation potential (Afzal *et al.*, 2011). Selected microbial communities characterized by a high hydrocarbon-degradation potential are often prepared for the purpose of bioremediation technologies (Cerqueira *et al.*, 2011). However, it is known that introduction of external organisms (consortia of isolated microorganisms) is not always possible – *e.g.* in the case of protected areas, which become contaminated. The same applies to physical removal of polluted soil, since the removal of the contaminated layer may also eliminate species of insects, molluscs and representatives of other organisms, which are under protection.

Furthermore, the proper functioning of a microbial consortium is based on its composition as well as the combinations of specific functions (defined by genes) which are provided by specific microbial species (Panicker *et al.*, 2010). Selection of specific traits (gene sequences) allows to monitor the changes in the structure of the consortium and it the general genetic poll of the remediated environment. Such studies may be the foundation for determining the natural attenuation potential of a given environment and to decide whether bioaugmentation or use of additional agricultural agents is necessary (Szczepaniak *et al.*, 2016). Usually, prokaryotic organisms oxidise PAHs using dioxygenases by introducing two oxygen atoms into the substrate and formation of cis-hydrodiols, which are then transformed into dihydroxyl compounds. This reaction is crucial for initiating the conversion of aromatic hydrocarbons into environmentally sage forms (Cébron *et al.*, 2008; Fuentes *et al.*, 2014).

Prior to the determination of the presence and abundance of genes responsible for the biodegradation of aromatic hydrocarbons, an indirect method was used based on the measurement of the number of cycles for the analysed gene (C_T gene) in comparison to an internal standard – the number of cycles encoding the 16S rDNA subunit (C_T16S). The employed method allowed to determine the relative changes in the expression of genes compared to the number of bacteria present in soil. The measured C_T16S value for the 16S RNA gene, which was used as an internal standard, was in the range of 6.2 to 7.3 for a given sample. (see Fig. 3). The presence of the gene encoding dioxygenases in Gram-negative bacteria (PAH-RHDα GN) was highest in soil originating from the fuel station (soil D) and from soil originating from the roadside (soil C). The relative number of these genes reached 0.54 ± 0.21 and 0.26 ± 0.14, accordingly, and these values were significantly different ($p < 0.05$). Whereas in soil originating from the forest area (soil A) and agricultural soil (soil B) the content of these genes in the soil metabiome gene pool was lowest and ranged from 0.08 to 0.14. The differences between these values were not statistically significant ($p > 0.05$).

A different result was obtained in the case of studies regarding the presence of the gene encoding the dioxygenase in Gram-positive bacteria (PAH-RHDα GP), as its number in the microbial gene pool was significantly lower in samples collected from the roadside (soil C) and soil collected from the fuel station (soil D) and ranged from 0.15 to 0.19. On the other hand, in soil originating from the forest area and agricultural soil its abundance did not exceed 0.08.

The relative number of genes encoding enzymes crucial for the degradation of aromatic hydrocarbons (xylE, Cat 2,3 and ndoB) was highest in case of soil originating from anthropogenic areas (soils C and D) and ranged from 0.3 to 0.45. In case of agricultural soil and soil originating from the forest area (soils A and B) these values did not exceed 0.10. The observations of several authors confirm that the origin of soils and their contact with contaminants results in the increase of genes responsible for the biodegradation of hydrocarbons in the gene pools. The obtained results confirm the observations of other authors regarding the uneven distribution of genes encoding enzymes decomposing hydrocarbons in different terrestrial environments (Cébron *et al*., 2008). This distribution is mainly based on the area of origin. In soils in which microorganisms had contact with contaminants (soils C and D) a higher ratio of genes associated with biodegradation processes was observed in comparison to non-contaminated soil. However non-contaminated soils were characterized by the presence of microflora capable of biodegradation processes and therefore the level of genes associated with biodegradation processes may increase after contact with contaminants, especially after a prolonged period, which confirms the high potential of soils to conduct natural attenuation during a long term period.

Conclusions

The selection of specific genes, which are relevant to the decomposition of hydrocarbons, allows to evaluate the biodegradation potential and monitor the changes in the microbial community structure and the overall gene pool in the remediated environment. Such monitoring is particularly justified in the case of freshly contaminated areas, providing solid foundation for additional, controlled bioaugmentation. In the case of permanent or long-term contaminations the detailed analysis of gene pool of the soil metabiome allows to determine the natural attenuation potential and decide whether bioaugmentation or application of agricultural agents is necessary. Such actions would facilitate the remediation of areas subjected to long-term anthropogenic activity. A precise diagnostic carried out in order to determine the genetic deficiencies or elimination of some consortium members may indicate that aside from the composition of a consortium, the combination of specific functions (defined by genes carried by those organisms) is a crucial factor.

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ORIGINAL PAPER

Comparison of Microbial Communities Associated with Halophyte (*Salsola stocksii*) and Non-Halophyte (*Triticum aestivum*) Using Culture-Independent Approaches

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Abstract

Halophyte microbiome contributes significantly to plant performance and can provide information regarding complex ecological processes involved in osmoregulation of these plants. The objective of this study is to investigate the microbiomes associated with belowground (rhizosphere), internal (endosphere) and aboveground (phyllosphere) tissues of halophyte (*Salsola stocksii*) through metagenomics approach. Plant samples were collected from Khewra Salt Mines. The metagenomic DNA from soil, root and shoot samples was isolated with the help of FastDNA spin kit. Through PCR, the 16S rRNA gene from four different *Salsola* plants and wheat plants was amplified and cloned in InsTAclone PCR cloning kit. Metagenomic analyses from rhizosphere, endosphere and phyllosphere of *Salsola* showed that approximately 29% bacteria were uncultured and unclassified. *Proteobacteria* and *Actinobacteria* were the most abundant phyla in *Salsola* and wheat. However, *Firmicutes*, *Acidobacteria*, *Bacteriodetes*, *Planctomycetes*, *Cyanobacteria*, *Thermotogae*, *Verrucomicrobia*, *Choroflexi* and *Euryarchaeota* were predominant groups from halophyte whereas *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Acidobacteria*, *Bacteriodetes*, *Planctomycetes* and *Verrucomicrobia* were predominant phyla of wheat samples. Diversity and differences of microbial flora of *Salsola* and wheat suggested that functional interactions between plants and microorganisms contribute to salt stress tolerance.

Key words: 16S rRNA gene approach, microbial communities associated with plants, microbiome of halophyte

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Introduction

Plants are colonized by different types of bacteria that can reach cell densities much greater than the number of plant cells. Microbial communities associated with a plant are collectively referred as plant microbiome. Rhizosphere is the zone surrounding the plant roots and is a hot spot for numerous microorganisms. The rhizosphere of halophytes harbors a variety of microorganisms (microbiome) that have ability to promote plant growth by increasing the availability and uptake of carbon, nitrogen and minerals from soil (Dodd and Perez-Alfocea, 2012). It is considered as one of the most complex ecosystems on Earth. Metagenomic techniques indicated that plant host genotype is an important factor structuring bacterial communities in plant leaves, roots and rhizosphere (Balint *et al.*, 2013). Based on metagenomic approaches, microbiome studies of different plants, *i.e.*, *Populus*, *Arabidopsis* and *Zea mays* revealed that overall structure of the microbial community may have variations in rhizo-, endo- and phyllosphere of same plant (Shakya *et al.*, 2013; Bonito *et al.*, 2014). Microbiome controls several important functions in the atmosphere, rhizosphere, phyllosphere, human and animal habitats. The phyllosphere of a plant considered nutrient poor as compare to rhizosphere. Microbial colonization of leaves is homogenous but is affected by leaf structures such as stomata and veins (Valenzuela-Encinas *et al.*, 2008). Phyllosphere microbiome is involved in nitrogen fixation, biodegradation of toxic compounds and pathogen suppression by production of antibodies and induction of systemic resistance in the host (Sundaram *et al.*, 2011; Bodenhausen *et al.*, 2014). *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* are the dominant phyla found in the phyllosphere of grasses and angiosperms suggesting that relatively few bacterial phyla colonize the phyllosphere (Bodenhausen *et al.*, 2013). Endophytic

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microorganisms are those that reside inside plant tissues at least part of their lives. They are generally nonpathogenic microbes causing no visible symptoms and promote plant growth by nitrogen fixation, mineral solubilization (P, Zn) and indole acid production. Bacterial endophytes and rhizosphere microbiome may provide the plant with different accessible nutrients such as nitrogen (N) and phosphorus (P) (Browne *et al*., 2009), phytohormones such as indole acetic acid (IAA) that promote plant growth (Dimkpa *et al*., 2012), suppress pathogens through competitive exclusion or production of antibiotics (Gupta *et al*., 2015), or may help plants to withstand salt, drought and heat (Rolli *et al*., 2015; Craita and Tom, 2013).

The distribution of saline soils on more than half a billion hectare worldwide warrants attention for their efficient, economical and environmentally acceptable management practices. Salt tolerance in plants is also connected with complex ecological processes within its rhizosphere and phyllosphere. Environmental factors have great effect on bacterial and archaeal abundance, community composition and its dynamics. So the phylogenetic analysis of plant associated halophilic bacteria is important to learn about their ecological functions, evolved mechanisms of saline adaptation and their potential uses in biotechnology (Ruppel *et al*., 2013; Sheng *et al*., 2014). Halophiles have novel enzymes with inherent ability to function under salt stress conditions (Delgado-García *et al*., 2014). Certain enzymes produced by halophiles are considered useful for bioremediation of pollutants in saline habitats (Dastgheib *et al*., 2011) and production of important biomolecules, *i.e*., exopolysaccharides and phytohornmones (Liszka *et al*., 2012). About 50% of the archaeal diversity and less than 25% of the total bacterial diversity has been recovered from salt affected soils. Halophilic strains of *Halomonas*, *Bacillus*, *Stenotrophomonas*, *Alkalimonas*, *Staphylococcus* and *Methylibium* have been isolated from halophyte roots, soil and desert habitats (Anton *et al*., 2002; Shi *et al*., 2012; Zhou *et al*., 2012). Microbial diversity analysis of communities by using metagenomic approaches has become a routine part of biological studies (Mason *et al*., 2014). Abiotic stresses such as temperature, pH, salinity and drought have effects on the plant microbiome, directly or indirectly, through the host and global microbial composition in the saline habitats is affected more by salinity than by other abiotic stresses (Ma and Gong, 2013).

Salt tolerant crops like kallar grass (*Leptochloa fusca*), *Suaeda fruticosa*, *Kochia indica*, *Atriplex amnicola* and *Salsola stocksii* have not only medicinal compounds that can be used to cure against disease such as cough, flu and cold but also used as food source (Ajmal and Qaiser, 2006; Khan, 2009). *Salsola* species are important biomass producers in barren lands of this area. This plant is a good source of fuel, fodder and even food during famines (Dagla and Shekhawat, 2005).

The objective of this study was to compare microbiome of *S. stocksii* (halophyte) and wheat (non-halophyte) using metagenomic techniques. Microbial diversity from phyllosphere, rhizosphere and endosphere of *S. stocksii* and wheat was compared. The identification of bacterial species through culture independent technique is especially important to understand the genetic potential of different community members constituting the microbiome and the interactions between them.

Experimental

Materials and Methods

Sampling of rhizospheric soil and plants (*S. stocksii* and wheat). Khewra salt mine is the world second largest salt mine, located near Pind Dadan Khan Tehsil of Jhelum District, Punjab, Pakistan (Ahmad *et al*., 2007). It has plenty of important salts including halite (NaCl), gypsum ($CaSO_4$. $2H_2O$) and sylvite (KCl). Geographically, it is located about 32°38’ North latitude, 73°10’ East longitude and an elevation of 313–360 above the sea level about 200 km from Islamabad. The rhizospheric soil, roots and shoots of four *S. stocksii* (Synonym: *Haloxylon recurvum*) were collected at vegetative stage from different localities of Khewra Salt Mines (Fig. S1). Wheat (*Triticum aestivum*) plants and rhizospheric soil were collected from wheat fields in Forman Christian College (A Chartered University), Lahore, Pakistan. All samples of soil and plants were brought to laboratory in black polythene bags under refrigerated condition. The rhizospheric soil and root samples were stored at –20°C for further processing.

Soil physical and chemical parameters. Each soil sample (400 g) was thoroughly mixed and sieved through a pore size of 2 mm. Physical properties (moisture content, pH, salinity and temperature) of soil samples from different plants were determined. Electrical conductivity (dS/m) was measured by 1:1 (w/v) soil to water mixtures at 25°C (Adviento-Borbe *et al*., 2006); pH was measured by 1:2.5 (w/v) soil to water suspension; moisture (%); temperature (°C) and texture class were determined by Anderson method (Anderson and Ingram, 1993). Organic matter (C_{org}) was calculated by the Walkley-Black method (Walkley and Black, 1934); phosphorous was estimated by extraction with sodium bicarbonate (Olsen *et al*., 1954) and calcium and magnesium were detected by atomic absorption spectrometry. Nitrate ions were measured by Raney-Kjeldahl method and potential acidity (H+Al) was determined by an equation based on the pH in SMP buffer solution (pH SMP). Cation exchange capacity (CEC) is capacity to retain and release cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) and sodium adsorption ratio (SAR) is the measure of the sodicity of soil which is calculated as the ratio of the sodium to the magnesium and calcium.

Isolation of metagenomic DNA and amplification of 16S rRNA gene. Metagenomic DNA from rhizosphere soil, root and shoot samples of *S. stocksii* and wheat was extracted with Fast DNA Spin kit for rhizospheric soil and roots using FastPrep® instrument (MP Biomedicals, USA). DNA was isolated from 0.5–1.0 g soil, sterilized root and shoot samples according to the procedure provided by the manufacturer. The concentration of metagenomic DNA was qualitatively determined on 0.8% (w/v) agarose gel and quantified using Nanodrop (NanoDrop 200c Thermo Scientific, USA). DNA was diluted to three different concentrations *i.e.*, 1:10, 1:25 and 1:50 using sterilized ddH_2O for use in PCR reactions. The metagenomic DNA samples were used as templates for PCR. The 16S rRNA gene was amplified using bacterial universal forward primer FD1 and universal reverse primer rP1 for rhizosphere and phyllosphere samples of *S. stocksii* (Akhtar *et al*., 2008) and primers P1 and P6 for wheat samples (Tan *et al*., 1997). For identification of archaea, forward primer 1A and reverse primer 1100A were used for amplification of 16S rRNA gene (Munson *et al*., 1997). Amplified PCR products were confirmed on 1% (w/v) agarose gel and were purified by using QIA quick PCR purification kit (QIAGEN, USA) before subsequently utilized for cloning and sequencing.

Cloning and sequencing of 16S rRNA gene. PCR products were ligated into pTZ57R/T vector using InsTAclone PCR cloning kit (Fermantas#K1213). Positive clones were selected using blue white screening and confirmed through double digestion of plasmids DNA with restriction enzymes *Hind*III and *Xba*I. Plasmid DNA samples were sequenced by M13 forward primer.

16S rRNA sequencing analysis. The sequence data was assembled and analyzed with the help of Chromus Lite 2.01 sequence analysis software. The chimeric sequences were eliminated; non-chimeric sequences were further analyzed and aligned using BIOEDIT (Hall, 1999). The gene sequences were compared to those deposited in the GenBank nucleotide database using the BLAST program. Phylogenetic affiliations and taxonomical hierarchy based on 16S rRNA gene were determined with 96% confidence by using CLASSIFIER tool (https://rdp.cme.msu.edu/classifier/classifier.jsp) of RDP-II database (Wang *et al*., 2007).

Nucleotide sequence accession numbers. Gene sequences obtained in this study were deposited in NCBI GenBank databse for accession numbers. Accession numbers for 16S rRNA gene sequences from *S. stocksii* rhizosphere were HG938313-HG938352, LN827740-LN827750, LN835771-LN835799 (Table S4), root endosphere LM644099-LM644131, LN555114-LN555147, LN827751-LN827759, LN835800-LN835828 (Table S6), phyllosphere LN879933-LN880052 (Table S8), from wheat rhizosphere LN880053-LN880164 (Table S3), root endosphere LN880218-LN880269 (Table S5) and phyllosphere LN880165-LN880217 (Table S7).

Calculation of diversity indices. An operational taxonomic unit (OTU) was defined as a 16S ribosomal DNA (rDNA) sequence group in which sequences differed by less than 3%. Phylotype richness (S) was calculated as the total number of OTUs. Shannon and Simpson indices are diversity measuring parameters which are commonly used to characterize species diversity in a community. Shannon index shows the uniformity of species and its abundance in OTUs while Simpson index is used to measure the number of species present in a community as well as the relative abundance of each species (Martin, 2002).

Statistical analyses. Principal component analysis is a multivariate statistical technique that uses ecological assessment because most environmental studies are characteristic of a large number of variables which make difficult to high light important trends in the data (Arndt *et al*., 2012). In this study, principal component analysis was done by using XLSTAT software.

Results

Rhizospheric soil characteristics. Soil in sampling site was encrusted with salts. Soil moisture content (%) of *S. stocksii* and wheat rhizosphere was 28 ± 4 and 20 ± 3. Electrical conductivity (dS/m) of *S. stocksii* and wheat rhizosphere measured by Adviento-Borbe method was 4.86 ± 0.22 and 3.51 ± 0.33. Soil samples were alkaline in nature with soil pH of *S. stocksii* and wheat rhizosphere 8.53 ± 0.21 and 7.71 ± 0.39. Soil temperature of *S. stocksii* and wheat rhizosphere was 23.5 ± 3°C and 32.50 ± 1.5°C (Table S1). Total organic matter ranged from 28.69 ± 3.39 to 34.55 ± 4.16 g/Kg. The available P, K, Ca and Mg contents were more in quantity in *S. stocksii* (halophyte) as compared to wheat (non-halophyte) rhizospheric soil samples. CEC values for *S. stocksii* and wheat rhizosphere were 71.1 ± 13.21 and 56.46 ± 8.51 mg/dm^3 and SAR values for *S. stocksii* and wheat rhizosphere were 13.45 ± 3.12 and 10.38 ± 2.51 respectively.

Calculation of diversity indices. Phylotype richness (S), Shannon diversity index (H), evenness (E_H) and Simpson index (D) were calculated. Phylotype richness (S) of the bacterial communities from the rhizosphere of *S. stocksii* and wheat was calculated as 98 ± 4 and 95 ± 5, Shannon diversity index (H) was 3.82 ± 0.31 and 2.65 ± 0.40, Evenness (E_H) was 0.56 ± 0.11 and 0.45 ± 0.08 and Simpson index (D) was 0.841 ± 0.14

Table I
Phylogenetic affiliation and abundance of bacterial and archaeal phyla.

Phylogenetic group	*S. stocksii* rhizosphere	Wheat rhizosphere	*S. stocksii* root endosphere	Wheat root endosphere	*S. stocksii* phyllosphere	Wheat phyllosphere
Total sequences	118	114	113	101	108	99
1. Bacterial sequences	114	114	107	101	100	99
1.1. Proteobacteria	35	33	31	28	24	36
1.1.1. Alphaproteobacteria	9	2	3	2	2	4
1.1.2. Betaproteobacteria	4	8	7	2	6	1
1.1.3. Gammaproteobacteria	17	17	19	22	14	25
1.1.4. Deltaproteobacteria	4	4	2	1	2	2
1.1.5. Unclassified proteobacteria	1	2	1	1	0	3
1.2. Actinobacteria	7	33	9	24	18	26
1.2.1. Actinobacteria	7	30	9	23	15	24
1.2.1. Unclassified Actinobacteria	0	3	0	1	3	2
1.3. Firmicutes	6	15	6	20	12	12
1.3.1. Bacilli	6	12	5	9	10	6
1.3.2. Clostridia	0	1	0	2	0	1
1.3.3. Negativicutes	0	2	1	9	2	5
1.4. Cyanobacteria	5	2	3	0	7	0
1.5. Bacteroidetes	7	4	7	6	5	7
1.6. Planctomycete	2	1	1	5	0	1
1.7. Acidobacteria	6	0	5	2	5	1
1.9. Chloroflexi	5	0	2	0	0	0
1.10. Thermotogae	1	0	0	0	0	0
1.11. Verrucomicrobia	5	0	3	1	4	0
1.12. Cyanophyta	0	1	0	0	0	0
1.13. Unclassified bacteria	35	25	40	15	23	16
2. Archaeal sequences	4	0	6	0	8	0
2.1. Euryarchaeota	4	0	6	0	8	0

and 0.729 ± 0.19 respectively (Table II). Phylotype richness (S) of the bacterial communities from the root endosphere of *S. stocksii* and wheat was calculated as 102 ± 8 and 94 ± 6, Shannon diversity index (H) was 3.39 ± 0.36 and 2.54 ± 0.28, Evenness (E_H) was 0.54 ± 0.12 and 0.55 ± 0.11 and Simpson index (D) was 0.812 ± 0.16 and 0.850 ± 0.12, respectively (Table II). Data analysis showed that root endosphere microbial community from *S. stocksii* had more diversity as compare to wheat root endosphere microbial community. Phylotype richness (S) of the bacterial communities from the phyllosphere of *S. stocksii* and wheat as calculated as 97 ± 6 and 91 ± 4, Shannon diversity index (H) was 3.46 ± 0.34 and 2.56 ± 0.34, Evenness (E_H) was 0.53 ±0.095 and 0.56 ± 0.11 and Simpson index (D) was 0.699 ± 0.13 and 0.779 ± 0.15, respectively (Table II). Shannon indices confirmed that microbial community from the rhizosphere, endosphere and phyllosphere of *S. stocksii* had more diversity as compared to wheat. These results also indicated that phyllosphere showed less microbial diversity as compared to rhizosphere and root endosphere from both *S. stocksii* and wheat.

Comparison of rhizosphere, endosphere and phyllosphere microbiome of *S. stocksii* and wheat at phylum level. From the rhizospheric soil of *S. stocksii*, 30% sequences of 16S rRNA gene were unclassified uncultured bacteria, 64% sequences showed homology with 10 bacterial phyla and 6% sequences with *Euryarchaeota*. *Proteobacteria* were the most abundant (28%), followed by *Bacteroidetes* (6%). Uncultured bacteria of phyla *Actinobacteria*, *Firmicutes* and *Acidobacteria* formed 15% of the total population density from the rhizospheric soil of *S. stocksii.* Members of phyla *Chloroflexi* (4%), *Verrucomicrobia* (4%), *Cyanobacteria* (3%), *Planctomycete* (3%) and *Thermotogae* (1%) were also identified from the rhizospheric soil of *S. stocksii* (Fig. 1A and Table I). Among the sequences of 16S rRNA gene from the rhizospheric soil of wheat, 23% sequences were unclassified uncultured bacteria. Among the 7 different phyla detected from the rhizosphere of wheat, sequences of *Proteobacteria* were most

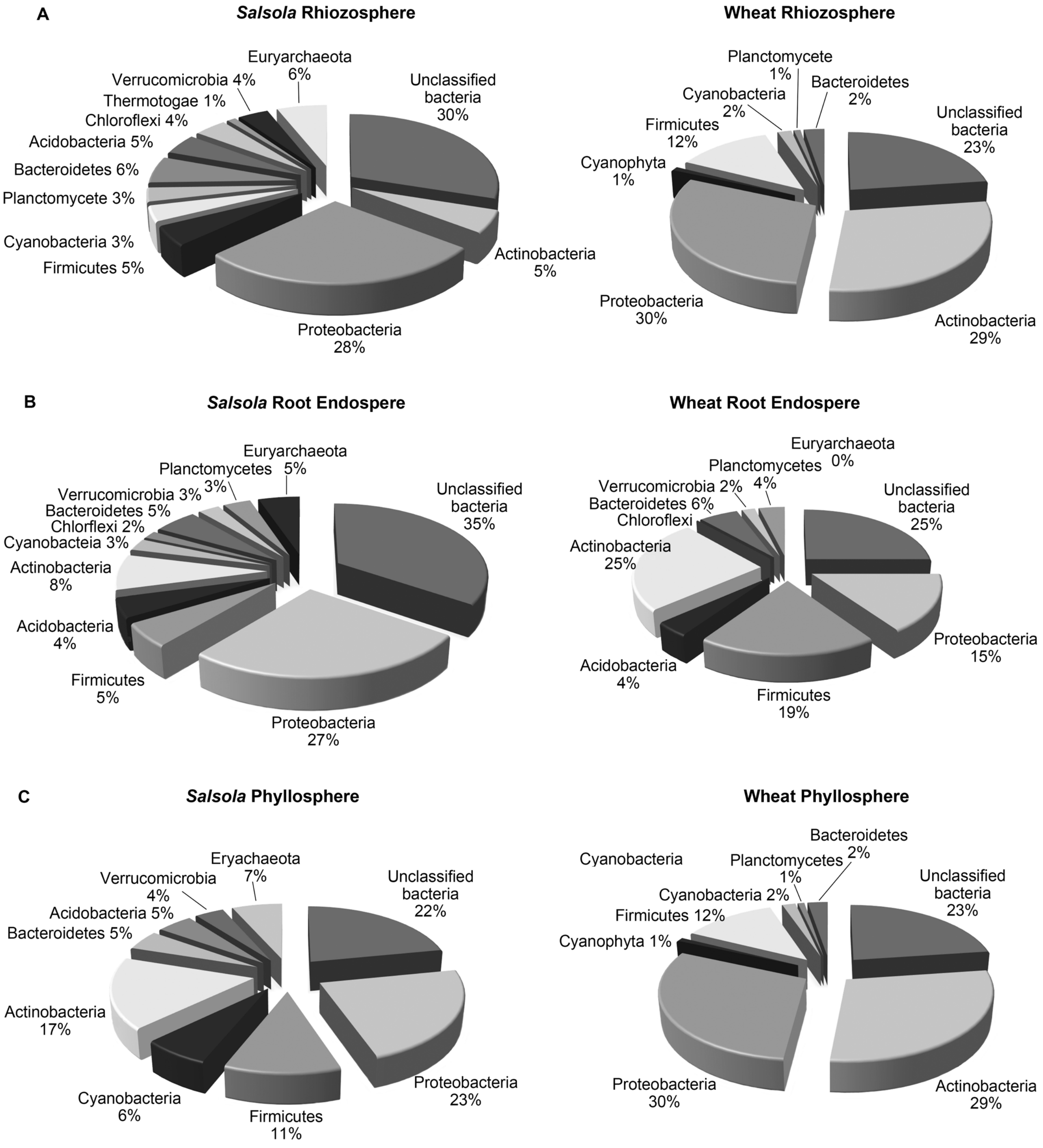

Fig. 1. Relative abundance of bacterial and archaeal phyla; (A) from rhizosphere (B) from root endosphere (C) from phyllosphere of *S. stocksii* and wheat.

abundant (30%) followed by *Actinobacteria* (29%), *Firmicutes* (12%), *Bacteroidetes* (2.69%), *Cyanobacteria* (2%), *Planctomycete* (1%) and *Cyanophyta* (1%).

Data analysis of 16S rRNA from the root endosphere of *S. stocksii* indicated that 35% sequences were uncultured unclassified bacteria, 60% sequences showed homology with 9 bacterial phyla and 5% sequences with Archaea. Among the bacterial phyla, *Proteobacteria* were the most abundant (27%) followed by *Actinobacteria* (8%). Bacterial sequences of *Firmicutes* (5%), *Bacteroidetes* (5%) and *Acidobacteria* (4%) were dominant in the root endosphere of *S. stocksii.* Members of the *Cyanobacteria, Verrucomicrobia* and *Planctomycete* formed 9% of total bacterial population. Sequences of *Chloroflexi* were found less abundant (2%) as compared to other bacterial phyla from the root endosphere microbiome (Fig. 1B and Table I). In case of wheat, 15% of sequences from the root endosphere showed homology with uncultured unclassified bacteria. Sequences of the phylum, *Proteobacteria* were the most abundant

Table II
Phylotype richness, diversity indices and evenness in microbial communities from rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat.

Clone library	Total number of usable sequences	Phylotype richness (S)	Shannon-Wiener index[1] (H)	Evenness[2] (E_H)	Simpson index[3] (D)
S. stocksii rhizosphere	118	98 ± 4	3.82 ± 0.31	0.56 ± 0.11	0.841 ± 0.14
Wheat rhizosphere	114	95 ± 5	2.65 ± 0.40	0.45 ± 0.08	0.729 ± 0.19
S. stocksii root endosphere	113	102 ± 8	3.39 ± 0.36	0.54 ± 0.12	0.812 ± 0.16
Wheat root endosphere	101	94 ± 6	2.54 ± 0.28	0.55 ± 0.11	0.850 ± 0.12
S. stocksii phyllosphere	108	97 ± 6	3.46 ± 0.34	0.53 ± 0.095	0.699 ± 0.13
Wheat phyllosphere	99	91 ± 4	2.56 ± 0.34	0.56 ± 0.11	0.779 ± 0.15

[1] Shannon-Wiener index was calculated as: H = –SUM[(pi) * ln(pi)] where Pi is the frequency of the species.
[2] Evenness was calculated as Hmax = ln(S)
[3] Simpson Index (D) was calculated as: $D = \Sigma(n/N)^2$ where n = the total number of organisms of a particular species and N = the total number of organisms of all species. The value of Simpson Index ranges between 0 and 1
Each value is the mean of four biological replicates (± SE) with significant differences ($P < 0.05$) among the bacterial communities of the analyzed soil samples.

(28%) followed by *Actinobacteria* (23%) and *Firmicutes* (19%). Members of the phylum, *Bacteroidetes* formed 6% of the total microbial population in the root endosphere of wheat. Sequences of the phyla *Acidobacteria* (2%), *Planctomycete* (5%) and *Verrucomicrobia* (2%) were also detected from the root microbiome.

Phylogenetic analysis of 16S rRNA gene sequences indicated that 22% sequences showed homology with uncultured unclassified bacteria, 71% sequences with 7 bacterial phyla and 7% sequences with Archaea from phyllosphere of *S. stocksii*. Among the retrieved sequences of 16S rRNA gene, sequences of *Proteobacteria* were the most abundant (23%) followed by *Actinobacteria* (17%) and *Firmicutes* (11%). Members of *Cyanobacteria* and *Bacteroidetes* formed 6% and 5% of the total population density from the phyllosphere of *S. stocksii.* Data analysis of 16S rRNA gene sequences showed that 5% sequences showed similarity with *Acidobacteria* and 4% sequences with *Verrucomicrobia* (Fig. 1C and Table I). Sequence analysis of 16S rRNA gene showed that 16% sequences corresponded to uncultured unclassified bacteria from the phyllosphere of wheat. Similar to rhizosphere microbial community, sequences of *Proteobacteria* were the most abundant (36%) followed by *Actinobacteria* (26%) and *Firmicutes* (12%). Together, *Bacteroidetes* and *Acidobacteria* constituted approximately 8% of the total microbial diversity in the phyllosphere.

Principle component analysis (PCA) was used to study potential differences in the microbial communities from the rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat. Two principle components explained 97% of the variability in the microbial diversity. Principle component 1 explained 87.30% of the data whereas principle component 2 explained 9.70% variations in the compositional data. This analysis revealed clear differences between overall microbiomes of *S. stocksii* and wheat as well as among rhizosphere, endosphere and phyllosphere of both *S. stocksii* and wheat (Fig. 2). Microbial communities from rhizosphere and root endosphere of *S. stocksii* were closely related to each other but significantly different from rhizosphere and root endosphere of wheat. There was no statistically significant difference between phyllosphere microbiomes of *S. stocksii* and wheat. At each site, certain bacterial and archaeal species prevailed better than others. The microbial communities expressed differently from point to point because of variations in environmental factors like salinity and pH differences in physicochemical characteristics compared to saline soil samples.

Comparison of rhizosphere, endosphere and phyllosphere microbiome of *S. stocksii* and wheat at class level. Microbial diversity at the class level showed significant difference in the microbiome of *S. stocksii* and wheat. At the class level, sequences from the *Gammaproteobacteria* was the most dominant class followed by *Actinobacteria*, *Betaproteobacteria*, *Bacilli*, *Alphaproteobacteria* and *Deltaproteobacteria* in the rhizosphere of *S. stocksii* while members of the class *Actinobacteria* were the most abundant in the rhizosphere of wheat followed by *Gammaproteobacteria*, *Bacilli*, *Betaproteobacteria, Deltaproteobacteria* and *Negativicutes* (Table I). Results showed that sequences belonged to the class *Gammaproteobacteria* was the most abundant in the root endosphere of *S. stocksii.* Sequences from *Actinobacteria*, *Betaproteobacteria*, *Bacilli*, *Alphaproteobacteria* were dominant in the saline environments. In case of root endosphere microbiome of wheat, sequences from the class *Actinobacteria* was the most dominant followed by *Gammaproteobacteria*, *Negativicutes*, *Bacilli*, *Betaproteobacteria* and *Clostridia*

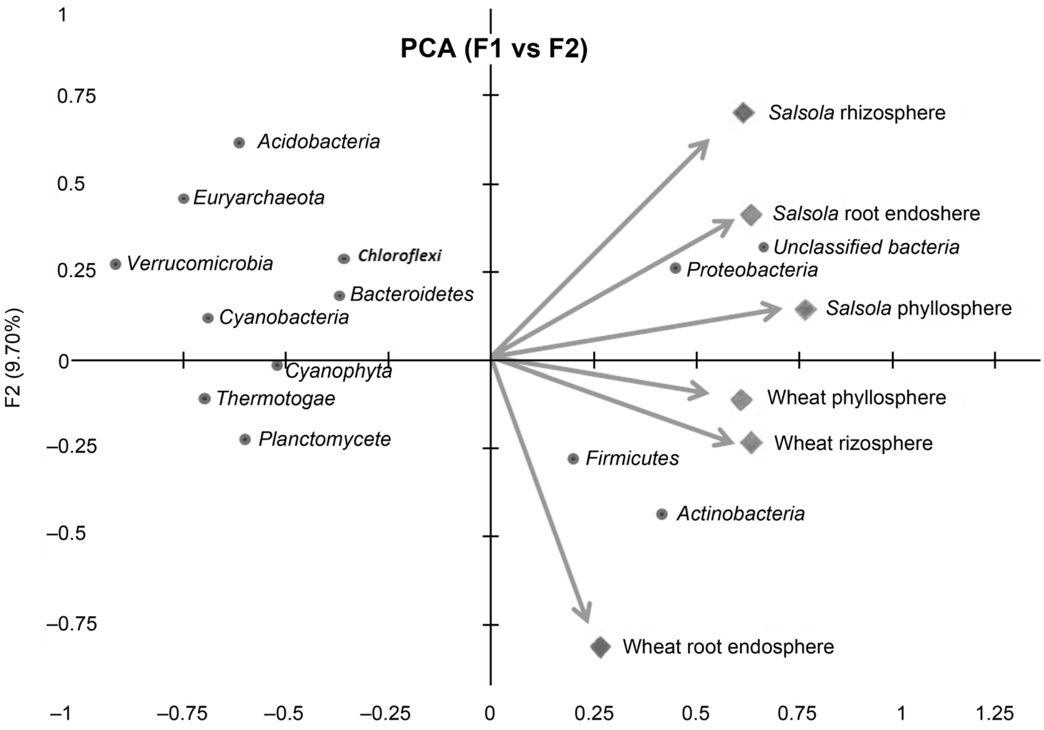

Fig. 2. Principal Component Analysis (PCA) of the rhizosphere, root endosphere, phyllosphere microbiomes of *S. stocksii* and wheat.

(Table I). It was observed that sequences from the class *Actinobacteria* were more dominant as compared to other bacterial classes (*Gammaproteobacteria*, *Bacilli*, *Betaproteobacteria* and *Alphaproteobacteria*) from the phyllosphere of *S. stocksii* while sequences belonged to the *Gammaproteobacteria* were most abundant in the phyllosphere of wheat followed by *Actinobacteria*, *Bacilli*, *Negativicutes*, Alphaproteobacteria and *Betaproteobacteria* (Table I).

Comparison of rhizosphere, endosphere and phyllosphere microbiome of *S. stocksii* and wheat at genus level. It was observed that 40% phylotypes were common in both plants whereas 33% in *S. stocksii* and 27% in wheat were different from each other (Fig. 3). Bacterial genera *Bacillus*, *Enterobacter*, *Flavobacteria*, *Gramella*, *Microbacterium* and *Pseudomonas* are commonly detected from halophyte and non-halophyte while salt tolerant bacterial and archaeal genera *Halococcus*, *Chromohalobacter*, *Rhodothermus*, *Desulfurella*, *Halomonas* and *Nesterenkonia* were identified only in the rhizosphere, endosphere and phyllosphere of *Salsola* and *Azospirillum*, *Aeromonas*, *Jatrophihabitans*, *Clostridium*, *Niastella* and *Paenibacillus* were dominant in the microbiome of wheat (Fig. 4).

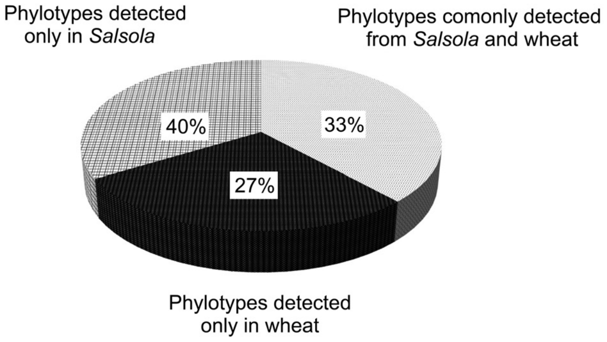

Fig. 3. Phylotype sequences detected from *S. stocksii* and wheat microbiomes.

The results showed that bacterial and archaeal genera *Halococcus*, *Halalkalicoccus*, *Haloferula*, *Chromohalobacter* and *Thermotoga* were detected only from the rhizosphere of *S. stocksii* while bacterial genera *Arthrobacter*, *Burkholderia*, *Brevibacillus*, *Citrobacter* and *Kribbella* were identified from the rhizosphere of wheat (Fig. 5A). Bacterial and archaeal genera *Halobacterium*, *Salegentibacter*, *Halovibrio*, *Halalkalicoccus* and *Halobacillus* were identified only from the root endosphere of *S. stocksii* while *Sporomusa*, *Pelosinus*, *Staphylococcus*, *Azospirillum* and *Curtobacterium* were dominant from the root endosphere of wheat (Fig. 5B). In case of phyllosphere microbiome of *S. stocksii,* bacterial and archaeal genera *Haloferula*, *Amphritea*, *Halomonas*, *Kocuria* and *Halococcus* were abundant. Sequences belonged to bacterial genera *Pantoea*, *Dendrosporobacter*, *Erwinia*, *Aeromonas* and *Paenibacillus* were detected only from the phyllosphere of wheat (Fig. 5C). Difference in bacterial and archaeal genera across rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat explained variations in saline and non-saline environments.

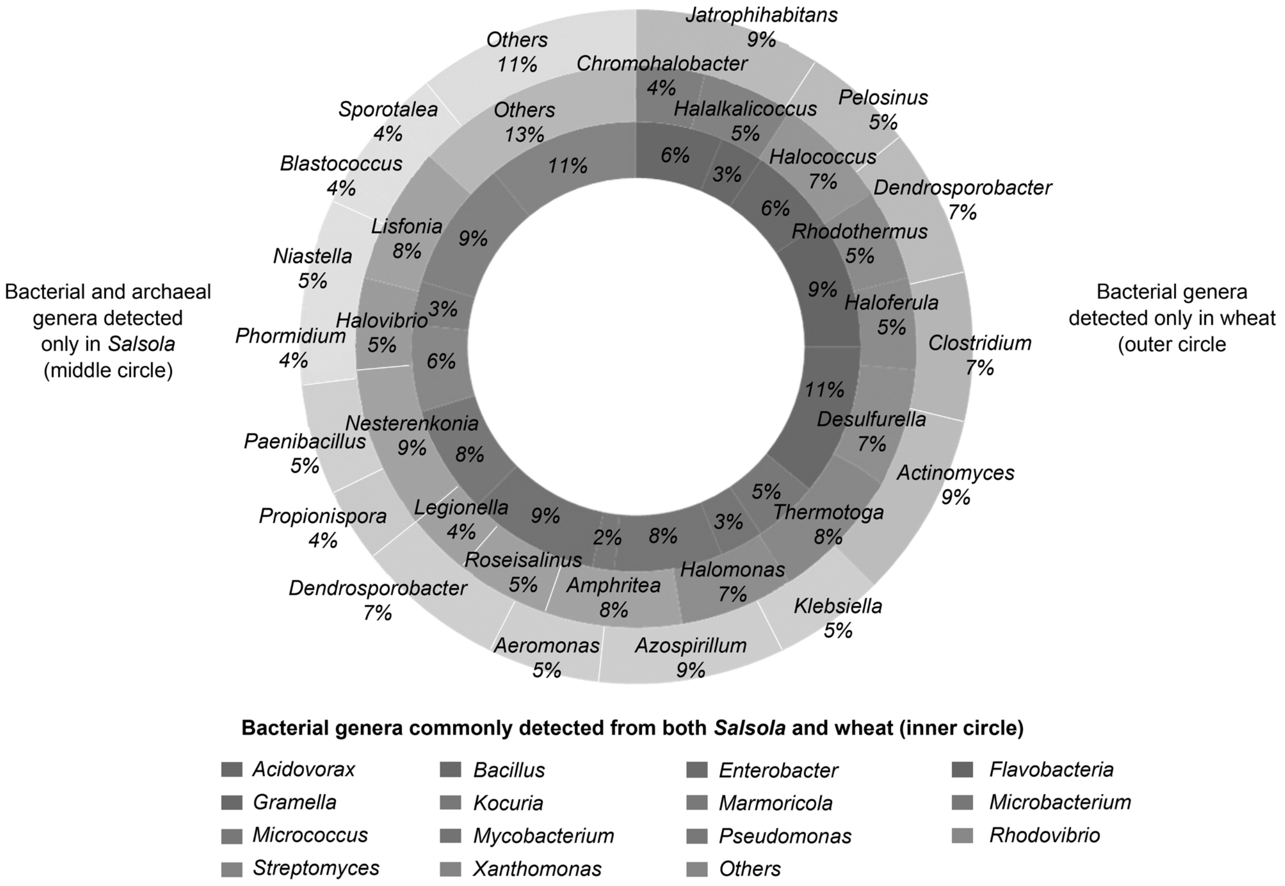

Fig. 4. Comparison of microbiomes of *S. stocksii* and wheat at genus level.

Discussion

In this study, we analyzed the microbial composition and community structure in the rhizosphere, endosphere and phyllosphere of *S. stocksii* (halophyte) and wheat (non-halophyte) by using metagenomic approaches. The study also focused on comparison of plant microbiome of *S. stocksii* and wheat.

Sequences analysis of *S. stocksii* and wheat microbiomes indicated that microbial communities present in the rhizosphere, endosphere and phyllosphere of *S. stocksii* had more diversity as compared to microbial communities identified from the wheat microbiome. In the present study, sequence analysis of 16S rRNA gene indicated that 10 bacterial phyla from rhizospheric soil and roots, 7 bacterial phyla from phyllosphere and leaves of *S. stocksii* whereas 7 bacterial phyla were detected from rhizospheric soil and roots, 5 bacterial phyla from phyllosphere and leaves of wheat. *Proteobacteria* was the most dominant phylum in the rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat. In case of *S. stocksii* rhizosphere, endosphere and phyllosphere, *Gammaproteobacteria* was the most abundant class followed by *Betaproteobacteria*, *Deltaproteobacteria* and *Alphaproteobacteria*. Sequences related to genera *Halomonas*, *Halospina*, *Amphritea*, *Halovibrio*, *Legionella*, *Chromohalobacter*, *Salicola* and *Shewanella* were abundant in the rhizosphere of *S. stocksii* while in case of wheat, *Pseudomonas, Klebsiella*, *Citrobacter*, *Kluyvera*, *Pantoea* and *Enterobacter* were abundant genera. Metagenomic approaches indicate that *Gammaproteobacteria* are a dominant class in moderate and high saline soils (Mwirichia *et al*., 2011; Lundberg *et al*., 2012). Genera (*Pseudomonas*, *Pantoea* and *Enterobacter*) belonging to *Gammaproteobacteria* were consistently dominant as compared to other *proteobacteria* (Bodenhausen *et al*., 2013). Sequences belonging to class *Alphaproteobacteria* were found to be more abundant in the saline habitats as compared to wheat rhizosphere. Bacterial genera; *Rhodobacter*, *Sphingomonas*, *Oceanicola* and *Roseisalinus* are widely distributed in the saline environments (Farias *et al*., 2011). In the phyllosphere, *Sphingomonas* species were widely distributed indicating nutrient poor environment. They have an important role against plant pathogens (Knief *et al*., 2012). Members of the *Betaproteobacteria* (*Massilia*, *Duganella*, *Burkholderia*, *Methylibium* and *Delftia*) and *Deltaproteobacteria* (*Cystobacter*, *Myxococcus* and *Desulfurella*) identified from the rhizosphere of both *S. stocksii* and wheat has been previously

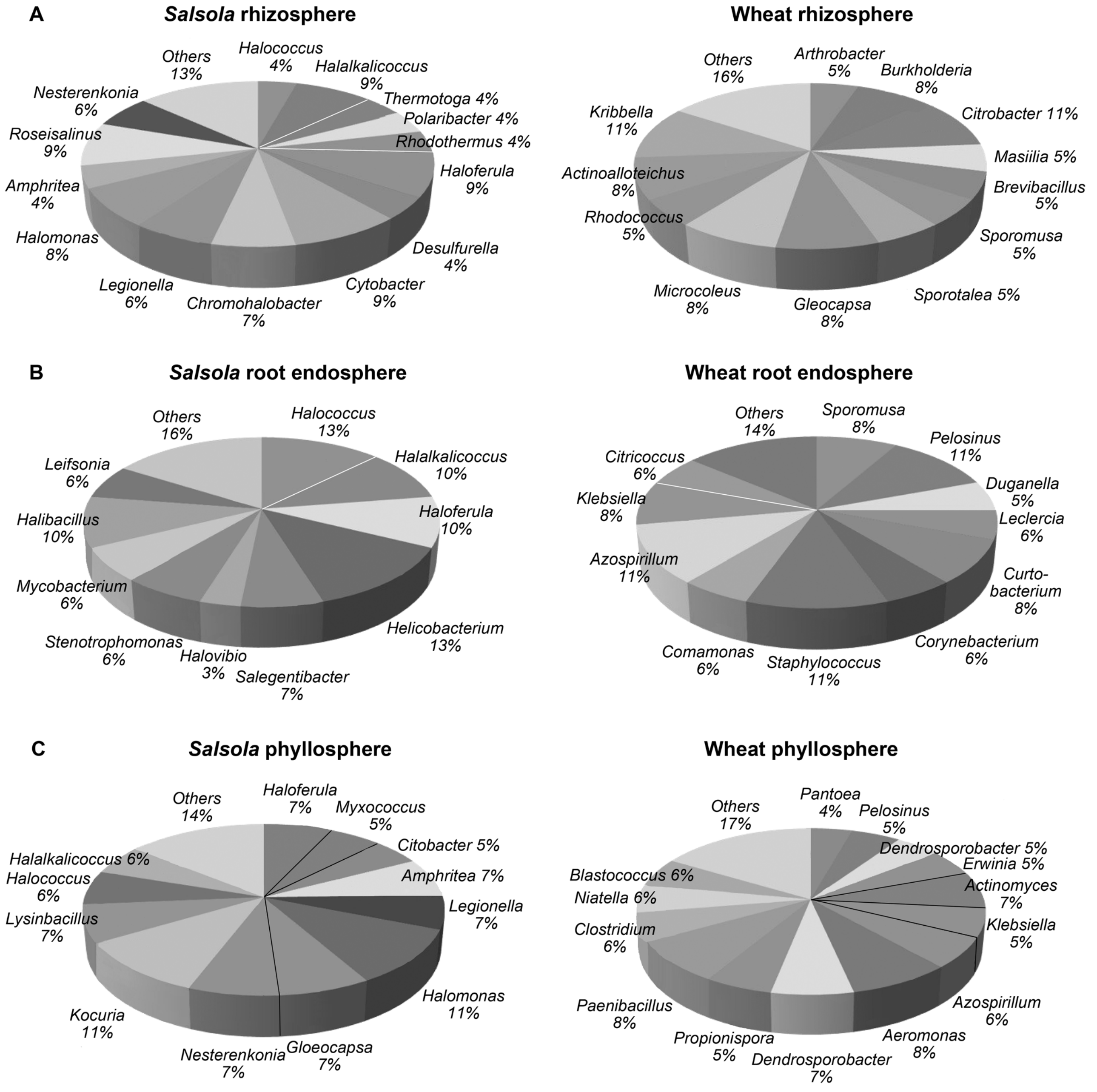

Fig. 5. Bacterial and archaeal phylotype sequences detected from rhizosphere, phyllosphere and endosphere of *S. stocksii* and wheat.

reported from saline environment and contaminated sludge samples (Valenzuela-Encinas *et al*., 2009).

Sequence analysis showed that members of *Actinobacteria* were abundant in the rhizosphere, endosphere and phyllosphere of wheat as compared to *S. stocksii*. Sequences related to genera *Nocardia*, *Microbacterium*, *Kocuria*, *Nesterenkoni*, *Marmoricola*, *Micrococcus*, *Frankia* and *Streptomyces* are commonly identified from the rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat. About 10% of the microflora from the rhizospheric soil and root endosphere of land plants was related to *Actinobacteria*, a phylum with diverse genera and ability to produce different secondary metabolites (Bulgarelli *et al*., 2012). *Actinobacteria* identified from phyllosphere have been known as biocontrol agents against fungal plant pathogens (Bodenhausen *et al*., 2013). Metagenomic analysis revealed that *Actinobacteria* are also found to be abundant in saline lands as well from marine environments (Tkavc *et al*., 2011). The third most abundant phylum in the rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat was *Firmicutes*. Sequences assigned to *Firmicutes* were more diverse in the rhizosphere and root endosphere of wheat as compared to *S. stocksii.* Among the sequences of *Firmicutes*; *Bacillus*, *Staphylococcus*, *Sporomusa*, *Clostridium*, *Sporotalea*, *Lysinibacillus*, *Salegentibacter* and *Pelosinus* were the dominant genera. A large number of bacteria related to *Firmicutes* have been isolated from low and moderate saline habitats (Lopez-Lopez *et al.*, 2010). *Bacillus* strains from halophytes have novel enzymes used for bioremediation of different pollutants in saline habitats (Liszka *et al.*, 2012). In the phyllosphere microbiome, *Bacillus* spp. behave as interesting biological control agents against plant pathogens. They cause induction of systemic resistance in the host plant and produce different antibiotics (Vasavada *et al.*, 2006; Krid *et al.*, 2010). Members related to *Cyanobacteria* were more abundant in the rhizosphere and root endosphere of *S. stocksii* as compared to wheat. Sequences retrieved from the phyllosphere showed that sequences related to *Cyanobacteria* were identified only from *S. stocksii. Prochloron*, *Phormidium* and *Gloeocapsa* were the dominant genera which have been previously reported from the soil and plant roots of saline environments (Mwirichia *et al.*, 2011).

Sequence analysis indicated that bacteria related to *Bacteroidetes* were abundant in phyllosphere as compared to rhizospheric soil and root endosphere of both *S. stocksii* and wheat. The dominant genera were *Flavobacteria*, *Gramella*, *Rhodothermus*, *Polaribacter* and *Salegentibacter. Bacteroidetes* are widely distributed in the saline and agricultural lands. They are mostly chemoorganotrophic and have abilities to degrade complex organic molecules (Vaisman and Oren, 2009). Sequences related to *Planctomycetes* were found in the rhizospheric soil and root endosphere but not detected from the phyllosphere of both plants. *Planctomycetes* have been identified as symbionts of marine sponges or algae. They have previously been studied from the marine and saline environments (Jogler *et al.*, 2011). Sequences belonging to *Acidobacteria* were abundant in the root and leaf endosphere as compared to rhizosphere of *S. stocksii* and wheat. Members of *Acidobacteria* were dominant part of microbial communities from medium saline soils and marine sediments (Ghosh *et al.*, 2010). *Chloroflexi*, *Verrucomicrobia*, *Thermotogae* were less abundant phyla which were detected only in the rhizospheric soil and root endosphere of *S. stocksii.* These phyla have previously been reported through metagenomic studies from saline and marine environments (Mukhtar *et al.*, 2016). Archaeal sequences belonging to phylum *Euryarchaeota* were abundant in the rhizospheric soil, phyllosphere and root and leaf endosphere of *S. stocksii. Halalkalicoccus*, *Halococcus* and *Halobacterium* were common genera in the rhizospheric soil, phyllosphere and root and leaf endosphere. Metagenomic analysis of marine environment indicated that members of *Euryarchaeota* have heterotrophic lifestyle. They have ability to break down complex lipids and protein molecules into fatty acids and amino acids to survive in marine habitats (Iverson *et al.*, 2012).

Conclusion

In the present study, halophyte (*S. stocksii*) microbiome was compared with wheat (non-halophyte) microbiome. Halophyte microbiome showed more diverse microbial communities as compared to wheat microbiome. *Proteobacteria* was the dominating phylum in the halophyte microbiome while *Actinobacteria* was the dominating phylum in the microbiome of wheat. Our results showed that about 36% of all identified genera were common in both *S. stocksii* and wheat while 29% were uniquely present in *S. stocksii* and 35% were present only in wheat. Halophilic bacterial genera *Amphritea*, *Chromohalobacter*, *Polaribacter*, *Nocardia*, *Salicola*, *Shewanella*, *Thermotoga*, *Steroidobacter*, *Halomonas* and *Halovibrio* and archaeal genera *Halalkalicoccus* and *Haloferula* have been reported for having important biological functions such as production of exopolysaccharides, nitrogen fixation and enrich carbon and nitrogen sources, production of pharmaceutical agents and antibiotic producing activity, bioremediation of heavy metals, degradation of cholesterol and rubber.

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Conflict of interest

The authors have no conflicts of interest to declare.

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ORIGINAL PAPER

Coomassie Blue G250 for Visualization of Active Bacteria from Lake Environment and Culture

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Abstract

Bacteria play a fundamental role in the cycling of nutrients in aquatic environments. A precise distinction between active and inactive bacteria is crucial for the description of this process. We have evaluated the usefulness of Coomassie Blue G250 for fluorescent staining of protein containing potentially highly active bacteria. We found that the G250 solution has excitation and emission properties appropriate for direct epifluorescence microscopy observations. It enables fast and effective fluorescent visualization of living, protein-rich bacteria, both in freshwater environment and culture. Our results revealed that the number of G250-stained bacteria from eutrophic lake was positively correlated with other standard bacterial activity markers, like number of bacteria containing 16S rRNA, bacterial secondary production or maximal potential leucine-aminopeptidase activity. In case of the *E. coli* culture, the percentage of bacteria visualized with G250 was similar to that of bacteria which accumulated tetracycline. Compared to other common methods utilizing fluorogenic substances for bacteria staining, the approach we evaluated is inexpensive and less hazardous (for example mutagenic) to the environment and researchers. It can be regarded as an additional or alternative method for protein rich, active bacteria staining.

Key words: active bacteria, aquatic bacteria, aquatic environment, bacterial culture, Coomassie Blue G250, fluorescence

Introduction

Coomassie Blue G250 is commonly used for the detection and visualization of proteins. It is the main chemical compound in the classical colorimetric Bradford assay (Bradford, 1976). The binding ability of G250 to proteins has been used for years for quantification of the concentration of proteins separated on polyacrylamide gels (Neumann *et al.*, 1994) or for the observation of protein rich particles under light microscope (CSP – Coomassie Staining Particles, Long and Azam, 1996). For protein detection not only absorbance of light by G250, but also its fluorogenic properties were used. For example Luo *et al.* (2006) proposed to use infrared fluorescence emission (excitation at 550 nm, emission at 720 nm) of G250 for protein detection after electrophoresis or western blotting.

Because of the high affinity of G250 to proteins, it might be, theoretically, a promising factor for the staining of bacterial cells containing proteins. Coomassie blue G250 should be particularly effective in staining live, active microorganisms with a high concentration of proteins such as ribosomal proteins of translationally active cells, structural proteins, enzymes of various metabolic pathways, and proteins placed in the cell membrane, outer membrane, cell wall and periplasmic space. Conversely, the debris of dead microorganisms, like for example empty bacteria envelopes, "cell ghosts", detected first by Zweifel and Hagström (1995), should be stained ineffectively because of their low protein content – the effect of fast protein degradation. Degradation rate in the case of dead bacterial cells exceeds 10% of intracellular proteins per hour (Gottesman and Maurizi, 1992).

There are several methods for the detection of active microorganisms. The most important of them are based on: the intracellular reduction of CTC (5-Cyano-2,3-ditolyl tetrazolium chloride), the detection of membrane integrity by LIVE&DEAD test, DNA staining by DAPI followed by propanol washing and staining by anionic or cationic membrane-specific dyes (Rodriguez *et al.*, 1992; Suller and Lloyd, 1999; Luna *et al.*, 2002; Zweifel and Hagström, 1995). However, the precise distinction of living, active bacteria from inactive ones is still an important subject of research. In addition, a majority of widespread methods of microorganism staining involve

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the use of chemical compound binding to nucleic acids, and are thus highly carcinogenic, posing a threat to researchers and the environment.

According to our knowledge, G250 is not commonly used for the direct visualization of the planktonic microorganisms. The reason for this could be that the absorbance of Coomassie Blue is inadequate for a quality visualization of small microorganisms, however it can be used for showing large protein-rich biofilms structure (Larimer *et al.*, 2016). The observed infrared light emission by Coomassie Blue (Luo *et al.*, 2006; Carlsson *et al.*, 2012) is not useful for visual observation and inconvenient for digital camera detection. However, our findings suggest that the fluorescence exhibited by this dye in the visible range of light spectrum could be promising for this purpose.

The main goal of this article was to evaluate the usefulness of G250 for the fluorescence staining of protein rich, potentially highly active bacteria. In particular: 1) characterization n of the fluorescence spectrum of Coomassie Brilliant Blue G250 in the visual light spectrum emission range; 2) testing the bacteria staining procedure on the *Escherichia coli* culture and natural aquatic microorganisms community; 3) comparing the effectiveness of bacteria staining by G250 with other methods commonly used for the evaluation of bacteria abundance and activity.

Experimental

Material and Methods

Study area and sampling. Environmental samples were taken from the pelagial zone of the deepest part of eutrophic Lake Mikołajskie (26 m max. depth, N 53°47’31.2”, E 21°34’56.3”) during two years of research in July, September 2010 and August 2011. The water was collected from six depth layers: 1) 0.5–2 m, 2) 3–5 m, 3) 7–9 m, 4) 11–13 m, 5) 16–18 m, and 6) 21–23 m. Within each layer, three one-liter subsamples were taken every 0.5 m and mixed together. Samples were subjected to further laboratory analyses within 2 h. To avoid the problem of underestimation of the number of attached bacteria, all the lake water samples were filtered through 3 μm polycarbonate filter (Millipore) to obtain sample fractions containing mostly free-living bacteria.

For *E. coli* staining with G250 and tetracycline, 3 days old LB broth culture of *E. coli* K12 strain, sensitive to tetracycline was used (incubation temp. 36°C, shaking).

The characteristics of CB G250 fluorescence spectrum. The spectrum of G250 was scanned in the 0.05% solution of G250 in sterile 0.2 μm filtered 0.1 × PBS in temp 24°C using the BioTek Synergy H1 Hybrid Reader (BioTek corp.). To confirm the results, we used Shimadzu Rf-1501 Spectrofluorometer (step 5 nm, delay 100 ms).

For testing the influence of Coomassie Blue G250 (Sigma) concentration on fluorescence emission intensity, the concentrations 0, 0.001, 0.003, 0.00625, 0.0125, 0.025, 0.05, 0.08, and 0.1% of G250 in 0.1 × PBS were measured using the maximum absorption 340 nm and the maximum emission 390 nm of G250. The measurement was done on Shimadzu Rf-1501 Spectrofluorometer.

For detecting the potential influence of proteins (albumin) on emission intensity of G250, solutions with increasing concentrations of 0, 10, 20, 40, 60, and 100 mg/l BSA (bovine serum albumin, BSA, Sigma-Aldrich) in double-distilled water were prepared. The G250 in PBS was added to each concentration (fin. conc. in BSA solution 0.05% G250 and 0.1% PBS). After 30 min of incubation in 24°C, sample fluorescence was measured (Ex. 340, Em. 390 nm). The spectrum of G250 (fin conc. 0.05%) in 40 mg/l BSA solution was scanned using the BioTek Synergy H1 Hybrid Reader (BioTek corp., step 5–10 nm, delay 100 ms).

G250 staining procedure. Half percent Coomassie Blue G250 (Sigma) stock solution in sterile, 0.2 μm filtered 1 × PBS (Sigma, in ddwater) buffer was prepared at temp. 20–24°C. A stock solution can be stored in 4–8°C, in darkness for several days. Before staining the samples, the 0.5% G250 stock solution was heated to the temperature of 20–24°C and filtered through a 0.2 μm polycarbonate filter (Millipore).

For staining, 1 ml of G250 stock solution was added to 9 ml of the sample (free-living bacteria fraction < 3 μm), mixed and incubated for 30 min in the dark, at a temperature of 20–24°C. After staining, 1 to 10 ml of stained samples (depending on the expected microorganism count number) were suspended on the surface of a 0.2 μm polycarbonate filter (Millipore), filter was washed 3 times with 5 ml 0.1 × PBS buffer and dried at 20°C for 1 h. After drying, the filter was mounted on a microscopic slide with one drop of epifluorescence Nikon immersion oil. For bacteria counting we used a computer image analyzing system composed of a Nikon epifluorescence E450 microscope, Nikon Digital Camera DXM 1200F and NIS elements software (Nikon). The bacteria were counted from digital images of 10–30 random fields for each membrane filter (from 50 to 100 bacteria per field, 1000–3000 bacteria cells per each membrane filter, picture area: 5510 μm^2). We recommend the use of UV2A microscope filter (UV2A, Ex. 330–380 nm, DM. 400 nm, Em. 420-α nm) for stained bacteria observation. In case of intense background fluorescence in UV2A filter, the B-2A filter (Ex. 450–490 nm, DM. 505 nm, Em. 520–∞ nm) could be used instead. Both filters are appropriate for the G250 fluorescence spectra.

The number of bacteria per 1 ml of the sample was calculated from the following equation: $BN = [(N \times F_{area}) / (M_{area} \times V)] \times 1.1$, where N the number of bacteria visible under epifluorescent microscope; F_{area} the area of filter with suspended bacteria; M_{area} the area of photographed filter surface; V the volume of filtered samples; 1.1 the G250 solution dilution coefficient.

Comparison of tetracycline and G250 staining of *E. coli* bacteria from culture. For the comparison of the staining results obtained using tetracycline and G250, three days old LB broth culture of tetracycline-sensitive *E. coli* K12 strain was used (in three repetitions).

E. coli culture for G250 staining was diluted 500 times in double 0.2 μm filtrated, sterile 0.1 × PBS buffer (pH 7.4), and then immediately stained according to the standard G250 staining procedure described above. Nikon UV2A filter (UV2A, Ex. 330–380 nm, DM. 400 nm, Em. 420-α nm) was used for observation. *E. coli* cells stained with G250 were observed as vivid blue cells among dark ones; the percentage of bright cells was calculated.

The test of tetracycline incorporation into *E. coli* cells was made according to Ammor *et al.* (2006), with a few modifications. Instead of the fluorometric measurement of incorporated tetracycline in bacteria suspension, the direct observation of *E. coli* cells was used. To 9 ml of the 500-times diluted samples of *E. coli* culture, we added 1 ml of tetracycline solution in deionized sterile water to obtain a final tetracycline concentration of 100 μg ml^{-1}. The samples were incubated for 2 h in 24°C. Subsequently, the bacteria were collected on 0.2 μm policarbonate filters (Millipore) and dried. After drying, the filter was mounted on a microscopic slide with one drop of epifluorescence Nikon immersion oil. Because tetracycline has a maximum excitation of 390 nm and emission of around 520 nm (Glazier and Horvath, 1995), tetracycline stained bacteria were visualized and photographed using the same filter as the G250 stained bacteria (Ex. 330–380 nm, DM. 400 nm, Em. 420–∞ nm, magnif. 1000 ×). *E. coli* with accumulated tetracycline were observed as bright green cells among dark ones; the percentage of bright cells was calculated.

Standard procedures for environmental sample analysis. The number of DAPI stainable free-living bacteria (DAPI BN) was determined by the direct counting of cells collected on 0.2 μm, black polycarbonate membrane filters (Millipore) under epifluorescence microscope (Porter and Feig, 1980). DAPI (4,6-diamidino-2-phenylindole) in final concentration 1 μg ml^{-1} was used (for 10 min., at 24°C). For bacteria counting, we used a computer image analyzing system composed of a Nikon epifluorescence E450 microscope, Nikon Digital Camera DXM 1200F and NIS elements software (Nikon). The bacteria were counted from digital images of 10 to 30 random fields for each membrane filter (from 50 to 100 bacteria per field, 1000 to 3000 bacteria cells per each membrane filter, picture area: 5510 μm^2, UV-2A Nikon fluorescence filter – Ex. 330–380 nm, DM. 400 nm, Em. 420-α nm).

Detection of microorganisms belonging to Domain Bacteria by fluorescence *in situ* hybridization (FISH) was carried out using the EUB338 (5'-GCTGCCTCC-CGTAGGAGT-3') CY3 labeled oligonucleotides developed by Amann *et al.* (1997). The bacteria were permeabilized (2% paraformaldehyde, 4°C, 18 h), retained on 0.2 μm polycarbonate filters, hybridized according to Knoll *et al.* (2001), and counterstained with DAPI. Fluorescently labeled cells were counted using CY3 Nikon filter and Nikon epifluorescence E450 microscope, as described above. The percentage of hybridized cells was used for the calculation of FISH stained bacteria abundance.

Secondary production of free-living bacteria was determined by means of thymidine (TdR, 90–97.5 Ci $nmol^{-1}$; NEN Du Pont) incorporation (Chróst and Rai, 1993).

To determine the percentage of free-living bacteria with intact cell membranes, LIVE/DEAD® BacLight™ Bacterial Viability Kit, Invitrogen Molecular Probes was used according to Invitrogen, Molecular Probes standard procedure (Luna *et al.*, 2002). The bacteria were stained in unpreserved samples within a few hours after the samples were taken. After staining, the bacteria were collected on 0.2 μm black polycarbonate membrane filters (Millipore) and dried. After drying, the filters were mounted in BacLight™ mounting oil on microscopic slides. For LIVE/DEAD® BacLight™ stained bacteria visualization we used the B2A Nikon filter (Ex. 450–490 nm, DM. 500 nm, Em. > 515 nm). The green (MEM+) and red (MEM-) colored bacteria (with integral and damaged membranes, respectively) were counted from digital images of 10–30 random fields for each membrane filter, as with DAPI staining. We calculated the percentage of MEM+ bacteria in the total, DAPI visible bacteria number.

Maximal potential leucine-aminopeptidase activity (V_{max} AMP) was measured fluorometrically (Chróst, 1990; Kiersztyn *et al.*, 2012). The plot of the reaction velocity (v) against substrate concentration [S] displayed a rectangular hyperbolic relationship, described by the equation $v = V_{max} \times [S] / (K_m + [S])$. Nonlinear regression analysis was applied to calculate the kinetic parameters of enzymatic reactions using Origin 8.6 software (OriginLab Corporation, Northampton, USA).

Dissolved Organic Carbon (DOC) concentration was measured as $C\text{-}CO_2$ concentration after complete burning in O_2 atmosphere using Shimadzu TOC 5050A analyzer (Tupas *et al.*, 1994).

The statistical analyses were performed using Origin 8.6 (OriginLab Corporation, USA) and Statistica (StatSoft, Poland). Pearson correlation matrices, multidimensional scaling (based on the correlation matrix of analyzed parameters), one-way ANOVA test and Mann-Whitney U test were used for data analysis.

Results

Analysis of the fluorescence spectrum of G250. In the visible range of light spectrum, 0.05% G250 in 0.1 PBS (pH 7.4) exhibited two excitation-emission maximum pairs: the first at Ex. 340 nm, Em. 390 nm and the second at Ex. 440 nm, Em. 490 nm. The normalized spectra of the relative fluorescence intensities of G250 are shown in Fig. 1A and Fig. 1B. The emission of fluorescence at 490 nm was less intensive than at 390 nm. In both emission-excitation pairs it was possible to observe G250-labeled microorganisms, under epifluorescence microscope equipped with a set of standard Nikon filters UV-2A (Ex.: 330–380; DM: 400; Em.: 420 – ∞) or B-2A (Ex.: 450–490 nm, DM: 505, Em.: 520 – ∞). The numbers of bacteria observed using the two different wavelengths were positively, significantly correlated ($r = 0.95$, $p = 0.004$) and stayed in the same range. The mean values of G250-stained bacteria number at all depths were $2.6 \pm 0.8 \times 10^5$ cell ml^{-1} and $3.9 \pm 1.2 \times 10^5$ cell ml^{-1} for UV-2A and B-2A filters, respectively, and were not significantly different from each other at the statistical probability level of $p = 0.05$ (using the one-way ANOVA test). Some typical microscopy images of bacteria from lake Mikołajskie stained with Coomassie G250 and the DAPI-stained bacteria are shown in Fig. 2.

Relationship of G250 concentration and G250 fluorescence. The relationship between the concentration and fluorescence emission intensity of G250 dye at Ex. 340 nm and Em. 390 nm is presented in Fig. 3. The highest fluorescence intensity was observed when Coomassie Blue G250 concentration was about 0.05%. This concentration was chosen as the final optimal concentration of G250 for the microorganism staining procedure.

Influence of BSA on G250 fluorescence. We did not observe significant linear correlation between relative fluorescence of G250 and albumin concentration ($r^2 = 0.34$, $p = 0.13$). In a solution containing BSA and G250 we observed an additional peak of albumin fluorescence at Ex. 280 and Em. 340. These values are distant from the maximum excitation / emission wavelength for G250.

Comparison of *E. coli* staining with tetracycline and G250. Sample microscopic images of *E. coli* cells observed after tetracycline treatment (A), stained with G250 (B) and unstained (C) are shown in Fig. 4. The

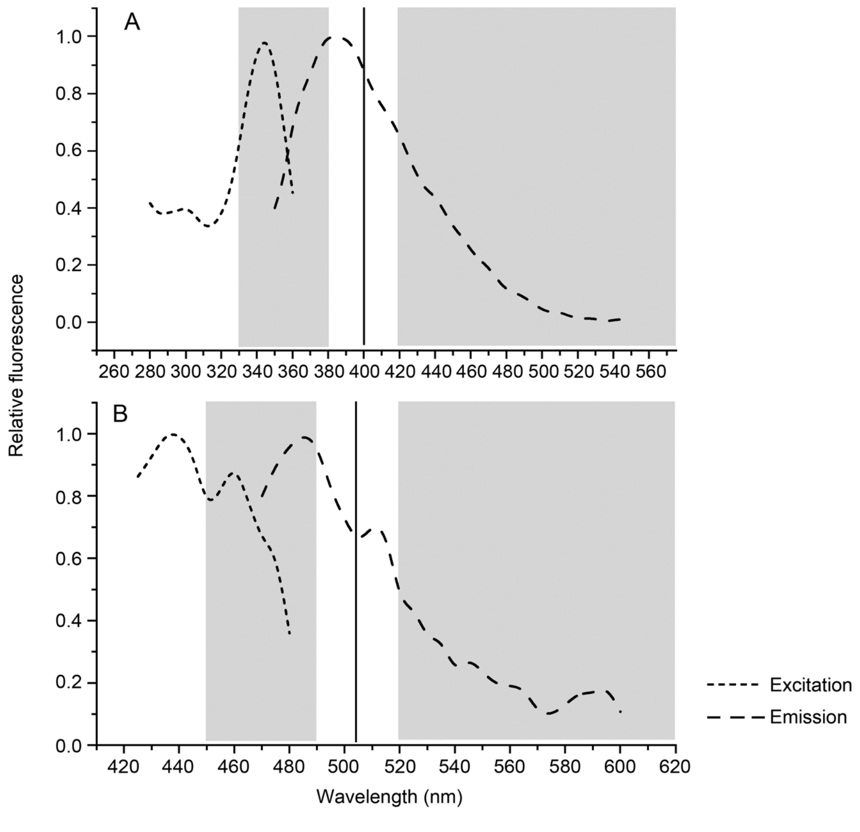

Fig. 1. The normalized, relative fluorescence spectrum of 0.05% Coomassie Blue G250 in 0.1 × PBS buffer excited by A) UV light and B) blue light. The grey boxes represent the excitation and emission parameters of epifluorescence filters used for microscopic observation of G250-stained microorganisms: Nikon UV2A, Ex. 330–380 nm, DM. 400 nm, Em. 420-α nm and Nikon B-2A, Ex. 450–490 nm, DM. 505 nm, Em. 520–∞ nm respectively. The vertical line shows the wavelength barrier of dichromatic mirror.

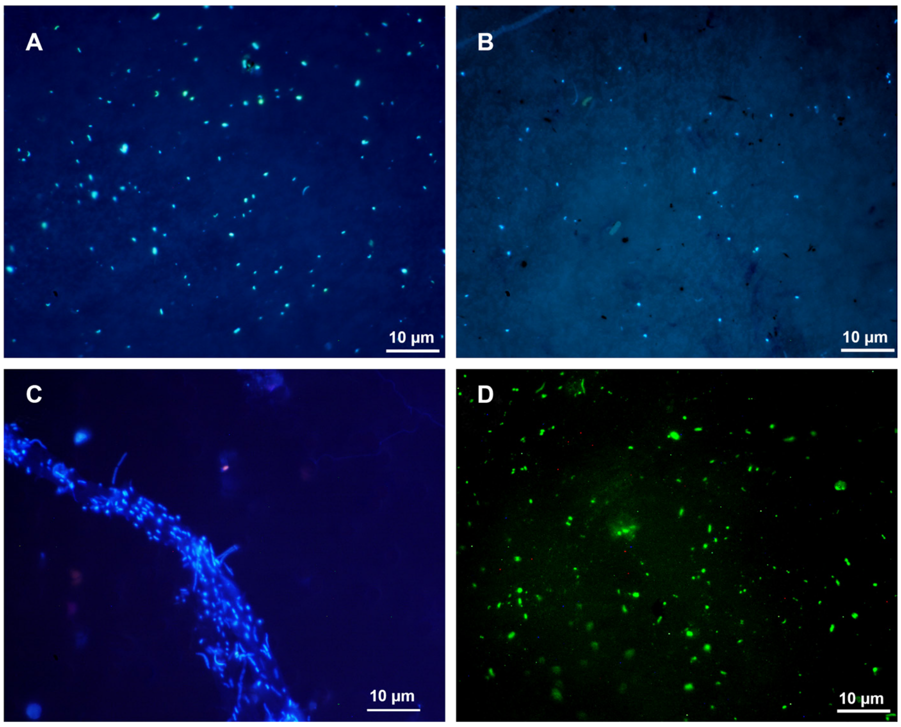

Fig. 2. Sample microscopic images of bacteria from Lake Mikołajskie: A) DAPI-stained bacteria (UV2A Nikon filter), B) G250-stained bacteria (UV2A Nikon filter), C) G250-stained bacteria (UV2A Nikon filter), D) G250-stained bacteria (B-2A Nikon filter). Mag. 1000×, Depth: 0.5–2 m, Lake Mikołajskie, August 2011. Image C) shows attached bacteria from unfiltered sample. The remaining images depict microorganisms from the 0.2–3.0 μm fraction.

staining of *E. coli* with G250 or tetracycline yielded similar images of vivid-bright cells of bacteria among dark ones (Fig. 4A, B). The mean percentages of bright cells among total visible cells were similar for G250 and tetracycline staining (40.2 ± 2.5% and 41.4 ± 3.2%, respectively; Mann-Whitney U test $p = 0.71$). For the unstained sample, an image of dark, hardly visible *E. coli* cells was obtained (Fig. 4C).

Comparison of the number of aquatic bacteria visualized by G250 staining with other indicators of potential bacteria activity. To evaluate the usefulness of G250 for aquatic, active bacteria detection, we compared the number of Coomassie Blue G250 stained bacteria (CB BN) with parameters characterizing the abundance and vitality-activity of microorganism communities like: DAPI stained cell abundance (DAPI BN), secondary bacterial production (Bacterial Prod.), the number of cells belonging to the domain Bacteria containing rRNA visualized using FISH staining procedure (FISH BN), maximal potential activity of aminopeptidase (V_{max}AMP), the live cell number (cells with intact cell membrane, MEM + BN), and DOC concentration. The matrix of linear correlation coefficients between these parameters is shown in Table I. We found

Table I

Matrix of Pearson correlation coefficients between the following parameters: DAPI BN – DAPI-stained cell abundance; Bacterial Prod. – secondary bacterial production; FISH BN – the number of Bacteria containing rRNA visualized using FISH staining procedure; VmaxAMP – maximal potential activity of Leucine aminopeptidase; MEM + BN – bacteria cells with intact cell membrane; CB BN – number of G250-stained bacteria; DOC con. – concentration of dissolved organic carbon. Stars indicate that the correlation is significant at the level of $p < 0.05$.

	DAPI BN	CB BN	Bacterial Prod.	Vmax AMP	MEM + BN	DOC conc.	FISH BN
DAPI BN	–						
CB BN	−0.62*	–					
Bacterial Prod.	−0.31	0.79*	–				
Vmax AMP	0.36	0.71*	0.82*	–			
MEM+ BN	−0.43	−0.36	−0.27	−0.14	–		
DOC con.	−0.26	0.69*	0.60*	0.57	0.30	–	
FISH BN	0.25	0.84*	0.52	0.56	−0.48	0.59	–

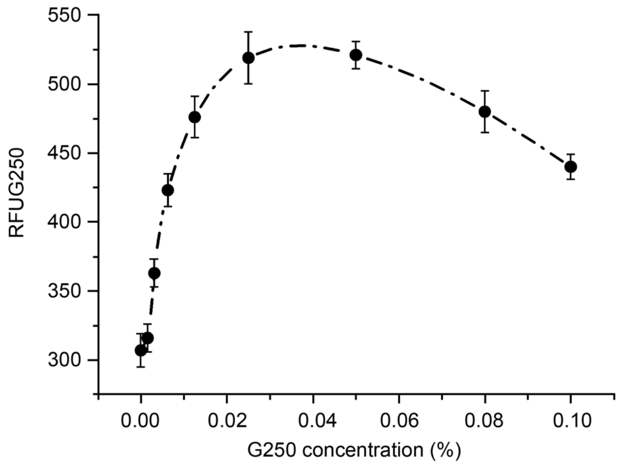

Fig. 3. Relationship between Coomassie Blue G250 concentration (in 0.1 × PBS, pH 7.4) and relative fluorescence emission intensity at 390 nm after excitation at 340 nm.

positive and significant correlations between CB BN and three bacterial activity indicators. These correlations were especially strong for secondary bacterial production ($r = 0.79$, $p < 0.001$) or FISH BN ($r = 0.84$, $p < 0.001$). We found no correlation of CB BN with MEM + bacteria number and a negative, significant correlation with DAPI BN ($r = -0.69$, $p = 0.007$). Fig. 5, reports the DAPI stainable bacteria number and Coomassie stainable bacteria number, together with bacterial production, at various depths during two years of research. The decrease in CB BN (but not DAPI BN) in August 2011 corresponded with a considerable decrease in secondary bacterial production. This suggests that only productive, live bacteria may be susceptible to effective staining with G250. Generally, the percentage of G250 bacteria in DAPI BN ranged from 1.9% to 82.7%, with a mean value of 36.8% and a median of 40.9%.

Discussion

This article evaluates the use of a well-known dye Coomassie Blue G250 for fluorescent visualization of active bacteria. To obtain the anionic form of G250 in our samples, we buffered the lake water samples with 0.1 × PBS (pH 7.4). The anionic form of G250 binds predominantly to arginine, lysine and histidine by electrostatic attraction, creating a complex, ensuring effective staining of proteins (Georgiou *et al.*, 2008). Given the well-known problem of fluorescence self-quenching (Penzkofer and Lu, 1986), we established experimentally the optimal concentration of G250 at 0.05% in PBS solution. Though the latter provided the most intense fluorescence at 340 nm excitation and 390 nm emission, concentrations lower and higher than 0.05% still allowed effective emission detection. We did not observe significant changes in fluorescence in the presence of, even high, concentrations of proteins (*i.e.*, BSA). We observed only slight emission light intensification (at 390 nm) with increasing BSA concentration, and additional BSA excitation-emission peaks (max. emission at 340 nm). It is interesting to note that the BSA emission peak is in the range of the G250 excitation maximum. This may be explained by the absorbance of BSA emission light by G250 (Katrahalli *et al.*, 2010), causing a slight increase in G250 fluorescence, especially under high BSA concentrations.

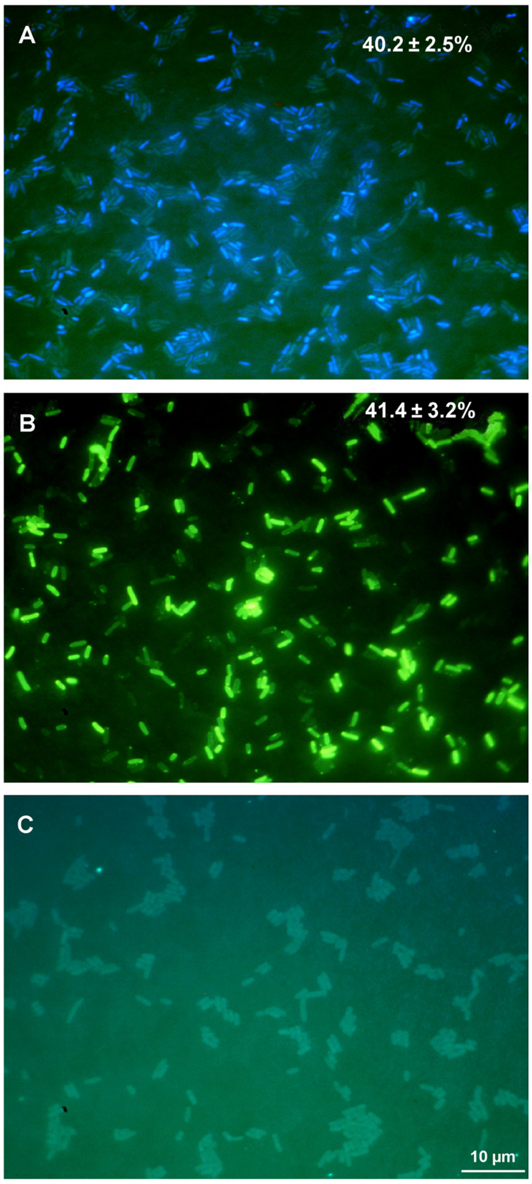

Fig. 4. Microscopic image of *E. coli* from three days' culture in LB medium: A) G250-stained, B) with tetracycline accumulated, and C) not stained. The percentages on image (A) and (B) indicate the fraction of bright cells in the total dark and bright cell numbers.

The G250 staining mechanism is different from that of the most common dyes used for bacteria visualization, like SYBR-I, SYBR-II, or DAPI, which is based on the conversion of the dye to its fluorescent form after

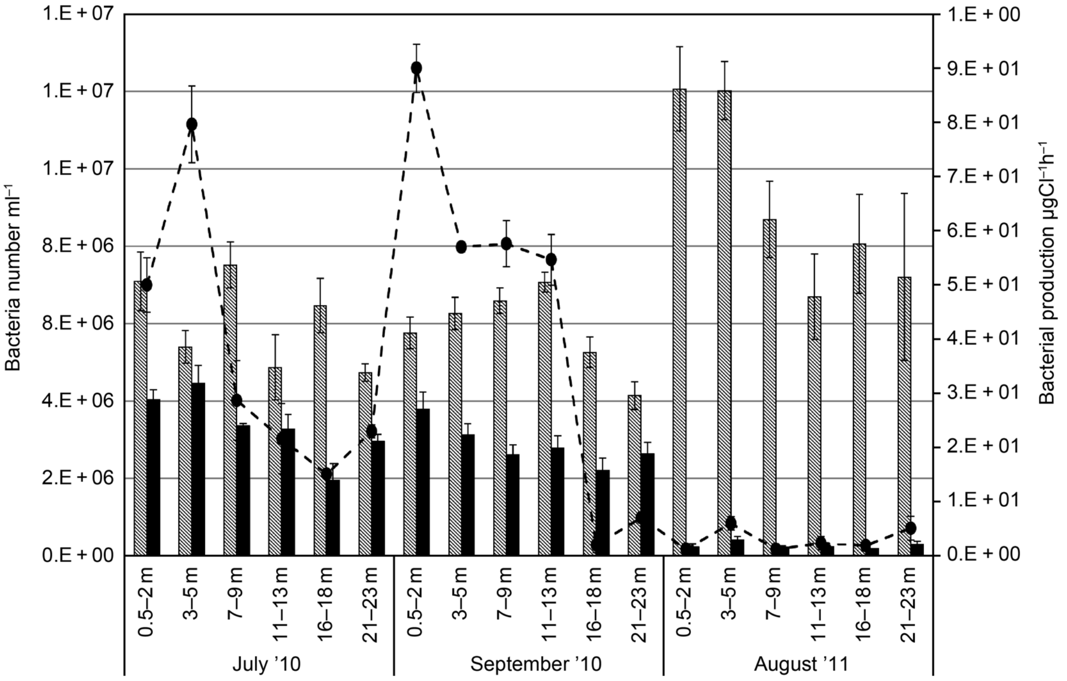

Fig. 5. The comparison of DAPI-stained bacteria number (dashed bars, left axis), G250-stained bacteria number (black bars, left axis) and bacterial secondary production (black dots, right axis) from different depths of lake Mikołajskie in July, September 2010 and August 2011.

interaction with DNA (Lebaron *et al.*, 1998). In the case of Coomassie blue G250, fluorescence intensity does not change after protein binding, and the visualization of protein rich bacteria is possible due to the contrast between the concentrated Coomassie bound to microorganisms and the background fluorescence. This background fluorescence is primarily a result of G250 binding to dissolved proteins remaining on the filter surface after sample filtration. A practical consequence of this fact is that the filter must be profusely washed prior to observation to provide high quality pictures.

Cellular proteins constitute from 15% to nearly 50% of the volume of bacteria, and more than 60% of bacterial dry weight (Simon and Azam, 1989). The protein synthesis rate calculated on the basis of H^3-leucin incorporation has been used for years as an indicator of live bacterial production (Bastviken and Tranvik, 2001) and bacterial incorporation of amino acids has been used for active bacteria detection by autoradiography (Simon, 1988). In dead bacterial cells, the protein content decreases rapidly after inhibition of protein synthesis due to the fast internal protein hydrolysis rate (more than 10% of internal proteins per hour) (Gottesman and Maurizi, 1992). This led us to consider protein richness as an indicator of bacterial cell activity and physiological condition. We tested the ability of G250 to selectively stain potentially live bacterial cells by comparing the staining results obtained by adding G250 and tetracycline to diluted samples of *E. coli* culture. We chose tetracycline for two reasons. First, it is a bacteriostatic and does not lead to fast disruption of affected bacteria (cells may still be visualized) – it only inhibits protein synthesis by binding reversibly to the 30S ribosomal subunit at a position that blocks the binding of the aminoacyl-tRNA to the acceptor site on the mRNA-ribosome complex. Second, tetracycline is fluorescent with an excitation maximum at 490 nm and emission maximum at 520 nm (Glazier and Horvath, 1995), enabling the observation of cells with accumulated tetracycline and measuring the accumulation rate of tetracycline in bacterial cells by detecting cell fluorescence (Ammor *et al.*, 2006). Only active, energy-dependent transport of tetracycline followed by binding to active ribosomes leads to intracellular accumulation of tetracycline at concentrations greater than those in the medium (Franklin and Snow, 2005). We observed similar percentages of tetracycline visualized and G250 stained *E. coli* cells in the total number of *E. coli* cells from culture, which suggests that G250 stains mainly active cells with a high protein synthesis rate.

The assumption that G250 primarily stains live bacteria is supported by environmental analysis. The analysis was conducted on the free-living bacterial fraction (< 3 µm lake water filtrate through 3.0 µm filter) to minimize potential measurement error arising from the strong heterogeneity of samples containing bacteria living in deep biofilm layers (Griebler *et al.*, 2001). Bacteria living in deep biofilm compartments require special treatment prior to their counting like, for example, gentle sonication to release them from the biofilm matrix. Such treatment might influence the measurement of some parameters used as activity indicators and had a negative influence on the vitality of the cells (Böllmann *et al.*, 2016). For example it could lead to protease release from damaged cells or change cell membrane integrity, influencing L&D test results. This does not mean that attached bacteria may not be visualized using G250. Without preliminary filtration it is possible to obtain images of well visible, G250-stained attached bacteria, as shown in Fig. 2C.

In contrast to the large *E. coli* cells from culture, which were visible (though barely) under epifluorescence microscope even without staining, the small (usually 0.2–1.0 µm of diameter, Simon, 1988) bacteria living in natural lake water, were visible and could be counted only after staining. We compared the number of G250-stainable bacteria with several parameters describing bacterial community abundance and activity (Table I), finding a positive correlation between G250-stained bacteria number and number of bacteria obtained using FISH. The FISH method allows visualization only of cells containing a large number of ribosomes. Such cells may generally be treated as translationally active, live cells (Karner *et al.*, 1997; Smith and del Giorgio, 2003; Freese *et al.*, 2006). The strong linear correlation between the number of FISH-detected and G250-detected bacteria supports the thesis that the G250-staining procedure visualizes bacteria which are, at the moment of staining or soon before it, in good vital condition, as suggested by high ribosome concentration. This thesis is also consistent with some of our other observations. First, the strong, significant correlation between G250-stained bacteria number and secondary production based on H^3-thymidine incorporation rate, which is used as an indicator of bacteria divisions (Fuhrman and Azam, 1982; Bengtsson *et al.*, 2012). Second, the fact that the rapid decrease in bacterial secondary production in August 2011 (Fig. 5) was accompanied by a parallel decrease in G250-stained bacterial abundance. Third, the significant positive correlation between the maximal potential activity of aminopeptidase and G250-stained bacteria number. Extracellular aminopeptidase is predominantly produced by bacteria, and is located in the periplasmic space or built into bacterial walls (Chróst, 1990; Kiersztyn *et al.*, 2012). Fourth, the strong correlation between G250-stained bacteria number and DOC concentration. DOC is the main source of carbon for free-living bacteria and an important factor of the bottom-up mechanism controlling bacteria activity in lake waters. All these observations lead us to conclude that G250 may reasonably be used for the staining of active bacteria.

Our conclusion also explains the fact that we did not find any positive correlation between the number of DAPI stained bacteria, live bacteria according to L&D test and G250. DAPI has been shown to stain not only active bacteria cells, but also empty bacterial envelopes (ghost cells) or micro-particles with DNA adsorbed (Zweifel and Hagström, 1995). On the basis of many activity markers, it has been found that in various aquatic environments the percentage of live cells in the total DAPI-stained cell count varies from a few percent up to 90%, depending on sampling period and environmental conditions (Haglund *et al.*, 2003; Warkentin *et al.*, 2007).

The lack of correlation between the number of live bacteria visualized by L&D test and that of G250 stained bacteria may be due to the specific properties of L&D test. This test is based on the assessment of cell membrane integrity and contains two dyes: red emission fluorescent propidium iodide which cannot penetrate the intact bacterial cell membrane and green emission SYTO® 9 which can penetrate the cell membrane easily. Both of them require nucleoid presence inside the cell for effective staining. Staining by propidium iodide is likely to cause an underestimation of the actual number of dead cells due to the fact that, apart from the predation of protozoa on bacteria, another important cause of bacterial mortality is viral lysis, resulting in DNA fragmentation (Shibata *et al.*, 1997). This problem is compounded by the fact that the presence of propidium iodide in a cell (suggesting that the cell is dead) might be an effect of small cell membrane damage during sample preparation. The lack of significant correlation between MEM+ cell abundance and bacterial production or aminopeptidase activity also point to the uncertainty of the L&D method for live bacteria detection in natural lake samples.

We conclude that the staining of bacterial cells using G250 offers a reasonably accurate assessment of the number of active bacteria in culture and freshwater environment. It may thus be regarded as a viable alternative or supplement to other microscopic methods of direct active bacteria visualization. G250 is inappropriate for staining bacteria with low activity and in dormant state. Its usefulness lies in its ability to visualize highly active bacterial cells – the fraction of the bacterial community mainly responsible for the global bio-geochemical processes in the natural environment.

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ORIGINAL PAPER

Bacterial Communities from the Arsenic Mine in Złoty Stok, Sudety Mountains, Poland

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Abstract

Investigations of bacterial communities and characterization of mineralogy of the environment in the Złoty Stok As-Au deposit were carried out. PXRD analysis revealed the presence of picropharmacolite as the most common secondary arsenic mineral in the mine. Total DNA was extracted from slime streams or slime biofilms samples to investigate the bacterial communities. PCR amplification of 16S rDNA was performed followed by subcloning of its products. Over 170 clones were analyzed by means of RFLP method. Eight group of clones representing different restriction patterns were identified. The nucleotide sequences of their inserts suggest that bacteria present in the mine environment belong to: *Flavobacteria*, *Sphingobacteriia*, *Bacteroides*, *Proteobacteria*, *Mollicutes* and *Firmicutes*. The metagenomic approach allows to demonstrate a higher diversity of microbiota than classical microbiological studies of cultivable isolates.

Key words: arsenic mine ecosystem, metagenomic approach, microbial community, supergene minerals

Introduction

Bacteria are able to grow and live as single (planktonic) cells but it is known that interactions between cells and cell-to-cell signalling exist. All processes in which a respective number of bacterial cells are involved and carried out is referred to as quorum sensing (Hammer and Bassler, 2003; Leggett *et al.*, 2014). This effect is controlled by chemical signal molecules termed autoinductors that are produced by bacteria and released to the environment. The concentration of those chemicals increases as a function of increasing cell-population density and after gaining minimal stimulatory concentration it leads to an alteration in gene expression (Miller and Bassler, 2001; Waters and Bassler, 2005). Thus, bacteria are able to form intricate multicellular communities, referred to as biofilms, which are the most ancient multicellular life forms on Earth (Pamp *et al.*, 2009; Römling and Balsalobre, 2012). These communities can form various sizes and shapes. Biofilms can be formed by cells surrounded by extracellular polymeric substances (EPS) (Wu and Xi, 2009), which are one of the components of the so-called matrix or extracellular matrix. Apart from that, proteins, extracellular DNA (eDNA), cell lysis products, water and organic matter from the surrounding environment are also involved in composition of the matrix (Narváez *et al.*, 2005; Pamp *et al.*, 2009; Wu and Xi, 2009). The matrix provides structural stability of the biofilm and allows cells to live as a community, protecting them from harmful physical and chemical factors like osmotic shock, UV radiation, predators or heavy metals and allows cells to interact with each other (Gonzales-Toril *et al.*, 2003; Jiao *et al.*, 2011). The structure of biofilms allows the bacteria living inside to function in an extreme environment. It is known that such communities can be found in mines, where nutrients are limited and pH is low (Drewniak *et al.*, 2008; Wu and Xi, 2009). Biofilms are very common in the environment and play a key role in many complex biochemical processes, which require cooperation. This microbial community also provides an ideal environment for horizontal gene transfer, which leads to genetic diversity as well as microbial evolution (Elias

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and Banin, 2012). The aim of this study was to initially characterize the ecosystem of Złoty Stok mine including the studies of its mineralogy and identification of bacterial communities (biofilm).

Experimental

Materials and Methods

Geology and mining history of the sample collection site. The Złoty Stok As-Au deposit is the largest gold deposit in the Polish part of the Western Sudetes. This deposit is located in the northern part of the Złoty Stok-Skrzynka shear zone. This zone is composed of mica schists, amphibolites, amphibole schists, myllonites and gneisses. These rocks are intercalated by marbles, serpentinites and coarse-grained gneisses. Main ore minerals, *i.e.* löllingite, arsenopyrite, pyrite, pyrrhotite and magnetite occur in black and green serpentinites and in diopside-tremolite rocks, which are connected with marble lenses. Impregnations of iron sulphides and arsenides also occur in mica schists, amphibolites and calc-silicate rocks. The precipitation of ore minerals is caused by regional metamorphic processes and migration of hydrothermal fluids from Kłodzko-Złoty Stok granitoid. The mining tradition in this area is very long. The first document confirming gold mining dates back to 1273. In 1709 the production of arsenic oxide started in Złoty Stok. The adit was built between 1916 and 1918. Gold mining and arsenic trioxide production were discontinued in 1962.

Sampling. Samples of supergene minerals and microbial communities were collected from a small drainage in the Gertruda adit. Microbial communities forming slime streamers or slime biofilms were found only in wet or completely submerged sites (Fig. 1). The occurrence of these microbial colonies is limited to the forefront of the mine. Five separate samples of slime streamers from different parts of the sampling area completely filled with water from the biofilm habitat were collected into sterile 50 ml polyethylene flasks. Supergene minerals coexisting with the microbial colonies were collected separately into small polyethylene containers.

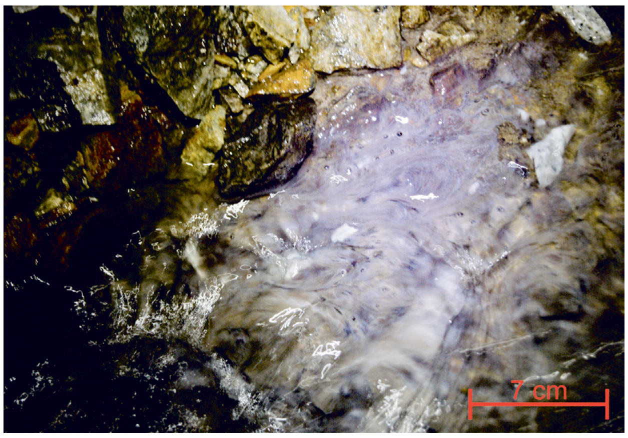

Fig. 1. Slime biofilm occurred only in wet places.

Surface morphology analyses of slime streamers and XRD analyses. Details of the surface morphology were studied with Sigma scanning electron microscopes (Carl Zeiss Microscopy GmbH, Jena, Germany) with an EDS detector (Bruker). We used an energy dispersion analyser (EDS) for qualitative chemical analysis. Powder X-Ray Diffraction (PXRD) analyses were made with an X'Pert Pro diffractometer in the Institute of Geochemistry, Mineralogy and Petrology, Faculty of Geology, University of Warsaw. The radiation was CoKα (1.73425, step of measurement = 0.02, 2.5131–75.9891°2Th).

Genomic DNA isolation. The DNA was isolated from environmental samples using a modified method developed by Ausubel and co-authors (Ausubel *et al.*, 2003). One gram of the sample material was placed into a sterile Eppendorf tube and centrifuged at 14.000 rpm at 4°C for 7 min. The supernatant was discarded and the pellet was suspended in 450 μl of TE buffer containing 4 mg/ml lysozyme and incubated for 1 h at 37°C on a horizontal shaker at 100 rpm/min. The lysate was supplemented with 50 μl 10% SDS and 10 μl of 20 mg/ml proteinase K and incubated for 1 h at 37°C. In order to remove polysaccharides 100 μl of 5 M NaCl solution was added and vortexed, followed by 80 μl of 10% CTAB and 0.7 M NaCl vortexing and incubation for 10 min at 65°C. Samples were extracted two times with an equal volume of chloroform (500 μl) and centrifuged at 14.500 rpm for 5 min at 4°C. The aqueous phases were transferred into fresh Eppendorf tubes and DNA was precipitated by adding 0.7 volume of isopropanol. After gentle mixing the samples were left for 20 min at –20°C. Precipitates were collected by centrifugation at 7.000 rpm for 10 min at 4°C. The pellets were washed with 70% ethanol, dried and resuspended in 40 μl of autoclaved double distilled water. The quality of genomic DNA was analysed by agarose gel electrophoresis.

Amplification and cloning of 16S rDNA sequences. The nearly complete 16S rDNA gene sequence was amplified by PCR with primers 16S f 5'-AGG CAG CAG TGG GGA ATA TT-3' and 16S r 5'-ACT TGA CGT CAT CCC CAC CT-3' (GeneAmp® PCR System 9700 – Applied Biosystems). PCR reactions were carried in a final volume of 25 μl. The reaction mixture contained 10 pmoles of each primer, 0.1 mmole of each dNTP, 1.0 U of *Taq* DNA polymerase, 2.5 μl of 10× reaction buffer (Novazym) and 100 ng of template genomic DNA. After initial denaturation at 94°C for 2 min. 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, polymerisation at 72°C

Table I
Similarities of the 16S rDNA clone sequences to sequences retrieved from databases.

Clone	Closest relative (accession no.)	Percent of homology	Phylogenetic classification
UPR_1 (KP772348)	Uncultured *Cytophagales* bacterium (FJ517047)	96.4	*Sphingobacteriia*
UPR_2 (KP772349)	*Pedobacter* sp. (KC252876)	97.5	*Sphingobacteriia*
UPR_3 (KP772350)	*Flavobacterium* sp. (KC969641)	97.1	*Flavobacteria*
UPR_4 (KP772351)	*Pseudomonas* sp. (JQ977323)	99.0	γ-*Proteobacteria*
UPR_5 (KP772352)	*Methylotenera versatilis* (NR074693)	96.8	β-*Proteobacteria*
UPR_6 (KP772353)	*Flavobacterium* sp. (JQ687101)	97.3	*Flavobacteria*
UPR_7 (KP772354)	*Mollicutes* bacterium (AY297808)	96.8	*Mollicutes*
UPR_8 (KP772355)	*Flavobacterium* sp. (KF499997)	95.9	*Flavobacteria*
UPR_9 (KP772356)	*Flavobacterium* sp. (KC969642)	96.3	*Flavobacteria*
UPR_10 (KP772357)	*Flavobacterium* sp. (JF694002)	98.5	*Flavobacteria*
UPR_11 (KP772358)	Uncultured *Hyphomicrobiaceae* bacterium (EU266801)	94.3	α-*Proteobacteria*
UPR_12 (KP772359)	Uncultured *Bacteroidetes* bacterium (JN656899)	97.0	*Bacteroides*
UPR_13 (KP772360)	*Fusibacter* sp.(AF491333)	95.2	*Firmicutes*
UPR_14 (KP772361)	*Pseudomonas frederiksbergensis* (KF424295)	97.5	γ-*Proteobacteria*
UPR_15 (KP772362)	*Flavobacterium* sp. (JF693993)	95.8	*Flavobacteria*
UPR_16 (KP772363)	*Fusibacter* sp. (KJ420408)	95.9	*Firmicutes*
UPR_17 (KP772364)	*Flavobacterium* sp. (JQ778313)	94.9	*Flavobacteria*
UPR_18 (KP772365)	*Aeromonas* sp. (KF278599)	98.8	γ-*Proteobacteria*

for 1 min followed by final polymerisation at 72°C for 4 min were applied. The PCR products were analysed on 1% agarose gels and cloned into p-GEM T Easy vector using a cloning kit (Promega – pGEM®-T Easy Vector Systems), following the manufacturer's protocol, and introduced into competent *Escherichia coli* cells, (supplied with the cloning kit). Transformants were selected by α-complementation test and grown overnight on plates with LB medium containing ampicillin (50 μg/ml), IPTG (200 mg/ml) and X-Gal (20 mg/ml). A total number of 170 white colonies were collected and plasmid DNA was isolated. The recombinant plasmids containing 16S rDNA gene fragments were amplified using vector primers: M13F (–47) 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3' and M13R 5'-TCA CAC AGG AAA CAG CTA TGA C-3'. The reaction mixture was prepared as described above.

Analysis of 16S rDNA sequences. The amplified 16S rDNA products were characterised by hydrolysis with endonuclease *Cfo*I at 37°C for 2 h. The digestion products were separated in 1.7% agarose gel electrophoresis for 90 min at 80 V. Bands were visualized by staining with ethidium bromide and UV illumination. RFLP patterns were grouped visually into OTUs (operational taxonomic units), and representatives of each OTU were selected for insert sequencing and analysis (Schloss and Handelsman, 2006; Schloss and Westcott, 2011; Römling and Balsalobre, 2012). Clones description was given as follows: UPR_X, where X is the clone number. They were sequenced with a BigDye® terminator v3.1 cycle sequencing kit on an ABI 3100 automated capillary DNA sequencer (Applied Biosystems, USA). The accession numbers of sequences generated in this study are listed in Table I. The sequences were analysed with the BLAST program in the NCBI Genbank database. For phylogenetic analysis, the sequences were aligned using ClustalW. The phylogenetic tree was constructed with MEGA 6 after initial analyses in Neighbour-Joining distance program.

Results

Supergene minerals present at the site of occurrence of microbial communities. The presence of picropharmacolite was confirmed by PXRD analysis. It is the most common secondary arsenic mineral in the Gertruda adit. It forms tiny spheroidal aggregates, up to 0.4 cm in diameter, comprising thin-acicular crystals up to 2 mm in length. Elongated crystals parallel to the *c* axis are the most common. This mineral is colourless or white, transparent to translucent with silky lustre. Aggregates of picropharmacolite growth can be seen separately on the surfaces of fissures inside of calc-silicate rocks, which contain weathered löllingite and arsenopyrite. Sometimes picropharmacolite forms white coatings up to several square centimetres. Qualitative EDS analysis corresponds to pure picropharmacolite without other elements (*e.g.* Co, Ni and Zn) which may substitute magnesium in the structure of this mineral. Hörnesite coexists with picropharmacolite. It forms white, ball-like aggregates composed of

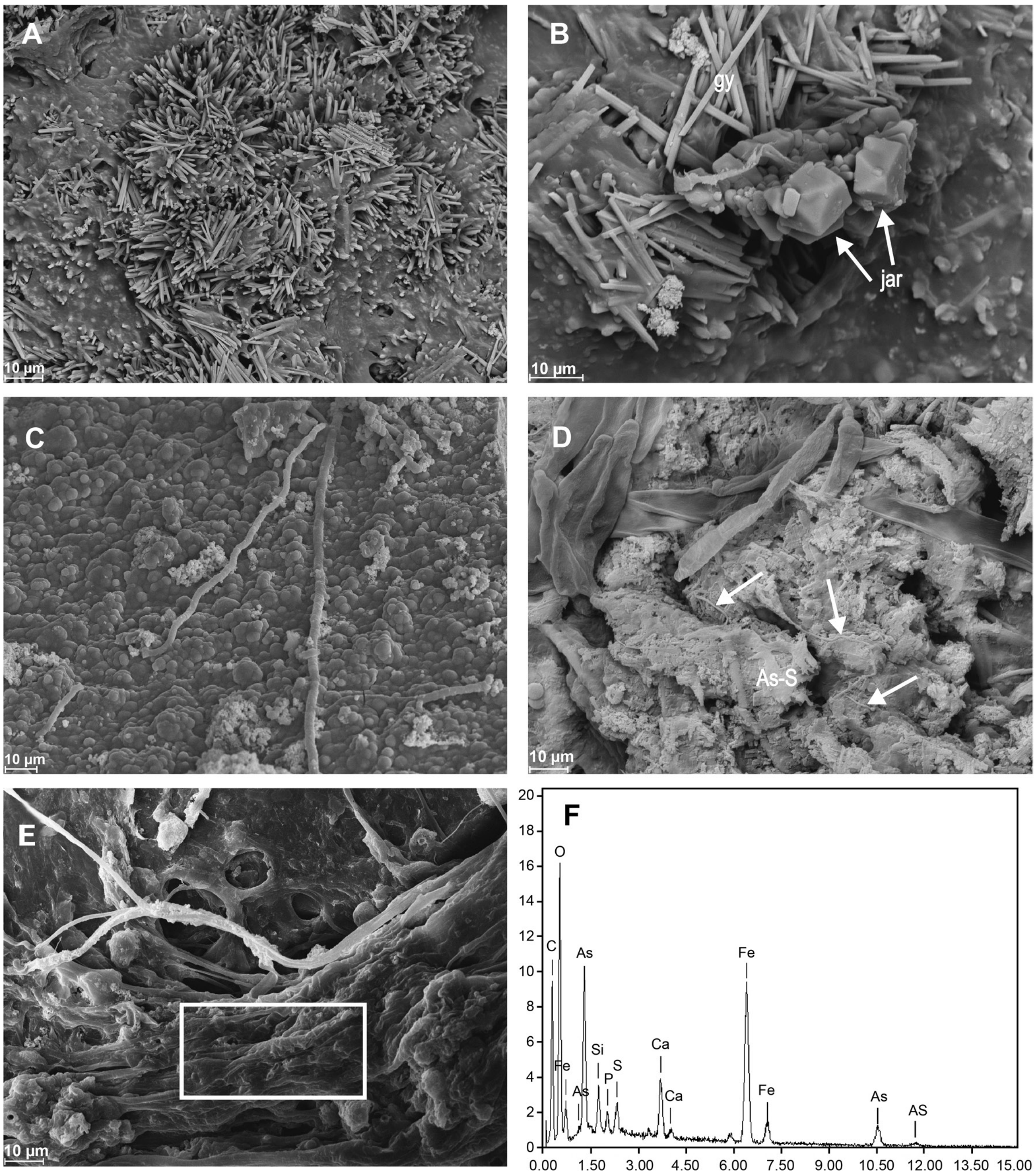

Fig. 2. A – Hörnesite crystals surrounded by a microbial mat; B – hydroniumjarosite (jar) and gypsum (gy) on the surface of a microbial mat; C – bacteria mineralised by pitticite, D – cryptocrystalline arsenic sulphide (As-S) on an old wooden mining construction; arrows indicate microbial colonies partially mineralised by As sulphide; E – a microbial mat mineralised by an unidentified Fe-Ca-As-S phase (the rectangle indicates the EDS analysed area); F – EDS spectra indicating the chemical composition of microbial structures visible in Fig. 2.

acicular crystals up to 0.2 cm long. Individual crystals are colourless or white and have perfect monoclinic morphology. EDS analysis confirmed high purity of the mineral. Accumulations of hörnesite are very often coated by microbial mats (Fig. 2A). Inside myllonites, which are connected with fault zones intersecting Złoty Stok, a hydroniumjarosite deposit is present. It forms yellow, dusty coatings on a mylonite surface. At high magnifications small pseudoregular crystals of this mineral are visible (Fig. 2B). EDS analysis shows only small amounts of potassium in cation positions. In the Gertruda adit scorodite formed two morphologically distinct types. The first type formed grey and green, dusty, cryptocrystalline masses. This scorodite replaced weathered löllingite-pyrite aggregates. The biggest accumulations of this type of scorodite are 10 cm in diameter. The other type is represented by spherical, green aggregates, which form botryoidal coatings. This type of scorodite always occurs in marginal parts of dusty scorodite accumulations. Both morphological types of scorodite were identified upon PXRD data. Kaňkite was identified in samples related with scorodite and pitticite.

This mineral forms light green botryoidal aggregates, with coatings of several square centimetres. Aggregates of kaňkite are composed of thin tabular crystals, which are visible at high magnifications. Pitticite is a product of primary arsenic ore weathering. This phase is amorphous hydrated Fe-arsenate-sulphate. In the Gertruda adit it forms compact masses with a botryoidal surface and resinous lustre. Pitticite accumulations are connected with microbial structures. We often observed mineralisation of microbial mats by this mineral phase (Fig. 2C). In the Gertuda adit ferrihydrite is the most common secondary iron mineral formed by weathering processes in primary ore minerals. It is usually present in sinters, less than 20 cm in length. Ferrihydrite is accompanied by goethite. The presence of both minerals was confirmed with the PXRD method. Peaks on X-ray powder diffraction patterns of ferrihydrite are often diffuse and of low intensity. It indicates low crystallinity of the mineral. Amorphous and cryptocrystalline arsenic sulphide precipitated from underground mine waters occupied by microbial colonies. This phase formed yellow dusty coatings on old wooden mining constructions. Coatings are composed of thin irregular crystals, which are visible at high magnifications (Fig. 2D). Microbial communities are very often mineralised by an unidentified Fe-Ca-As-S phase. It formed a thin film on the surface of colonies. The EDS analysis revealed the presence of iron, calcium, arsenic and sulphur as major elements (Fig. 2F). Small amounts of phosphorus were probably related to the bioactivity of microorganisms. Silicon was probably related to amorphous silica, which precipitated on the surface of the colony.

The analysis of 16S rDNA sequences. A total number of 170 cloned 16S rDNA sequences were analysed with the RFLP (restriction fragment length polymorphism) method. Eighteen clones revealing different from each other digestion patterns (Fig. 3) were reamplified and sequenced. Nucleotide sequences were deposited in GenBank. Seven sequences were closely related to the sequences of a division that belonged to *Flavobacteria*. Others were apparently related to seven other divisions: two clones to *Sphingobacteriia*, one clone to *Bacteroides*, three clones to γ-*Proteobacteria*, one to α-*Proteobacteria* and one to β-*Proteobacteria*. Another clone belonged to *Mollicutes* and two clones were classified as *Firmicutes*. The richer division fell into the *Flavobacteria*, which were closely related to *Flavobacterium* sp. (Table I). A phylogenetic tree was constructed using the neighbour-joining method with the sequences representing all known divisions (Fig. 4). The evolutionary history was inferred by means of the neighbour-joining method (Saitou and Nei, 1987). The optimal tree is shown, where the total branch length is 1.66576087. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsesnstein, 1985). The evolutionary distances were computed with the p-distance method (Nei and Kumar, 2000) and are expressed as units of the number of amino acid differences per site. The analysis involved 37 amino acid

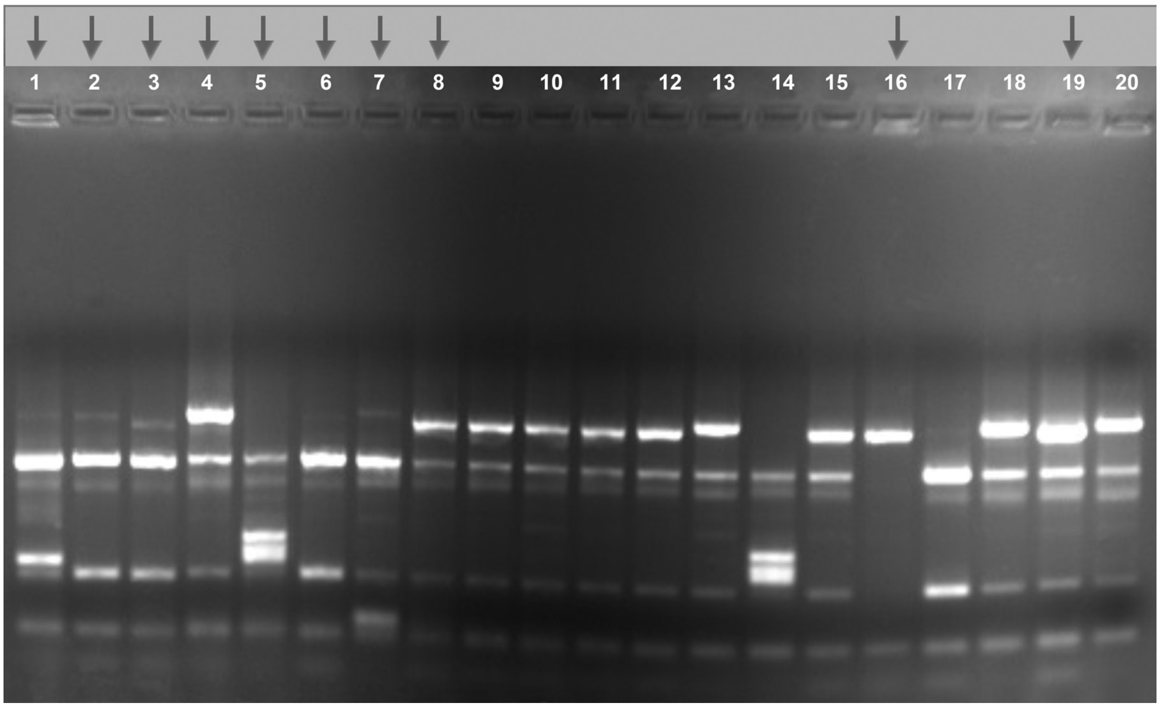

Fig. 3. Example of one gel sample with the restriction patterns of a 16S rDNA gene amplified by PCR and digested with endonuclease *Cfo*I. Each path refers to different microorganism clones that were analyzed. Clones with unique patterns (marked by arrows at the top) were collected and sequenced.

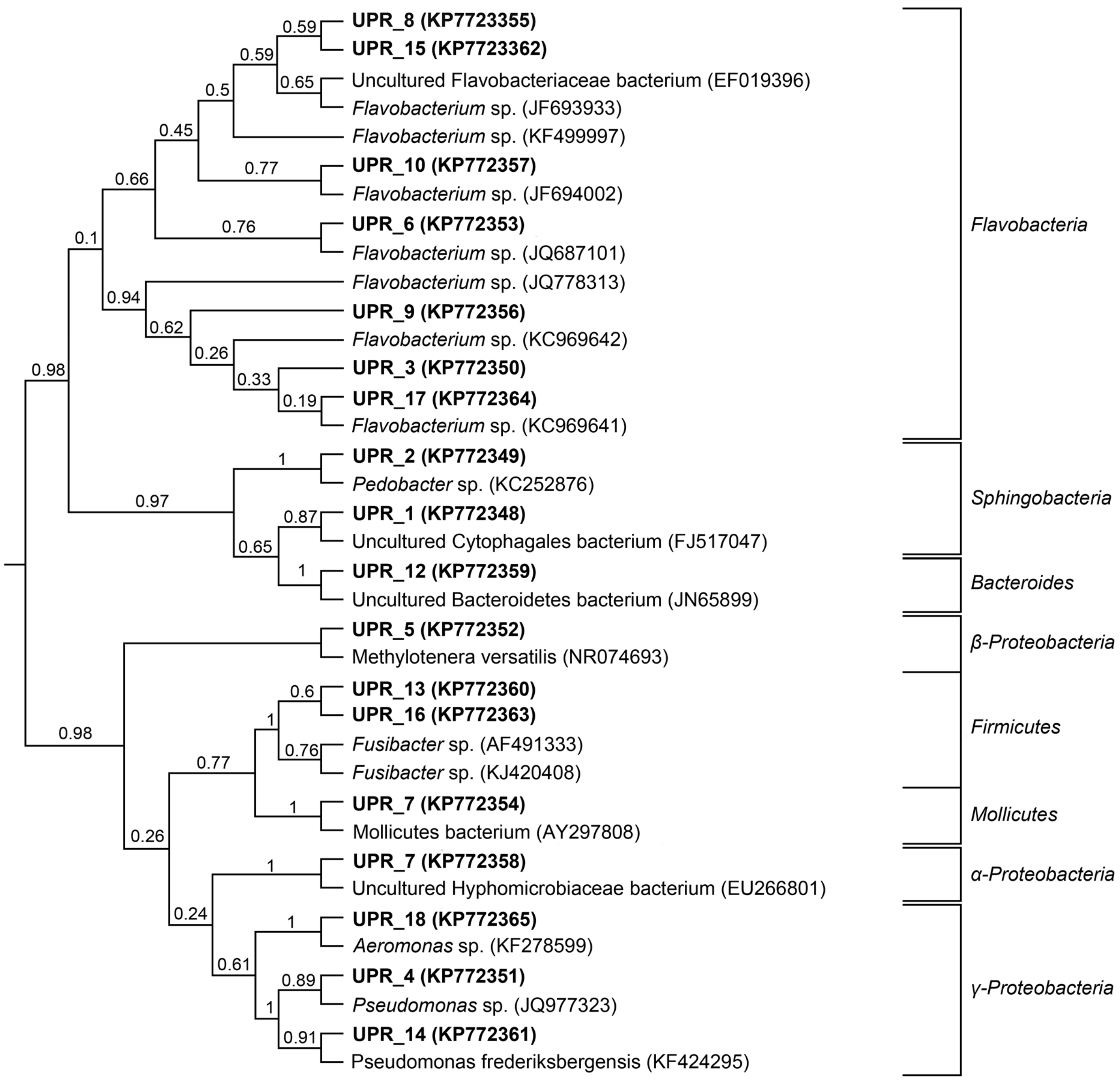

Fig. 4. A phylogenetic tree based on analyses of 16S rDNA sequence. The clone numbers of uncultured microorganisms are indicated in uppercase boldface type (UPR).

sequences. The coding data was translated assuming a standard genetic code table. All positions with gaps and missing data were eliminated. In total there were 92 positions in the final dataset. Evolutionary analyses were conducted with the MEGA6 (Tamura *et al.*, 2013).

Discussion

An approach based on the cloning of 16S rDNA fragments obtained by the amplification of DNA isolated directly from environmental samples allows to determine the diversity of bacteria which can survive in the harsh environment and, perhaps, may influence its geochemistry. Our results are different from those presented by Drewniak *et al.* (2008; 2010; 2012) which were limited to cultured strains capable of utilizing inorganic arsenic species. The data obtained by the Authors clearly demonstrate the presence and cultivability of bacteria contributing to arsenic geochemistry. The sequence analysis revealed the presence of a wide range of bacterial phylogenetic groups, including *Flavobacteria*, *Sphingobacteriia*, *Bacteroides*, *Proteobacteria*, *Mollicutes* and *Firmicutes*. The presence of picropharmacolite was confirmed by PXRD analysis as the most common secondary arsenic mineral in the mine. The microbial colonies occupied amorphous and cryptocrystalline arsenic sulphide precipitated from underground mine waters. They were able to form a yellow dusty coating on old wooden mining construction. The presence of iron, calcium, arsenic and sulphur as major elements revealed by the EDS analyses. The activity of microorganisms is also related with the presence of a small amount of phosphorus in that site. To present a broad picture of these processes and the microbial mat community larger areas of the mine should be analysed. Therefore, it is necessary to conduct further research on the isolation of these bacteria.

The results of sequencing of 16S rDNA fragments obtained from isolation of total genomic DNA from biofilm shows richer composition of bacterial community in the environmental sample than in an approach based on cultivation methods. A metagenomic approach clearly shows that some bacteria cannot be cultivated, even by using selective medium. Thus, analyses of environmental samples should be based on metagenomic and molecular biology techniques. Analyses of environmental samples, to see a 'bigger picture', of all processes that take place in the environment should not be limited only to one type of analysis. To understand how such different groups of microorganisms are able to live together, especially during extreme conditions, it is necessary to conduct complex analyses of the environment.

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ORIGINAL PAPER

MRSA in Pig Population

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) is widespread worldwide in different types of animal species and as a zoonosis takes a great risk for human health not only as a food toxicoinfection, but also as a highly resistant pathogen causing serious soft tissue infectious, septicaemia and even death. One of the most affected food-producing animal species is swine in the production of which new antibiotics in big amounts are used more and more continuously, increasing antimicrobial resistance. In this study several commercial pig farms and pigs with different age groups as well as farm workers and samples from environment were examined with the purpose of detecting MRSA prevalence and evaluating antimicrobial resistance. A total of 85 isolated MRSA strains were characterised by conventional microbial and molecular methods. MRSA was found in all farms. MRSA prevalence in different pig age groups and farms varied from none to 79.2% reaching higher values among 3–3.5 (26.6%) and 4–4.5 (31.9%) old pigs. The 98.7% of 74 further investigated MRSA isolates were resistant to penicillin, 94.9% to tetracycline, 45.6% to cephalexin and 10 different *spa* types were found among which *spa* type *t011* was the most widespread. To the best of our knowledge, this is the first time MRSA was researched in sow milk and the first description of the presence of MRSA in several age groups of pigs in Latvia.

Key words: *Staphylococcus aureus*, antimicrobial resistance zoonosis, MRSA in pig farms

Introduction

Staphylococcus aureus is an important cause of food poisoning, pneumonia, wound infections and nosocomial bacteraemia for humans (Tiemersma *et al.*, 2004). The methicillin resistance of *S. aureus* is mediated by positive *mec*A gene, which encodes penicillin-binding protein 2a (PBP2a) (Chambers, 1997). Among food animals, pigs have been implicated as one source of potential infections to humans, including farmers, slaughterhouse workers, and veterinarians who are in frequent contact with MRSA-colonized pigs (Voss *et al.*, 2005; Huijsdens *et al.*, 2006; Wulf *et al.*, 2008). A subsequent worrisome report indicated that 40% of pigs from the Netherlands carried MRSA CC398 in their nostrils (de Neeling *et al.*, 2007; van Duijkeren *et al.*, 2008). This observation has been confirmed by a number of studies in other countries, including Belgium (Denis *et al.*, 2009), Denmark (Guardbassi *et al.*, 2007), Germany (Whitte *et al.*, 2007), the USA (Smith *et al.*, 2009), and Singapore (Sergio *et al.*, 2007). Especially pigs and also pig farmers and their families were found to be colonized with MRSA and in the Netherlands contact with pigs is now recognized as a risk factor for MRSA carriage (Van Duijkeren *et al.*, 2008).

In addition, there is rather little knowledge of MRSA carriage related to the age of pigs. Therefore this study is the first description of the presence of MRSA in several age groups of pigs in Latvia. The aim of the study was to find out the occurrence of MRSA in several age groups of pigs, in environment and evaluate antimicrobial resistance and see if there any differences or similarities to other European countries.

Experimental

Materials and Methods

Farm characteristics. During the present study three Latvian pig farms were sampled from October to March. These three farms were selected with different

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Table I
Characteristic of farms.

Cryteria	Farm A	Farm B	Farm C
Number of sows	250	1200	2000
Number of fattening pigs	1500	8000	12000
Batch monitoring systems (weeks)	3	3	3
Weaning age (days)	28	28	30
Separate building with separate air supply	No	Yes	Yes
Sows condition score	2	3.5	3
Suckling piglets condition score	2.5	3.5	3
Fattening pigs condition score	2.5	3.5	3.5
Evidence of scars and purulent lesions	No	No	Yes
Signs of cannibalism	Yes	No	Yes
Reduced fertility (small litter 7–8), weak and lot of stillbirth	Yes	No	No
Dirty, wet cages and pens	Yes	For fattening pigs	Yes, 24°C for suckling piglets
Slatted floors	Yes	Except 4–4.5 month age group	Yes
Lack of straw	Yes	Except 4–4.5 month age group	Yes
Antibiotic usage	For treatment	For treatment and prophylaxis	For treatment

amount of pigs. All three farms were closed pig farms without any other commercially bred farm animals presented and were located in different areas of Latvia. These farms had farrow-to-finish pig production with size varying from 1500 to 12000. Each farmer also completed a questionnaire on farm size, internal and external biosecurity measures and antimicrobial drug use over the preceding 6 months. The characterising of each pig complex is described in Table I. The body condition of swine was scored according to Stockmanship standards (Carr, 1998). Evaluation of animal welfare, hygiene, and microclimate conditions in pig complexes were based on Council Directive 2008/120/EC of 18 December 2008 laying down minimum standards for the protection of pigs and microclimate standards according to Muirhead (Muirhead *et al.*, 2013) suggestions.

Sample collection. Pigs were divided into four groups: pre-weaned piglets with sows, 3–3.5 month old piglets, 4–4.5 month old piglets and fattening pigs (shortly before slaughter) (see Table II). There were collected nasal (n = 305) and rectal (n = 305) samples from all farms. There were taken milk samples (n = 69) and air samples (n = 22). In total amount 305 pigs and 716 microbiological samples were investigated.

Samples were taken from randomly selected healthy pigs. Nasal and rectal samples were collected with sterile transport swabs (Meus, IT). Milk samples were collected in 50 ml amount sterile tubes without preservative. Air samples were collected using Baird-Parker Agar plates according to Koch's sedimentation method (Boucher *et al.*, 2010). The number of sampled environment, workers and pigs per age category per farm is shown in Table II. One swab from each worker was taken from both nares. Environmental samples were obtained in every compartment in. All microbiological samples were stored in 4°C and first isolation was made during 24 hours after sample collection.

Microbiological examination. Microbial examination was performed in the Latvia University of Agriculture (LUA), Faculty of Veterinary Medicine. Samples from transport swabs were transferred on Baird-Parker Agar with egg yolk supplement (Becton, Dickinson, USA), and incubated in 37°C for 24 hours according to LVS EN ISO 6888-1:1999 A1:2003 'Microbiology *S. aureus* and other species – Part 1: Technique using Baird-Parker agar medium – Amendment 1: Inclusion of precision data. After incubation positive colonies were inoculated on Mannitol Salt Agar (MSA) plates (Biolife, IT) at 37°C for 24 hours and suspended in Brain Heart infusion (BHI) (Acumedia manufacturers). *Staphylococcus* coagulase tube test (Becton Dickinson,

Table II
Investigated pigs, milk and air samples in each complex.

Group of pigs/sample type	Number of investigated pigs/samples			
	Farm A	Farm B	Farm C	Total
Suckling piglets with sows	32	32	32	96
3-3.5 month old piglets	15	25	24	64
4-4.5 month old piglets	24	24	24	72
Fattening pigs	25	24	24	73
Milk	18	25	26	69
Air	5	9	8	22
Workers	4	4	7	15

USA) was done by using BHI suspension after 24 hours incubation period at 37°C. Coagulase positive samples with positive reaction on MSA plates were determined as *S. aureus*-like and were inoculated on CHROMagar Staph aureus plate (Becton Dickinson, USA) in 37°C for 24 hours. Isolates were confirmed to be *S. aureus* by examining of previous tests. Samples were categorised positive, if at least one *S. aureus* positive colony-forming unit was isolated. Positive colonies from CHROMagar Staph aureus plate were inoculated on CHROMagar MRSA plate (Becton Dickinson, USA). Samples were categorised positive if at least one MRSA positive colony-forming unit was isolated. These samples were categorised as MRSA-like and were stored at –20°C until further use.

MRSA identification. MRSA identification and further examination was performed in Riga Stradins University, Institute of Oncology and in LUA Laboratory of Molecular Biology and Microbiology. One suspected positive MRSA-like colony per sample was then confirmed by PCR and typed by *spa* typing.

Animals and human were considered positive when MRSA was isolated and confirmed with multiplex-PCR form at least one anatomical sampling site. The dominant pig *spa*- and SCC *mec*-type was defined as the type that was most abundantly present in pigs per farm.

DNA was isolated by E.Z.N.A. Bacterial DNA Kit following manufacturer's instructions. DNA amount was verify by ND-1000 spectrophotometer. Polymerase chain reaction (PCR) was performed by HotStarTaq® Plus Master Mix Kit following manufacturer's instructions. The primer sequences for the *mec*A genes were: mecA F: 5'-GTAGAAATGACTGAACGTCCGA TGA-3' and *mec*A R: 5'-CCAATTCCACATTGTTTC GGTCTAA-3'. Amplification of DNA was performed in a Applied Biosystems 2720 thermal cycler using the following conditions: initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 1 min), following final extension at 72°C for 10 minutes. The amplicons were separated in a 2% agarose gel. After electrophoresis fragments were checked out by UV transilluminator visualization and photographed for visual prove. MecA positive samples were 310 base pair long. *spa* typing was performed as has been described (Shopsin *et al*., 1999). The *spa* gene typing was performed through the Ridom Spa server (www.spaserver.ridom.de).

Antimicrobial susceptibility testing. Randomly selected 74 MRSA positive samples were tested for antimicrobial susceptibility by the disk diffusion method using Oxoid™ (Thermo Scientific) Antimicrobial Susceptibility Disks, following recommendations for Clinical and Laboratory Standards Institute (CLSI) for inoculum preparation, inoculation and incubation (CLSI, 2010). The interpretation of results was done according to the information provided by Thermo Scientific instruction for each type of antibiotic discs. The following antimicrobial agents were tested: Amoxycillin/clavulanic acid (2:1 AMC; 30 µg), Penicillin V (PV; 10 µg), Oxacillin (OX; 1 µg), Cephalexin (CL; 30 µg), Ciprofloxacin (CIP; 5 µg), Tetracycline (10 µg; 30 µg), Clindamycin (DA; 2 µg), Erythromycin (E; 15 µg), Gentamicin (CN; 10 µg), Trimethoprim/sulphamethoxazole 1:19 (Cotrimoxazole) (SXT; 25 µg), Meropenem (MEM; 10 µg), Vancomycin (VA; 30 µg). After 24 h of incubation at 37°C, inhibition zones were measured in millimetres on Mueller-Hinton agar plates (Oxoid, UK) and interpreted according to the manufacturer directions.

Data statistical analysis. Animals and human were considered positive when MRSA was isolated and confirmed with multiplex-PCR form at least one anatomical sampling site. The dominant pig spa- and SCC *mec-type* was defined as the type that was most abundantly present in pigs per farm. Statistical analysis was conducted using software SPSS 16 (SPSS, INC., Chicago, IL, USA). The analysis of contingency tables based on statistics of Chi-square test for independence was performed to determine whether there is a significant association between different farms, slaughterhouses and pig age groups. The Chi-square test was used to analyse whether the different farms or slaughterhouses and pig age groups were related to *S. aureus* and MRSA prevalence. Hypothesis of independence was tested at significance level 0.05. Cramér's V coefficient was used to measure the strength of the association between the variables as post-test after chi-square has determined significance. Cramer's V varies between 0 and 1, showing little association between variables close to 0 and indicating strong association between variables close to 1. Bayes' theorem was used to calculate probability to find staphylococci in samples taken from infected pigs.

Results

We isolated 11.9% MRSA positive samples (85 from 716) samples and identified 10 different *spa* types from all MRSA isolates.

In the present study microorganisms, as shown in Fig. 1, varied significantly (χ^2 p value < 0.05), *S. aureus* prevalence at the farm level ranged from 22.9% to 65.4% and MRSA prevalence ranged from 4.2% to 44.2% The highest prevalence of all staphylococci were seen in Farm C: 65.4% *S. aureus* and MRSA 44.2% positive samples. The lowest prevalence of staphylococci was seen in Farm B: *S. aureus* 22.9% and MRSA 9.5%.

The prevalence of *S. aureus* (Fig. 2.) in different age groups varied from 33.3% in suckling piglets group to 53.4% in 4–4.5 month old piglet group, but prevalence

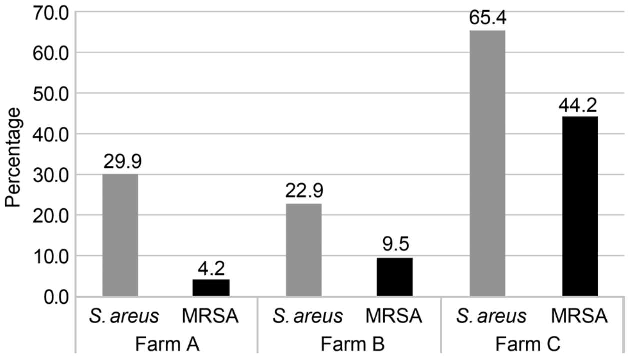

Fig. 1. *S. aureus* and MRSA prevalence in pig farms.

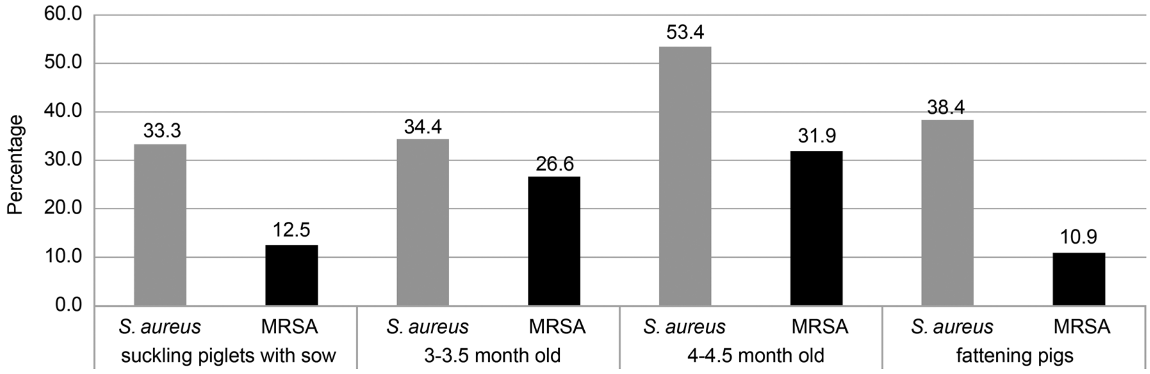

Fig. 2. *S. aureus* and MRSA prevalence between age groups.

of MRSA varied from 10.9% in fattening pig group to 31.9% in 4–4.5 month old pig group. The highest prevalence of MRSA positive samples were seen in 4–4.5 and 3–3.5 month old pigs, but prevalence of *S. aureus* was similar in all age groups except 4–4.5 month age group, where it was for 15.0% to 21.1% higher as in the other groups.

Only 13.3% workers (2 from 15) were MRSA positive. MRSA prevalence in different farms and pig age groups varied from zero to 79.2% (see Table III). In farm A, where the prevalence of MRSA was lower, MRSA positive pigs were found only in 4–45 month (8.3%) and in fattening pig group (8.0%). In farm B MRSA was not detected in 3–3.5 month pig age group, but the highest amounts of positive pigs were seen in suckling piglet group (15.6%). The highest prevalence of MRSA positive pigs were detected in farm C (44.2%) with the highest evidence in 3–3.5. month age group (70.8%) and in 4–4.5. month age group (79.2%). Only several milk samples were positive- in farm B (4.0%) and in farm C (7.7%). There were no positive MRSA samples taken from environment.

As seen in Fig. 3, 19.7% of all samples were MRSA positive (nasal samples 8.2% and 5.6% rectal samples) and 5.9% of all MRSA positive samples were seen in both rectal and nasal samples. Analysing data we found out, that MRSA and *S. aureus* positive sample distribution depending from sample source is similar. Taking only nasal or rectal samples for MRSA testing decreases probability to find microorganism for 10.2% to 41.7%. According to Bayes' theorem the probability of finding infected pigs with MRSA taking rectal samples is 0.28, nasal samples – 0.42, but in both samples 0.30 and the probability of finding infected pigs with *S. aureus* taking only nasal samples is 0.15, only rectal samples – 0.50, and for both samples – 0.35.

In our study in 7 cases from one animal two different MRSA *spa* types were isolated. There were seen two different MRSA *spa* type combinations: *spa* type *t808* and *t1985* in farm C in 3–3.5 month age group and 4–4.5 month age group.

We isolated 74 MRSA isolates with 10 different *spa* types (see Table IV and Table V). MRSA distribution depending from *spa* type, sample origin and resistance

Table III
MRSA prevalence in farms.

Farm	Suckling piglets with sow*		3–3.5 month old*		4–4.5 month old*		Fattening pigs*		Total prevalence	Milk*		Workers*	Environment*
A	0/32	0	0/15	0	2/24	8.3%	2/25	8.0%	4.2%	0/18	0	2/4	0/5
B	5/32	15.6%	0/25	0	2/24	8.3%	3/24	12.5%	9.5%	1/25	4.0%	1/4	0/9
C	7/32	21.9%	17/24	70.8%	19/24	79.2%	3/24	12.4%	44.2.%	2/26	7.7%	0/7	0/8
Total	12/96	12.5%	17/64	26.6%	23/72	31.9%	8/73	10.9%	18.7%	3/69	4.3%	3/15	0/22

* MRSA positive samples from all tested

Table IV
MRSA origin.

Spa type	Farm	Number of Antimicrobial resistance profile	Antimicrobial resistance profile	Origin (n)
t011	C	2	Pen-AmCl-Tetr	Fattening pigs (n = 1), 3–3.5 month old (n = 2)
		3	Pen-AmCl-Cip-Tetr	Suckling piglets (n = 1)
		4	Pen-AmCl-Cef-Tetr	4–4.5 month old (n = 3)
		8	Pen-AmCl-Cef-Tetr-Clin-Ery-Ge-Tri-Me-	4–4.5 month old (n = 1)
		11	Pen-Cef-Tetr	4–4.5 month old (n = 13), suckling piglets (n = 1), sows (n = 2), milk (n = 1)
		12	Pen-Cef-Tetr-Clin	4–4.5 month old (n = 1)
		13	Pen-Cef-Tetr-Me	4–4.5 month old (n = 1)
		20	Pen-Tetr	Fattening pigs (n = 1), 4–4.5 month old (n = 5), 3–3.5 month old (n = 11), suckling piglets (n = 2)
		21	Tetr	4–4.5 month old (n = 1)
	A	4	Pen-AmCl-Cef-Tetr	4–4.5 month old (n = 1)
	B	4	Pen-AmCl-Cef-Tetr	Suckling piglets (n = 1)
		11	Pen-Cef-Tetr	Suckling piglets (n = 1)
		23	Pen-Cef-Tetr-Clin-Ery	Fattening pigs (n = 1)
t1333	B	1	Pen	Fattening pigs (n = 1)
		24	Pen-Tetr-Ery	Fattening pigs (n = 1)
		25	Pen-Tetr-Clin-Ery	4–4.5 month old (n = 1)
t808	C	4	Pen-AmCl-Cef-Tetr	3–3.5 month old (n = 1)
		20	Pen-Tetr	4–4.5 month old (n = 3), 3–3.5 month old (n = 1)
t899	A	1	Pen	Fattening pigs (n = 1)
		20	Pen-Tetr	4–4.5 month old (n = 1)
		5	Pen-AmCl-Tetr-Tri-Me	Fattening pigs (n = 1)
t400	B	1	Pen	milk (n = 1)
		8	Pen-AmCl-Cef-Tetr-Clin-Ery-Ge-Tri	sow (n = 1)
		20	Pen-Tetr	sow (n = 1)
		25	Pen-Tetr-Clin-Ery	Suckling piglets (n = 1)
		26	Pen-Tetr-Clin	4–4.5 month old (n = 1)
		27	Pen-Cef-Tetr-Clin-Ery-Ge	Suckling piglets (n = 1)
t1580	C	11	Pen-Cef-Tetr	Fattening pigs (n = 1)
t1985	C	11	Pen-Cef-Tetr	Suckling piglets (n = 1)
		13	Pen-Cef-Tetr-Me	3–3.5 month old (n = 1)
		20	Pen-Tetr	Suckling piglets (n = 1), 3–3.5 month old (n = 1)
t693	A	13	Pen-Cef-Tetr-Me	Worker at farm (n = 1)
t2383	C	16	Pen-Tetr-Clin-Ge	4–4.5 month old (n = 1)
t1255	B	11	Pen-Cef-Tetr	Farm worker (n = 1)

* Pen-penicillin, AmCl – amoxicillin with clavulanic acid, Cef – cephalexin, Cip – ciprofloxacin, Clin – clindamycin, Ery – erythromycin, Ge – gentamycin, Me – meropenem, Tetr – tetracycline, Tri – trimethoprim sulphonamide.

against several antibiotics are shown in Table IV. The amounts of different *spa* types were higher in farms with greater pig production (Farm C) and higher MRSA prevalence (Farm C). We found 3 *spa* types in farm A, in farm B – 4, but in farm C – 5 *spa* types. MRSA *spa* type *t011*, that was one of the most widespread (51 from 74), which was evident in all three farms while other *spa* types were seen in only one farm (Table V). Analysing MRSA strains, we found out, that spa types *t899* belong to *ST9*, *t400* to *ST528*, but *t011* to *ST398*.

Antimicrobial susceptibility testing on the selected MRSA isolates revealed the presence of 19 different antibiotic profiles. Amounts of MRSA *spa* type distribution among samples are shown in Table VI and Fig. 4.

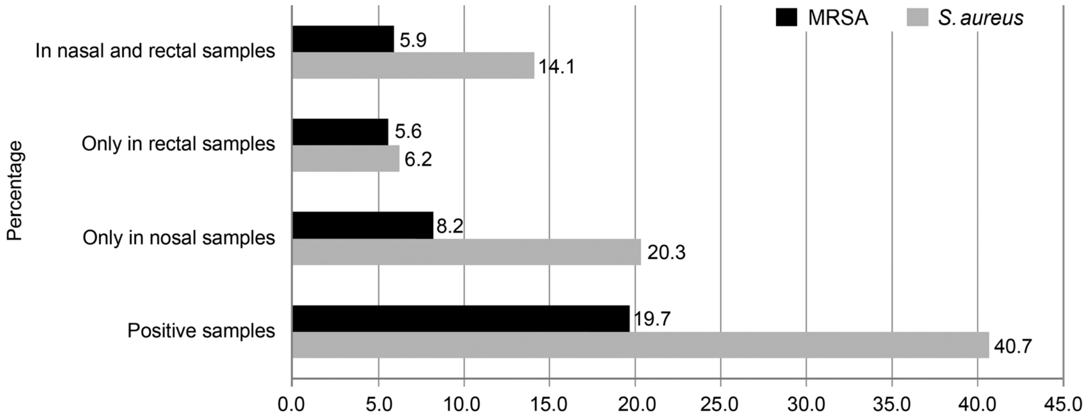

Fig. 3. *S. aureus* and MRSA findings depending from sample type.

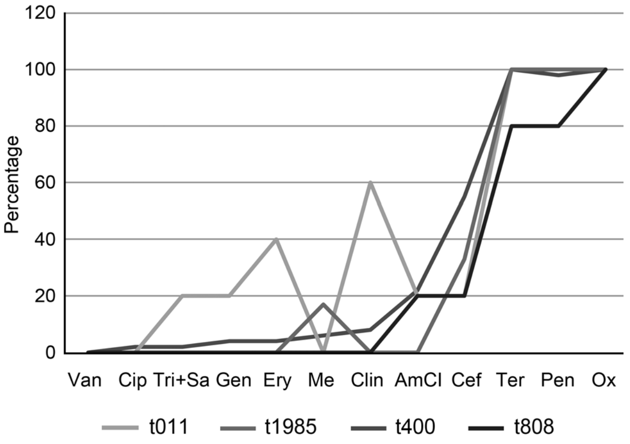

Fig. 4. Antimicrobial resistance of four MRSA most spread *spa* types.

As seen from our study 69% of MRSA isolates belong to *spa* type *t011* (n = 51), and other most widespread *spa* types are *t808* (7%, n = 5), *t1985* (5%, n = 4) and *t400* (5%, n = 4). Two to eleven different antibiotic resistance profiles were seen depending on MRSA *spa* type (Table VI). The most spread *spa* types also were the ones with the highest antibiotic profile heterogeneity, for example *spa* type *t011* integrated in to more than one half (n = 10) of antibiotic profiles (Table VI), while the lowest heterogeneity was evident among MRSA *spa* type *t808* (n = 2). MRSA isolate t1255 from farm worker, which belongs to MRSA isolates from pig origin, had the highly widespread antibiotic type Pen-Cef-Tetr.

Sixty-four percent of the isolates belonged to the two most prevalent antibiotic resistance profiles (Table VII).

MRSA *spa* type *t011* was almost evident in all most frequently spread antibiotic resistance profiles, but *spa* type *t1985* was evident in antibiotic type Pen-Tetr, Pen-Cef-Tetr and Pen-Cef-Tetr-Me. Most of all MRSA isolates showed multidrug resistance.

The highest antimicrobial resistance was seen against four (34% of all MRSA isolates), three antibiotics (32.9% of all MRSA isolates) and five (17.7% of all MRSA isolates) antibiotics. Lower amounts of isolates were resistant to six (5.1%) and seven (1.3%) antibiotics.

98.7% of all MRSA isolates were resistant to penicillin, 94.9% to tetracycline, 45.6% to cephalexin, 19.90%

Table V
MRSA spa type distribution in farms.

Farm	*Spa* types
A	*t011* (n = 1), *t899* (n = 3), *t693* (n = 1)
B	*t011* (n = 3), *t1333* (n = 3), *t400* (n = 4), *t1255* (n = 1)
C	*t011* (n = 47), *t808* (n = 5), *t1580* (n = 1), *t1985* (n = 4), *t2383* (n = 1)

Table VI
Characterisation of most frequently spread MRSA spa types.

Spa type	% of all MRSA isolates	The highest resistance for current antibiotics	Total amount of different antimicrobial resistance profiles
t1985	5	Pen, Tetr, Cef, Me	3
t011	69	Pen, Tetr, Cef, Am-Clav	10
t808	7	Pen, Tetr, AM Cl, Cef	2
t400	5	Pen, Tetr, Clin, Ery	4

Table VII
Characterisation of most frequently found antibiotic types.

Antibiotic types most frequently found	Number of MRSA isolates	% from all MRSA isolates	Number of different MRSA *spa* types
Pen-Tetr	28	38	5 (*t011, t808, t899, t400, t1985*)
Pen-Cef-Tetr	19	26	4 (*t011, t1580, t1985, t1255*)
Pen-AmCl-Cef-Tetr	6	8	2 (*t011, t808)*
Pen	3	4	3 (*t1333, t899, t400*)
Pen-AmCl-Tetr	3	4	1(*t011*)
Pen-Cef-Tetr-Me	3	4	3 (*t011, t1985, t1255*)

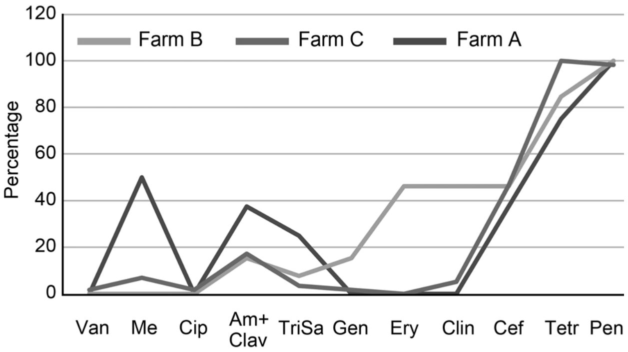

Fig. 5. Antimicrobial resistance in farms.

to amoxicillin combined with clavulanic acid, 11.4% to clindamycin, 10.0% to meropenem, 7.6% to erythromycin, 6.3% to trimethoprim sulphonamide, 3.8% to gentamycin and 1.3% to ciprofloxacin.

All isolates were sensitive to vancomycin, but 9 MRSA isolates were intermediate to vancomycin, *t011* (n = 5), *t693* (n = 1), *t899* (n = 2) and *t400* (n = 1).

Comparing MRSA *spa* type antibiotic resistance, we found out, that even between one *spa* type, there are differences in antibiotic resistance profiles, therefore we calculated average % resistance from all isolated one type MRSA *spa* types and showed results in graphics (Fig. 4).

All four most prevalent *spa* types in our samples differ each from another, but a common tendency can be seen, that is that all these types are almost 80% to 100% resistant to tetracycline and penicillin. *spa* type *t011* is the most widespread compared to other *spa* types, but has a moderate antibiotic resistance profile, while *t400* is mostly resistant to seven of the 12 tested antibiotics.

Among all the most widespread MRSA *spa* types can be found isolates that are more or less, resistant to penicillin, cephalexin and tetracycline. MRSA resistance was seen even to antibiotics that are not frequently used or are not allowed to be used for food chain animal treatment, such as, gentamycin, ciprofloxacin, cephalexin and meropenem. In some rare cases MRSA isolates showed (not showed in figures) intermediate sensitivity to vancomycin.

Comparing antibiotic resistance in farms (Fig. 5), greater resistance appears against meropenem and amoxicillin combined with clavulanic acid in Farm A, but in Farm B there is greater resistance against gentamycin, erythromycin and clindamycin. However, there is no evident difference in antibiotic resistance among farms related to farm size and pig number.

Discussion

This study investigated the transmission and distribution of *S. aureus* and MRSA in individual pigs throughout the production cycle, environment and pig industry workers. Significant findings include the identification and detailed characterisation for the first time of MRSA isolates from Latvian pigs and demonstrated MRSA colonization status between several pig age groups. In addition, this study studied MRSA distribution and antibiotic resistance level tendencies between different pig farms depending from production amounts. It documented tendency in the MRSA distribution and antibiotic resistance profiles according to MRSA *spa* types. Antimicrobial use is also thought to be a factor in the emergence and transmission of MRSA in pigs and deserves further investigation.

However, it is acknowledged that many factors, in addition to pig colonisation status in farms, are likely to be important influences on carrier status. Such factors might include antimicrobial medication for treatment and prophylaxis, animal welfare aspects and pig density and contact availability to each another. In our study MRSA was not found in environmental samples, but two *S. aureus* isolates were found (two from air in 3–3.5 old pig group in farm C, where the MRSA prevalence in pigs was higher among all farms), but other investigations have shown MRSA distribution in barn spaces in Germany (Friese *et al.*, 2012) MRSA appears in 23 of 27 investigated pig barns (85.2%) and the prevalence in dust samples appeared 100% whereas in EFSA report (EFSA, 2009c) the prevalence in dust samples was 0%. In other studies testing dust and farm air were used filtration methods using specific equipment, but we used Koch's sedimentation method, and it could be a reason for such a low MRSA detection level. Failure to detect airborne MRSA and *S. aureus* in farms by the used Koch's sedimentation method in our study does not guarantee the absence of these bacteria in the air.

MRSA appearing in air samples reveals the difficulties in reducing the spread of bacteria within an animal house. It can be concluded that very effective cleaning and disinfection of the stables including all ventilation systems before stocking with new pigs is necessary to avoid transmission of MRSA between subsequent fattening groups of animals within breeding farms by contaminated premises. Depending on the ventilation system and construction of the industrial house the dissemination of MRSA through the whole building *via* air seems possible. Occurrence of MRSA in the air may lead also to colonisation of negative animals without direct contact with MRSA carriers (Friese *et al.*, 2012).

In our study the prevalence of *S. aureus* and MRSA varied in each age group. The results indicated differences between the farm types with respect to within farm associated MRSA *spa* type. The average MRSA prevalence in farms (4–44.2%) were little bit lower or similar to other studies in Italy (EFSA, 2009; Batisti *et al.*, 2010) where MRSA prevalence in pig herds warried from 38% to 52%, in Belgium (Pletnickx *et al.*, 2013) 40–84%. Moreover, the holding size was found to be a significant factor influencing the prevalence (Battisti *et al.*, 2010). Larger farms have showed a higher risk.

This study found the average carriage rate of MRSA was at its highest in 3–3.5 and 4–4.5 old pigs. Burns with co-authors (2014) found the average carriage rate of *S. aureus* was at its highest on day 2 after farrowing, followed by a decrease prior to weaning and similar findings were reported by other authors (Smith *et al.*, 2009; Weese *et al.*, 2010; Broens *et al.*, 2011; Verhegghe *et al.*, 2011). In our study the prevalence of MRSA and *S. aureus* was highest in 3–3.5 and 4–4.5 month old pigs and decreased among fattening pigs, but in Burns study with co-authors (2014) the prevalence of MRSA continued to increase during the 100 day investigation period. According to Weese (2010) and Dewale (2013) increase of MRSA positive pigs recorded at weaning was due to the commingling of positive and negative pigs, stress during weaning, age related susceptibility and contamination of other sites on farms. In our study depending from farms pigs in 3–4.5 month age were moved to fattening buildings and once again regrouping was carried out and different holding conditions appeared causing additional stress. Weaning, regrouping and moving may be a point at which controls could be implemented in order to reduce the transmission of MRSA.

Burns and co-authors have stated (2014) that more than 1/3 of *S. aureus* isolates were resistant to tetracycline and erythromycin, a similar situation to that seen in our study, moreover 46% of MRSA isolates were resistant to cephalexin and 11% to clindamycin that were not used for pig treatment in farms, but resistance to penicillin, that were used us a first choice antibiotic several years ago and tetracycline that were administrated in these farms during the sample taking process, reached 99% and 95%. Antibiotic usage for prophylactic purposes does not decrease MRSA distribution. The MRSA distribution in farm B, where antibiotics are used for prophylactic purposes, are little lower as in Farm C, where antibiotics were used for treatment only but quite higher than in farm A, where also antibiotics were used for treatment purposes only. Similar parallels from our study to Italian study (Normanno *et al.*, 2015) are seen in antibiotic resistance profiles, where mostly MRSA isolates were multidrug resistant, including resistance to clindamycin, tetracycline, erythromycin. A study from Denmark (Witte *et al.*, 2007) showed that *spa* type *t034* were the most widespread, whereas other authors (Tenhagen *et al.*, 2009; Broens *et al.*, 2011; Crombe *et al.*, 2012; Friese *et al.*, 2012; Pletinckx *et al.*, 2013) and our study most frequently found *spa* type *t011*, that was isolated from all farms. MRSA *t011 spa* type was also found in Belgium poultry farm (Nemati *et al.*, 2008), in Denmark (Agerso *et al.*, 2012), in Italy (Normanno *et al.*, 2015). *spa* type "t899" that we detected in our study, was found in Normanno's research (Normanno *et al.*, 2015). According to Fishers' test value (1.1×10^{-11}) statistically significant differences were found regarding the origin of samples, and MRSA *spa* types isolated from farms. One another *spa* type *t1333* that was found in our study, was one of the most frequently found MRSA *spa* type in a study by Tehnagen and co-authors (2009). Our study agrees with Pletinckx and co-authors (2013), where different antimicrobial resistance profiles per *spa* type and per farm appear.

Human infectious caused by MRSA are one of main causes of morbidity and mortality in industrialized countries. In addition to the traditional routes of MRSA infection, it has recently been demonstrated that direct transmission to humans takes a place *via* contact with farm animals (Wendlandt *et al.*, 2013). A lot of studies on spread of MRSA in farm animals and their carcases have included pigs, which are currently the most important reservoir of MRSA (Gomez-Sanz *et al.*, 2010; Overesch *et al.*, 2011).

The high presence of MRSA in pigs is a potential professional hazard for these working in the meat production chain (workers in farms and slaughterhouses, transportation workers and veterinarians). It is known that people working several hours per week in direct contact with pigs colonized or infected with MRSA animals are exposed to high risk of nasal colonization (Voss *et al.*, 2005; van Loo *et al.*, 2007; Witte *et al.*, 2007; Denis *et al.*, 2009; Moodley *et al.*, 2011). The general population shows a high prevalence (approximately 30%) of *S. aureus* nasal colonization, whereas MRSA nasal colonization levels are lower (0.7–1.5%) depending on geographical area (Wertheim *et al.*, 2004; Gorwitz *et al.*, 2008; Munckhof *et al.*, 2009). Human colonization implies that carriers become a staphylococcal reservoir and may transfer the infection to others or animals, especially pigs. In addition, subclinical carriage of MRSA by humans is considered a risk factor for subsequent occurrence of clinical disease (Cohn and Middleton, 2010; Jordan *et al.* 2011). Many studies have investigated MRSA nasal colonization among personnel in contact with animals, especially farmers and veterinarians (van Cleef *et al.*, 2014). In our survey, the anterior nares 1 out of 15 investigated workers from farms were colonised by MRSA, that were higher than in other studies: in China no MRSA in 107 slaughterhouse workers (Cui *et al.*, 2009) in Switzerland no MRSA in 179 slaughterhouse workers (Huber *et al.*, 2010), in Netherlands 5.6% of nasal carriage from 195 pigs in slaughterhouse, which was higher than the prevalence 0.1 among the general population of the country (van Cleef *et al.*, 2010). High concentration of MRSA in barns may also be an issue of occupation health. It has been proved in several studies that humans working in pig farms carry MRSA of the same sequence type as swine (Cunny *et al*., 2009; Van Den Broek *et al*., 2009). Considering the MRSA and *Staphylococcus aureus* occurrence in worker nares sample, this fact is not surprising since the stuff do not wear any respiratory masks and are in close physical contact with pigs. In addition, their hands, equipment, clothes and boots can serve as infection transmitters and contaminants.

Antimicrobial resistance is increasing worldwide in human bacterial pathogens and zoonotic agents and this may cause a risk for effective treatment of infections in humans. Multidrug resistance was prevalent in our MRSA isolates in all groups – from pigs and worker in farms. Most of all isolates displayed resistance to two or more classes of antimicrobials and some of them were resistant or with intermediate sensitivity to vancomycin, that indicate development of resistance to that antibiotic. These findings are in agreement with other studies of high MRSA prevalence and antimicrobial resistance in isolates from pigs, pork and humans (Batisti *et al*., 2010; Jackson *et al*., 2013). As expected 99% of isolates were resistant to penicillin and 95% to tetracycline, but quite high resistance appears to cephalexin (46%), amoxicillin combined with clavulanic acid (10%), clindamycin (11.4%), meropenem (10%) and erythromycin (7.6%). Several studies have showed 100% resistance to MRSA isolates (van Duijkeren *et al*., 2008; Batisti *et al*., 2010; Fesler *et al*., 2012; Crombe *et al*., 2013) and Pletinckx (2013) have found high resistance to trimethoprim, lincomycin and ciprofloxacin.

Finally, it is known that MRSA prevalence and *spa* types differ according to farm density and animal welfare conditions. In farms with higher amount of pigs, several evident scars and purulent lesions on joints MRSA prevalence and thought different MRSA spa types were found higher as in others. The pigs used in our study originated from different farms in our country, and this could be the reason for the wide heterogeneity of the MRSA *spa* types we found. An unexpected finding was that in farms, where pig condition score was lower and reduced sow fertility was in presence, staphylococcal colonisation rate was lower than in farms where pig condition score were higher and better animal welfare conditions were evident. In addition no significantly lower staphylococcal colonisation in farm B, compared to other farms, were seen, despite antibiotic usage for prophylactically purposes.

As far as we know, this is the first report documenting the prevalence and characteristics of MRSA in farms and stuff involved in pig industry in Latvia and MRSA detection in sow milk.

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SHORT COMMUNICATION

Genes Controlling 2-deoxyglucose Induced Lysis and Formation of Reactive Oxygen Species in *Schizosaccharomyces pombe*

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Abstract

Schizosaccharomyces pombe cells of strains each carrying a deletion of one of the genes *snf5*, *ypa1*, *pho7* and *pas1* and of a strain overexpressing gene *odr1*, have been previously shown to grow in presence of the toxic glucose analogue 2-deoxyglucose (2-DG). Here we report that these genes control 2-DG induced lysis and are, with the exception of *odr1*, also involved in control of formation of reactive oxygen species (ROS) upon exposure of cells to H_2O_2. Lysis of deletion strains, but not of strain overexpressing *odr1*, is dependent on glucose concentration of the medium whereas ROS formation is glucose independent.

Key words: 2-deoxyglucose, fission yeast, glucose signaling, lysis, ROS formation

Reactive oxygen species (ROS) are harmful by-products of basic cellular metabolism in aerobic organisms and are mainly known to be formed under oxidative stress (Apel and Hirt, 2004). They act as signalling molecules and are involved in many different biological processes in various organisms including mammalian cells and yeast (Apel and Hirt, 2004; Ikner and Shiozaki, 2005; Herrero *et al.*, 2008; Ray *et al.*, 2012; de la Torre-Ruiz *et al.*, 2015). One of the agents which induces oxidative stress and ROS formation in higher cells is 2-deoxyglucose (2-DG). It is a glucose analogue, which, in yeast and mammalian cells is phosphorylated to toxic 2-DG-6-phosphate, which in turn interferes with many processes including glycolysis, protein glycosylation, cell wall synthesis, growth and others (Brown, 1962; Farkas *et al.*, 1969; Biely *et al.*, 1971; Krátký *et al.*, 1975; O'Donnell *et al.*, 2015). Knowledge of mode of action of 2-DG is still poorly understood but is, however, of special interest because it exhibits anticancer activity (Pelicano *et al.*, 2006).

Budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* are widely used model systems for eukaryotic cells with fission yeast in many aspects closer related to higher organisms than *S. cerevisiae* (Hoffman *et al.*, 2015). 2-DG has several effects on yeast cells including inhibition of growth and induction of lysis (Johnson, 1968). To identify genes involved in the control of 2-DG action in fission yeast, we recently identified, by screening a haploid deletion library, four genes (*snf5*, *ypa1*, *pho7*, and *pas1*) which when deleted, grow on plates containing the toxic 2-DG. By transforming cells with a wild type gene library we identified, in addition, a gene *odr1* which when overexpressed also exhibits similar resistance to 2-DG as the deletion strains (Vishwanatha *et al.*, 2016).

In this study we show that these genes are involved in the control of 2-DG induced cell lysis, and in the control of ROS formation induced by the oxidative stress inducing agent H_20_2.

In a first series of experiments we grew cells of the 2-DG resistant strains and the control strains given in the Table I in liquid minimal medium (MM) in the

Table I
List of 2-DG resistant and control strains.

Strain name	Genotype	Plasmid
wild type	*972* h^-	
pREP4X	*ura4D18* h^-	*pRep4X*
pODR1	*ura4 D18* h^-	*pODR1*
parent	*ade6 M210 ura4 D18 leu1* h^+	
snf5Δ	*ade6 M210 ura4 D18 leu1 snf5::KanMX* h^+	
ypa1Δ	*ade6 M210 ura4 D18 leu1 ypa1::KanMX* h^+	
pho7Δ	*ade6 M210 ura4 D18 leu1 pho7::KanMX* h^+	
pas1Δ	*ade6 M210 ura4 D18 leu1 pas1::KanMX* h^+	

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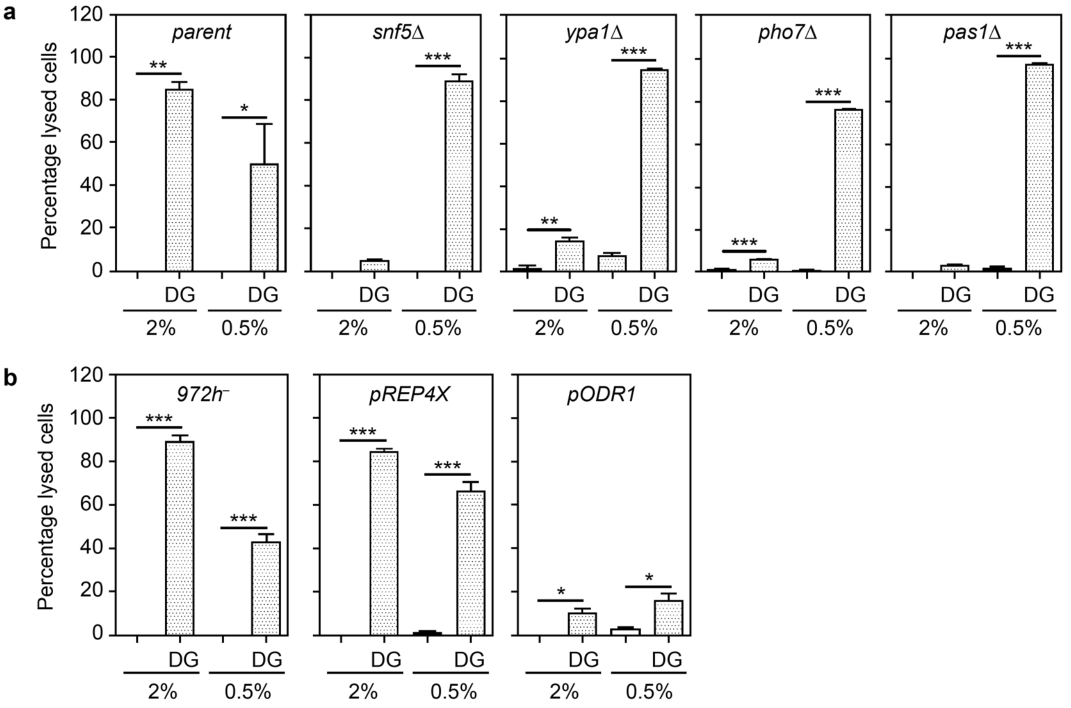

Fig. 1. Microscopic examination and quantification of lysis of 2-DG resistant strains.

Cells of the deletion strains *snf5*Δ, *ypa1*Δ, *pho7*Δ and *pas1*Δ (a) and the *odr1* overexpressing strain *pODR1* (b) were pregrown in MM (Vishwanatha *et al.*, 2016) containing the supplements leucine, adenine and uracil (50 µg/ml) to log phase (OD_{600} 0.5–0.8), pelleted and resuspended in the same medium containing 2% and 0.5% glucose with or without 2-DG (0.25 mg/ml) (abbreviated as DG in figure) (≥ 99% pure, Sigma – Aldrich, USA) and grown for 4 hrs at 30°. Cells were concentrated and stained with Evans Blue. Cell lysis was examined under a bright field microscope with cells immobilized on concanavalinA (conA) coated coverslips. The cell number was counted and the proportion of lysed cells was expressed as percentage of the total cells counted. Statistical analyses were performed using GraphPad Prism 5® (GraphPad Software Inc., La Jolla, CA, USA) and statistical significance was determined either using one-way ANOVA, followed by Bonferroni post hoc test or by paired t test as applicable. Significance was accepted at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). The pairs of data sets analysed for significance are marked by lines. The mean ± SEM is plotted. Asterisks represents the degree of significance of the differences between pairs of data sets analysed.

presence and absence of 2-DG and examined the cells microscopically.

Knowing that glucose can modify effects of 2-DG (McCartney *et al.*, 2014; Vishwanatha *et al.*, 2016), we tested cells at high (2%) and low (0.5%) glucose concentrations. As shown in Fig. 1 cells of the control strains lyse in the presence of 2-DG as reported previously (Megnet, 1965; Johnson, 1968). Lysis is more efficient in cells grown in a medium containing high glucose than cells grown in the presence of low glucose. Cells of the four deletion strains lyse in the presence of 2-DG when grown at the low glucose concentration but not so at the high concentration. This indicates that the four genes are involved in the control of 2-DG induced lysis and that this control is glucose dependent. Independent of the glucose concentration, cells of the strain containing the overexpressed gene *odr1* are not lysed by 2-DG.

Oxidative stress in *S. pombe* is greatly affected by glucose and its signalling/sensing pathways (Palabiyik *et al.*, 2012; 2013). Glucose starvation induces oxidative stress, activating a stress induced mitogen activated protein (MAP) kinase pathway resulting in an increased expression of Atf1-dependent stress response genes (Madrid *et al.*, 2004; 2006; 2013; Kato *et al.*, 2013). Glucose signalling mediated by PKA is also affected by glucose starvation (Gupta *et al.*, 2011). Also, a 2-DG resistant mutant, *ird11* was reported to be defective in oxidative stress response (Suslu *et al.*, 2011; Palabiyik *et al.*, 2012). Knowing this and the fact that the 2-DG resistant mutants we identified are defective in glucose signalling (Vishwanatha *et al.*, 2016), we examined whether cells of the deletion strains are also altered in ROS formation upon oxidative stress. As shown in many studies ROS formation can be visualized by staining cells with 2', 7' Dichlorofluorescein diacetate (DCFDA) which is converted in the cells to the fluorescent 2', 7' Dichlorofluorescein (DCF) (Wu and Yotnda, 2011). Since the control cells of the parent strain are lysing in the presence of 2-DG we could not directly test the effect of 2-DG on ROS formation. For this reason we cultivated cells in the presence of non-lethal concentration and absence of H_2O_2 which is well known to induce oxidative stress (Apel and Hirt, 2004; Roux *et al.*, 2009). As shown in Fig. 2 H_2O_2 induces ROS formation in the wild type (*972 h*⁻) and the control strains (pREP4X and parent). Its production is more prominent in cells grown at a low rather than at a high glucose concentration. In the deletion strains ROS formation

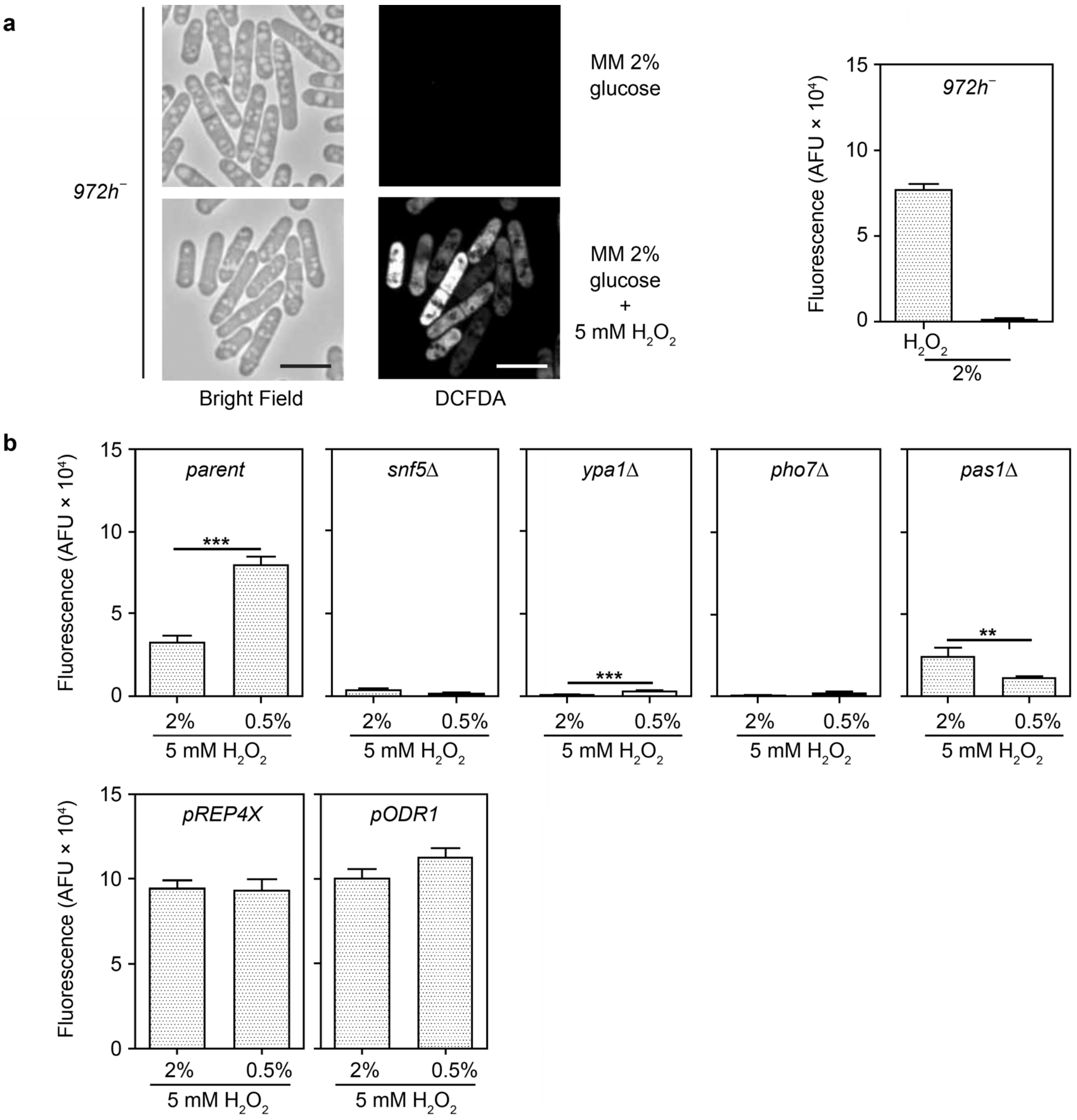

Fig. 2 Induction of ROS formation by H_2O_2 in 2-DG resistant *S. pombe* strains.

Cells were cultured in MM as descibed in Fig. 1 in the presence and absence of 5 mM H_20_2 (SD Fine Chemicals) for 4 hrs at 30°, pelleted, resuspended in 200 µl of Fluorobrite™ DMEM (Life Technologies, USA) containing 25 µM DCFDA (Sigma Aldrich, USA), immobilized on con A coated coverslips and examined microscopically for ROS formation. (a) A representative 5 second fluorescent exposure image after background correction along with bright field image of wild type *972h⁻* grown in the presence and absence of H_2O_2. Scale bars represent 10 µm. Corresponding quantification of fluoresence from the wild type is plotted as bar graph adjacent to the image. It was achieved by measuring fluoressence from at least 120 cells using Image J. Mean of integrated density values obtained after estimation was plotted as arbitrary fluorescence units (AFU). Statistical significance was determined as in Fig. 1 and the values are plotted as mean ± SEM. (b) Quantitated fluoresence achieved as given for (a) for the deletion strains and strain pODR1 along with control strains.

is drastically reduced both in cells grown at a high and a low glucose concentration indicating that the four genes *snf5*, *ypa1*, *pho7* and *pas1* are also responsible for ROS formation upon oxidative stress. Cells of strain pODR1 are unable to quench ROS formation. This may indicate that gene *odr1* is not involved in the control of ROS formation and may possibly have a function leading to the detoxification of 2-DG.

With these experiments we show that the genes *snf5*, *ypa1*, *pho7* and *pas1* are involved in 2-DG induced lysis and ROS formation upon oxidative stress. To our knowledge these functions of the genes have not yet been reported, either for yeast or for any other organism. Even though some biochemical functions of their gene products are known (Vishwanatha *et al.*, 2016) we have no straightforward explanation how they control lysis and ROS formation. However these findings are of interest since 2-DG is known to induce oxidative stress in cancer cells (Coleman *et al.*, 2008). Together with our previous result (Vishwanatha *et al.*, 2016), they suggest interplay between oxidative stress controlling mechanisms and glucose signalling. For the genes *snf5*, *ypa1* and *pas1* human orthologues are known (van Slegtenhorst *et al.*, 2005; Monahan *et al.*, 2008; Goyal and Simanis, 2012; Vishwanatha *et al.*, 2016). It remains to be seen whether deleting these orthologues also inhibit ROS formation in cancer cells. The finding that gene *odr1* is preventing 2-DG induced lysis but does not prevent ROS formation is in accordance with our previous speculation that gene *odr1* might be 2-DG specific and may have a similar function as *S. cerevisiae* Dog1, which acts as a 2-DG-6 phosphate phosphatase (Randez-Gil *et al*., 1995; Vishwanatha *et al*., 2016).

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SHORT COMMUNICATION

Clonal Analysis of Clinical and Environmental *Pseudomonas aeruginosa* Isolates from Meknes Region, Morocco

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Abstract

From 123 clinical and environmental *Pseudomonas aeruginosa* isolates, 24 strains were selected for their similar antibioresistance, virulence and biofilm formation profiles, to examine their diversity and occurrence of clones within two hospitals and different natural sites in Meknes (Morocco). Pulsed-field gel electrophoresis, using DraI enzyme, didn't reveal a close relationship between clinical and environmental isolates nor between strains of the two hospitals. 19 genotypes were obtained, including two virulent environmental clones and three clinical clones virulent and resistant to antibiotics. Intra-hospital transmission of high-risk clones detected, in and between wards, constitutes a great public health concern.

Key words: *Pseudomonas aeruginosa*, clonal transmission in hospital, genetic diversity, genotyping of environmental and clinical isolates

Pseudomonas aeruginosa is one of the major opportunistic human pathogens known to cause severe nosocomial infections often associated with high morbidity and mortality, particularly in immunocompromised or vulnerable patients (Armour *et al.*, 2007; Kerr *et al.*, 2009). Typing methods are essential in tracking sources, pathways of spreading infections and studying population structure. Phenotypic methods as biotyping, serotyping, pyocin typing, phage typing and antibiotyping are not discriminatory enough to identify strains belonging to same genotype. Thus, molecular typing methods are required to investigate diversity of *P. aeruginosa* collections (Speert, 2002). Molecular typing is an important tool in epidemiological surveillance and outbreak investigations of human *P. aeruginosa* infections. Although many typing systems have been developed and show a variety of discriminatory powers, analysis of macrorestriction fragment patterns created by pulsed-field gel electrophoresis (PFGE) is one of the most powerful discriminating methods to type *P. aeruginosa* (Romling *et al.*, 1994a; Johnson *et al.*, 2007). PFGE has demonstrated its efficacy, efficiency, and usefulness for *P. aeruginosa* genotyping. It has been used to detect spread of *P. aeruginosa* clones within wards, within hospitals, among hospitals in the same city, and within state borders (Romling *et al.*, 1994b; Landman *et al.*, 2002). The purpose of present study was to assess genetic diversity and eventual relatedness among a collection of phenotypically close *P. aeruginosa* strains isolated from different natural environmental sites, and distinct pathological specimens from different wards of two major public hospitals in Meknes (Morocco).

A total of 123 environmental and clinical *P. aeruginosa* strains were isolated in Meknes city from June 2012 to June 2014. These isolates originated from different environmental sites and various pathological specimens of patients admitted at two major hospitals in Meknes region. Environmental samples included soil (n = 20), aliments (n = 3), amurca olive (n = 6), rivers water (n = 16), wells (n = 5) and public swimming pools (n = 5). Clinical samples included distal bronchial levy protected (n = 6), pus (n = 35), urine (n = 18), Pleural

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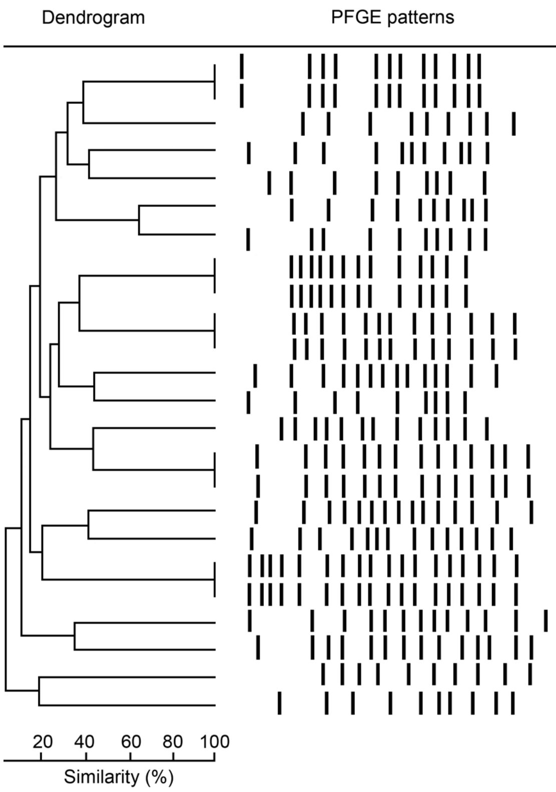
fluid (n = 4), biopsy (n = 2), blood culture (n = 1), bronchial aspirate (n = 1) and vaginal levy (n = 1). These isolates were investigated in previous studies for antimicrobial resistance against 14 antipseudomonal drugs (Maroui *et al.*, 2016a), some secreted and cell-associated virulence factors (production of β-haemolysin, caseinase, lipase, lecithinase, pyocyanin and pyoverdin; ability to swim, swarm, and twitch) and biofilm formation kinetics (Maroui *et al.*, 2016b). Their antibiograms, structural and biochemical profiles were compared to determine whether these isolates were related. Strains showing distinct antibiotypes, phenotypic expression of studied virulence factors and biofilm formation kinetics were first discarded. Remaining strains (24 isolates) which presented closely profiles were selected for further typing by a molecular test.

Genetic relationship among isolates was evaluated using PFGE following a standardized protocol developed by Durmaz *et al.* (2009), and using the restriction enzyme Dra I (Promega, Madison, WI, USA). Dice similarity coefficient was calculated between pairs of lanes, and strains were grouped using the dendrogram construction utility Dendro UPGMA (Biochemistry and Biotechnology Department, Rovirai Virgili University, Tarragona, Spain) (http://genomes.urv.cat/UPGMA/index.php). The Dice similarity coefficient of ≥ 80% was used to define genetically related strains.

Nineteen distinct genetic profiles determined by PFGE were detected among *P. aeruginosa* studied strains (Fig. 1). Clinical strain genotypes were not similar to that of environmental isolates. Furthermore, clinical strains showed 10 patterns indicating none identical genotype identified when pulsotypes obtained for Military hospital and Regional hospital strains were compared. Among Military hospital sample two strains from cardiology ward isolated at distant period (35 days) shared the same clonal profile (clone 12), while remaining isolates presented distinct clonal profiles (clones 2, 13, 16, 17 and 18). For Regional hospital isolates, two strains isolated in burn ward at distant period (4 months) belonged to the same clone (clone 15), one strain isolated from intensive care unit and another from burn ward (after 2 months) shared the same genotype (clone 8), while other strains presented distinct clonal profiles (clones 14 and 19). Pulsotypes analysis of environmental strains showed 9 genotypes. Two strains from amurca olive isolated in the same habitat at distant period (one month) belonged to the same clone (clone 1), one isolate detected in soil and another from well water of sciences faculty of Meknes at distant period (6 months) shared the same clonal profile (clone 7), while other strains presented distinct genotypes (clones 3, 4, 5, 6, 9, 10 and 11).

Analysis of PFGE patterns revealed that examined *P. aeruginosa* strains showed a great genetic variabil-



Strain code	Origin	Isolation site	Isolation date	Ward*	Gender*	PFGE type
2	Env.	Amurca olive	11/01/2013	–	–	C1
42	Env.	Amurca olive	15/02/2013	–	–	C1
BP601	MH	Biopsy	21/02/2014	Intensive care	M	C2
41–1	Env.	River Boufekrane water	07/02/2013	–	–	C3
11	Env.	River Bouishak water	02/02/2013	–	–	C4
47	Env.	Public pool water	15/07/2013	–	–	C5
38	Env.	River Bouishak soil	25/12/2012	–	–	C6
22	Env.	Sc. Fac. well water	14/07/2012	–	–	C7
25–1	Env.	Sc. Fac. soil	31/01/2013	–	–	C7
Pu241	RH	Pus	28/10/2013	Burn	M	C8
102–1	RH	Pus	30/08/2013	Intensive care	M	C8
43	Env.	Public pool water	15/07/2013	–	–	C9
31–1	Env.	River Ouislane water	15/02/2013	–	–	C10
30	Env.	River Ouislane water	15/02/2013	–	–	C11
Pu301”	MH	Pus	28/01/2014	Cardiology	M	C12
Pu505	MH	Pus	06/03/2014	Cardiology	M	C12
Pu601	MH	Pus	22/03/2014	Thoracic	M	C13
115–1	RH	DBP	07/05/2013	Intensive care	M	C14
Pu141	RH	Pus	03/07/2012	Burn	M	C15
Pu234	RH	Pus	29/10/2012	Burn	M	C15
Pu402	MH	Pus	30/04/2014	Oncology	F	C16
Pu304	MH	Pus	27/05/2014	Oncology	M	C17
Pu305’	MH	Pus	03/06/2014	Thoracic	M	C18
Pu247	RH	Pus	04/11/2013	Intensive care	M	C19

Fig. 1. Dendrogram of environmental and clinical *P. aeruginosa* isolates after DNA digestion with Dra I enzyme and PFGE.
*, for clinical isolates; DBP, distal bronchial levy protected; Env., Environmental; F, Female; M, male; MH, Military hospital; RH, Regional hospital; Sc. Fac., Sciences Faculty.

ity, regardless of the strains origin. Most *P. aeruginosa* selected isolates belonged to distinct genotypes (79.2% of polymorphism). Environmental isolates showed more polymorphism (81.8%) than clinical ones (76.9%). PFGE did not reveal a close relatedness between clinical and environmental isolates nor between strains of the two hospitals. High level of heterogeneity recorded in this *P. aeruginosa* strains collection is in agreement with several studies suggesting that this species exhibits a nonclonal population structure (Picard *et al.*, 1994; Wiehlmann *et al.*, 2007; Pirnay *et al.*, 2009; Maatallah *et al.*, 2011).

Higher diversity of environmental isolates can be explained by their origin, since most of these strains were isolated in various habitats geographically scattered in and around Meknes city. Even more, a genetic variability was also observed even among strains isolated from the same natural site at the same time. Only two clones with two strains each were identified, the first concerns strains isolated with one month interval from amurca olive directly discharged in nature by an olive oil factory. These isolates showed the same antibiotype and virulence profile and formed denser biofilm. They would be from the same parent strain that was able to resist and grow on amurca olive. The second clone originated from sciences faculty of Meknes with one strain from well water and other isolated in soil after 6 months. These isolates showed the same virulence profile and biofilm formation kinetics, but distinct antibiotypes. This observation can be explained by the circulation of clones between these geographically close biotopes. These isolates would have arose from the same ancestor strain, and the imipenem and meropenem resistant one could evolved in response to changing environmental conditions and developed this resistance.

Clinical isolates were also highly diverse, the level detected (76.9%) is higher than that reported by Selim *et al.* (2015) signaling 50% of polymorphism in *P. aeruginosa* isolates from Egypt. However it remains slightly lower than that obtained by Freitas and Barth (2004) indicating 71 PFGE types in 81 clinical isolates from 3 Brazilian hospitals. Obtained polymorphism is also lower than that reported by Lim *et al.* (2009) signaling 93.7% of polymorphism among 48 *P. aeruginosa* isolates from 6 Malaysian hospitals. In fact, tested clinical strains were isolated from various samples of different patients at distinct times in two hospitals. Three clones with two strains each were identified, each clone contains strains showing the same virulence profile and biofilm formation kinetics, but distinct antibiograms. These clones contain virulent and carbapenem resistant strains, with a particular attention to a high risk clone (clone 15) from burn ward that includes 2 multi drug resistant isolates harboring *bla*$_{\text{VIM-2}}$ gene. In fact, metallo-β-lactamases VIM have a potential for horizontal transfer and are among the most clinically threatening carbapenemases (Gupta, 2008).

Findings of isolates showing the same genotype but having distinct antibiotypes and others with same antibiograms but belonging to distinct genotypes were also highlighted by Freitas and Barth (2004) as well as Selim *et al.* (2015). Among various features of *P. aeruginosa* its capability to customize its genome to adapt and fit the needs for thriving in any environmental niche (Mathee *et al.*, 2008). Intra-clonal phenotypic diversity detected in this study may be explained by the remarkable plasticity of *P. aeruginosa* genome. Indeed, genomic dynamism mainly by microevolution events (mutations) or acquisition or discard of genomic segments *via* horizontal gene transfer and recombination modulate *P. aeruginosa* strain's phenotype and differentiate it (Mathee *et al.*, 2008; Bezuidt *et al.*, 2013).

We have detected two transition cases of clones between patients admitted to the same ward and 1 case of clone transmission between 2 distinct wards. Persistence of these resistant and virulent *P. aeruginosa* strains within hospital can be explained by their resistance and high biofilm formation. Indeed, biofilm was identified as a mechanism promoting persistence of clonal groups (Sommerfeld Ross and Fiegel, 2012), and more antimicrobial selection pressure could allow their persistence and silent spread in hospital wards. Because of difficulty in accessing patient histories, the method of transmission between patients or wards could not be determined.

Clinical implications of this study are of concern. Indeed, intra-hospital persistence and dissemination of multi drug resistant *P. aeruginosa* high-risk clones is a serious epidemiological problem. This result causes alarm and should serve as the basis for nationwide strategies to improve infection prevention and control measures in hospitals, aiming to bring down costs and damages caused by this life-threatening microorganism.

To our knowledge, this study is the first genotyping of environmental and clinical *P. aeruginosa* isolates in Morocco. Genetic technique supported by some phenotypic tests has enabled us to conduct a detailed characterization of *P. aeruginosa* strains isolated from distinct samples at particular times. Our findings are in agreement with several studies showing high discriminatory power of PFGE method, and failure to obtain reliable results based only on phenotypic methods (Muller and Gubina, 2000; Freitas and Barth, 2004; Yousefi *et al.*, 2013; Selim *et al.*, 2015).

In conclusion, we detected a great genetic variability and a clear distinction between clinical and environmental isolates. We also described clonal transmission of high-risk *P. aeruginosa* in and between wards in Meknes hospitals which can be deemed a major public health concern.

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SHORT COMMUNICATION

KPC-2-producing *Klebsiella pneumoniae* ST11 in a Children's Hospital in Poland

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Abstract

Four *Klebsiella pneumoniae* isolates from children hospitalized over 10 months in an intensive care unit in a children's teaching hospital in Poland were analyzed. All of the isolates belonged to a single pulsotype and sequence type (ST) 11, and produced the KPC-2 carbapenemase and extended-spectrum β-lactamase (ESBL) CTX-M-15. They were resistant to a variety of antimicrobials, and their β-lactam resistance patterns were typical for KPC producers. This is one of few cases of identification of KPC (or carbapenemase)-producing *K. pneumoniae* in a pediatric center in Poland.

Key words: carbapenemase, epidemiology, KPC-2 producing *K. pneumoniae* ST11

In recent years resistance to carbapenems has become a matter of the highest concern in medicine of bacterial infections (Bush, 2010; Nordmann and Poirel, 2014). It has been associated largely with carbapenemase-producing *Enterobacteriaceae* which express various carbapenem-hydrolyzing enzymes, including *Klebsiella pneumoniae* carbapenemases (KPCs) (Munoz-Price *et al.*, 2013; Nordmann and Poirel, 2014). KPCs hydrolyze virtually all β-lactams of clinical use (Mehta *et al.*, 2015), and are produced by many species, predominantly by *K. pneumoniae*. Strains with KPCs disseminate rapidly and cause outbreaks; since the late 1990s these have spread in the United States, followed by Israel from 2005, and then worldwide (Munoz-Price *et al.*, 2013; Nordmann and Poirel, 2014). In large part, this has been due to clonal expansion of *K. pneumoniae* strains belonging to the sequence type (ST) 258 and related clones, forming the clonal group (CG) 258 (Chen *et al.*, 2014; Mathers *et al.*, 2015). bla_{KPC} genes, mainly $bla_{\mathrm{KPC-2}}$ or $bla_{\mathrm{KPC-3}}$, are carried by Tn*4401*-like transposons of some structural polymorphism (Naas *et al.*, 2008; Baraniak *et al.*, 2015). Most KPC-producing bacteria also express other β-lactamases and contain genes conferring resistance to other antimicrobials, such as aminoglycosides, fluoroquinolones or co-trimoxazole (Nordmann and Poirel, 2014). Therefore, infections caused by multidrug-resistant KPC producers have scarce treatment options and are associated with high mortality rates (Tumbarello *et al.*, 2015).

KPC-2-producing *K. pneumoniae* ST258 emerged in Poland in 2008 (Baraniak *et al.*, 2009) and by the end of 2009 it caused a large outbreak in Warsaw and its region, Mazowieckie (Baraniak *et al.*, 2011). At the same time sporadic cases of other STs of *K. pneumoniae* with KPC-2, namely ST11 and ST23, were observed in Warsaw and Kielce, respectively. All $bla_{\mathrm{KPC-2}}$ genes identified were located within the Tn*4401*a transposon variant. In 2010–2014 still the most affected region was Mazowieckie but new KPC outbreaks occurred in four other areas: Świętokrzyskie, Lubelskie, Podlaskie and Śląskie (Baraniak *et al.*, 2017). The outbreak organisms were *K. pneumoniae* ST258 or ST512 and they produced KPC-3 encoded by Tn*4401*a or Tn*4401*b elements. According to the National Reference Centre for Susceptibility Testing (NRCST) data, since 2014 the situation has had a tendency to stabilize at a low prevalence level in most of the regions mentioned above (D. Żabicka, A. Baraniak, M. Gniadkowski, W. Hryniewicz, unpublished data).

In this study four KPC-positive *K. pneumoniae* isolates from children were analyzed. All patients were hospitalized between February and November

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2016 in an intensive care unit (ICU) in a large tertiary pediatric center in Kraków, and included chronologically: a 7-years-old female with aspiration pneumonia, a 2-months-old male with respiratory distress syndrome, a 9-months-old female with multiple organ failure, and a 6-years-old female with pneumonia. The *K. pneumoniae* isolates were recovered mainly from tracheal or bronchial aspirates; one isolate, 2214/16, was cultured from blood. The isolates were identified by the hospital microbiology laboratory, using the Phoenix system (BD Biosciences), and owing to carbapenem resistance these were sent to the NRCST in Warsaw for reference diagnostics and surveillance purposes. The NRCST has confirmed carbapenem resistance by the Carba NP test (Nordmann *et al.*, 2012), and the positive combined disk test with phenylboronic acid (Doi *et al.*, 2008) and PCR (Navon-Venezia *et al.*, 2006) revealed the KPC presence.

The isolates were typed using pulsed-field gel electrophoresis (PFGE) (Seifert *et al.*, 2005), and produced identical DNA banding patterns. The following multilocus sequence typing (MLST) (Diancourt *et al.*, 2005) classified them into ST11 (http://bigsdb.web.pasteur.fr/klebsiella). Amplicons containing the $\mathit{bla}_{\text{KPC}}$ genes were digested by the *Rsa*I restriction enzyme (Thermo Scientific) which distinguishes the $\mathit{bla}_{\text{KPC-2}}$- and $\mathit{bla}_{\text{KPC-3}}$-like genes (Lopez *et al.*, 2011). All isolates carried the $\mathit{bla}_{\text{KPC-2}}$-like alleles, which turned out to be $\mathit{bla}_{\text{KPC-2}}$ by sequencing performed for the representative isolate 2214/16. A PCR mapping assay that discerns various polymorphs of the Tn*4401* transposon (Naas *et al.*, 2008; Baraniak *et al.,* 2015) demonstrated the presence of the Tn*4401*a variant exclusively. Specific PCRs for major β-lactamase types (Baraniak *et al.*, 2011) allowed detecting additionally ESBLs of the CTX-M-1 group and TEM-like enzymes in all of the isolates, identified by sequencing as CTX-M-15 and TEM-1, respectively, in the isolate 2214/16. Susceptibility of the KPC-producing *K. pneumoniae* was tested by MIC Test Strips (Liofilchem®) and by the broth microdilution method in the case of colistin (http://eucast.org). The results were interpreted according to the EUCAST guidelines (http://eucast.org). The isolates showed multi-drug resistance patterns, with uniform resistance to all β-lactams, gentamicin and ciprofloxacin, susceptibility only to amikacin, tigecycline and colistin (Table I).

K. pneumoniae is a relatively frequent cause of nosocomial outbreaks, including those in neonatal or pediatric wards that are a matter of special concern (Paczosa and Mecsas, 2016). The constantly and rapidly increasing resistance of this pathogen remarkably magnifies the problem. The NRCST data indicates that the carbapenemase-producing multi-drug-resistant *K. pneumoniae* strains have been rarely observed in pediatric centers in Poland so far (D. Żabicka, A. Baraniak, M. Gniadkowski, W. Hryniewicz, unpublished data); therefore, the cases analyzed in this study might signalize the risk of their expansion into these environments. The high genetic relatedness of the four isolates suggests the epidemic character of the KPC infections in the ICU. However, the infection cases were separated in time from each other, and identification of each case was followed by implementation of enhanced infection control measures. It is possible that the outbreak was mediated by unidentified carrier(s) or a hidden environmental source, but the repeated introduction of the KPC-2-producing *K. pneumoniae* ST11 organism cannot be totally excluded either. Since November 2016 to the moment of writing this report (March 2017) no new KPC cases have been recorded in the hospital.

Interestingly, the isolates were not related to any of the outbreak or sporadic KPC-producing *K. pneumoniae* isolates ever studied in detail in Poland so far, predominantly representing various lineages of the CG258 group (Baraniak *et al.*, 2009; 2011; 2017). These comprised two KPC-3-positive ST512 isolates from another hospital in Kraków from 2012 (Baraniak *et al.*, 2017), as well as the only two "Polish" ST11 isolates with KPC-2 from 2009 from Warsaw (Baraniak *et al.*, 2011). ST11 is a truly pandemic *K. pneumoniae* clone, and an evolutionary precursor of ST258 (Chen *et al.*, 2014). It has been identified in many countries with a variety of resistance mechanisms, including diverse β-lactamases, and for example in Poland it has

Table I
Susceptibilities of the *K. pneumoniae* isolates obtained in the study.

Isolate	MIC[1] (μg/ml) of:																
	AMX	AMC	PIP	TZP	CAZ	CTX	FEP	ATM	IMP	MEM	ERT	AMK	GEN	CIP	TET	TGC	CST
2213/16	>256	>256	>256	>256	>256	>256	64	>256	>32	>32	16	4	32	>32	>256	1.5	0.25
2214/16	>256	>256	>256	>256	>256	>256	128	>256	>32	>32	>32	4	32	>32	>256	1.5	0.125
6465/16	>256	>256	>256	>256	>256	>256	128	>256	>32	>32	>32	4	32	>32	>256	1.5	1
6973/16	>256	>256	>256	>256	>256	>256	128	>256	16	>32	>32	4	32	>32	>256	1.5	0.125

[1] Abbreviations: AMC, amoxicillin-clavulanic acid; AMK, amikacin; AMP, ampicillin; AMX, amoxicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; ERT, ertapenem; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MEM, meropenem; PIP, piperacillin; TET, tetracycline; TGC, tigecycline; TZP, piperacillin-tazobactam.

been responsible for a spectacular on-going outbreak of New Delhi metallo-β-lactamase (NDM) producers (Baraniak *et al.*, 2016). KPC-producing *K. pneumoniae* ST11 has been identified in several countries, being the predominant KPC producer in China (Qi *et al.*, 2011; Cheng *et al.*, 2016; Hu *et al.*, 2016) and playing a significant role in Spain (Oteo *et al.*, 2016) or Brazil (Pereira *et al.*, 2013; Andrade *et al.*, 2014). The emergence of the KPC-2-producing ST11 in Kraków might be due to a new KPC introduction to Poland; considering the relatively low prevalence of KPCs in the country and especially in Kraków, the on-site transmission of a KPC-encoding plasmid from a CG258 member to ST11 seems to be less likely.

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SHORT COMMUNICATION

Genetic Characterization of Human Enteroviruses Associated with Hand, Foot and Mouth Diseases in Poland, 2013–2016

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Abstract

The objective of the present study was to describe the molecular characteristics of enteroviruses associated with hand, food, and mouth disease (HFMD) in Poland. Clinical material from HFMD cases, that occurred during 2013–2016 were examined. It has been showed that coxsackievirus A6 (CVA6), CVA10 and CVA16 were circulating in the country. Phylogenetic analysis showed that Polish CVA6 strains were divided into two distinct clusters suggesting two independent introductions. This is the first report of CVA6 infections associated with HFMD in Poland. These results emphasize the need for continuous monitoring of HFMD and facilitation of the diagnosis using molecular approaches.

Key words: coxsackieviruses, genotyping of HFMD enteroviruses, hand, foot and mouth disease (HFMD), human enteroviruses (HEVs)

Hand, food and mouth disease (HFMD) is a common infection characterized by fever, stomatitis and a vesicular rash affecting the hands, feet, and occasionally the buttocks. The disease is usually mild and self-limiting, but serve neurological and systemic syndromes that can be fatal occur in some patients. HFMD is common in children younger than 5 years old, but can also occur in older children and adults. It is caused by human enteroviruses (HEVs) belonging to the *Picornaviridae* family. The most common etiologic agents are coxsackievirus A16 (CVA16) and enterovirus 71 (EV71), but other enteroviruses, mainly belonging to the species HEV-A (CVA2, CVA4-8, CVA10, CVA12), but also HEV-B (echoviruses: E4, E7, E9, E11, E25, E30 and CVB1-5, CVA9, EV84) have been associated with illness (Lei *et al.*, 2015).

Recently, the switch of HFMD etiology has been suggested by the increased epidemics of serotypes other than EV71 and CVA16, including CVA6, CVA10, and CVA12. Serve HFMD in children and atypical HFMD in adults has been reported in association with CVA6 (Lott *et al.*, 2013). The switch of HFMD etiology requires a precise virus genotyping in the surveillance for a better HFMD control.

There are no published data available on the incidence of HFMD in Poland, there is no active public health surveillance for HFMD and it is not a notifiable infection. Whereas EV71 has been occasionally detected in clinical samples (Wieczorek and Krzysztoszek, 2016). To better understand the molecular characteristics of enteroviruses circulating in Poland, particularly the diversity of those viruses associated with HFMD, we examined clinical material from HFMD cases, that occurred during the period 2013–2016, in three regions of Poland.

Since November 2013 to March 2016, a total of 28 specimens (10 stools, 18 throat swabs) were collected from 27 clinically diagnosed HFMD cases (Fig. 1) from 3 voivodoships. Patient's age ranged from 5 months to 17 years (Table I). The clinical samples were tested with diagnostic pan-enterovirus RT-PCR. Viral RNA was extracted from 140 μl of the sample using QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. RT-PCR was carried out using pan-enterovirus primers based on the WHO manual (WHO, 2004). This set of primers produces a product of 114 bp and has been designed to detect and amplify a genome segment present at the 5'NCR of the enterovirus genomes. Out of

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Fig. 1. Child with atypical hand, food and, month disease associated with coxsackievirus A6 infection, Wroclaw, Poland, February 2016.

28 samples, 20 (71.4%) were positive for enteroviruses using RT-PCR, 10 stools (100%) and 10 swabs (55.5%).

Viruses have been isolated from throat swabs and stool specimens by conventional cell culture method using WHO recommendations. Isolation was performed on RD cells (human rhabdomyosarcoma). A volume of 200 µl of the sample was inoculated into tubes with RD cells. The tubes were incubated at 36°C and were examined daily. After 7 days, the tubes were frozen and thawed and re-passaged, and anther 7-day examination was performed. Each specimen underwent three passages. The study found that among the samples with positive RT-PCR results 6 (30%) were positive. Enterovirus was isolated from the stools of 6 patients and not even one throat swab. Complete cytopathic effect (CPE) has occurred after 12 to 23 days post-inoculation (Table I).

To identify the enterovirus type in positive samples, RT-PCR specific for a partial sequence of the viral pro-

Table I
Laboratory findings in clinical specimens and epidemiologic data for patients with HFMD, Poland 2013–2016.

No.	Sample identification number	Sex/age	Region	Sampling date	Specimen type	Isolation result	5'NCR RT-PCR	HEV-A RT-PCR
1	672/PL30/2013	f/1 y	wielkopolskie	XI.2013	stool	+ (23d)	+	+ CVA10
2	1HFMD/PL14/2014	f/9 m	mazowieckie	VII.2014	throat swab	–	+	–
3	2HFMD/PL14/2014	m/9 y	mazowieckie	VII.2014	throat swab	–	–	–
4	3HFMD/PL14/2014	f/2 y	mazowieckie	VII.2014	throat swab	–	+	–
5	4HFMD/PL14/2014	f/17 y	mazowieckie	VII.2014	throat swab	–	–	–
6	0610/PL14/2014	m/2 y	mazowieckie	X.2014	stool	+ (17d)	+	+ CVA6
7	5HFMD/PL14/2014	f/1 y	mazowieckie	X.2014	stool	+ (14d)	+	+ CVA6
8	6HFMD/PL14/2014	m/3 y	mazowieckie	X.2014	stool	+ (15d)	+	+ CVA16
9	7HFMD/PL14/2014	m/9m	mazowieckie	X.2014	stool	–	+	+ CVA6
10	8HEMD/PL14/2015	f/5 y	mazowieckie	III.2015	stool	+ (13d)	+	+ CVA16
11	9HFMD/PL14/2015	f/2 y	mazowieckie	III.2015	stool	+ (12d)	+	+ CVA16
12	20HFMD/PL02/15	m/11 y	dolnośląskie	IV.2015	throat swab	–	–	–
13	10HFMD/PL14/2015	m/4 y	mazowieckie	VI.2015	stool	–	+	+ CVA6
14	11HFMD/PL14/2015	m/2 y	mazowieckie	VI.215	stool	–	+	+ CVA6
15	19HFMD/PL02/2015	f/15 y	dolnośląskie	VI.2015	throat swab	–	+	–
16	18HFMD/PL02/2015	m/7 y	dolnośląskie	VII.2015	throat swab	–	+	–
17	16HFMD/PL02/2015	f/5 m	dolnośląskie	VIII.2015	throat swab	–	–	–
18	17HFMD/PL02/2015	m/11 y	dolnośląskie	VIII.2015	throat swab	–	+	–
19	24HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	stool	–	+	–
20	24HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	throat swab	–	–	–
21	28HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	throat swab	–	+	+ CVA6
22	13HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	throat swab	–	+	+ CVA6
23	14HFMD/PL02/2015	m/2 y	dolnośląskie	XI.2015	throat swab	–	+	+ CVA6
24	15HFMD/PL02/2015	m/1 y	dolnośląskie	XI.2015	throat swab	–	+	–
25	12HFMD/PL02/2015	m/1 y	dolnośląskie	XII.2015	throat swab	–	–	–
26	21HFMD/PL02/2016	m/7 m	dolnośląskie	II.2016	throat swab	–	+	+ CVA6
27	22HFMD/PL02/2016	m/13 y	dolnośląskie	II.2016	throat swab	–	–	–
28	23HFMD/PL02/2016	f/16 y	dolnośląskie	III.2016	throat swab	–	–	–

tein 1 (VP1) region, was performed by using Superscript III (Invitrogen) followed by a second amplification reaction with nested primers for species A and B VP1 sequences and PCR cycling times and temperature as previously described (Leitch *et al.*, 2009). Amplified products were analysed in 1.5% agarose gels, GelRed-stained, and examined under a UV DNA transilluminator. The resulting DNA templates were processed in cycle sequencing reaction with a BigDye 3.1 according to manufacturer's protocol. The product of sequencing reaction was run in an automated genetic analyser (Applied Biosystems, model 3730). The resulting sequences were manually edited using BioEdit program and examined in terms of closest homologue sequence using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of isolated strains (region VP1) were aligned with the reference strains. A phylogenetic tree was computed using the neighbor-joining method with bootstrap 1000 replicates. Molecular and phylogenetic analyses were conducted using MEGA version 6.06 (Tamura *et al.*, 2013) (http://www.megasoftware.net/). Sequences have been assigned GenBank accession numbers KX865266 to KX865274.

Out of 20 positive samples, 13 were positive in reaction characteristic for species A and none in reaction for species B. A total of 3 different serotypes of species A were identified by partial VP1 sequencing (CVA10, CVA6, CVA16). The typed strains included 6 cell culture

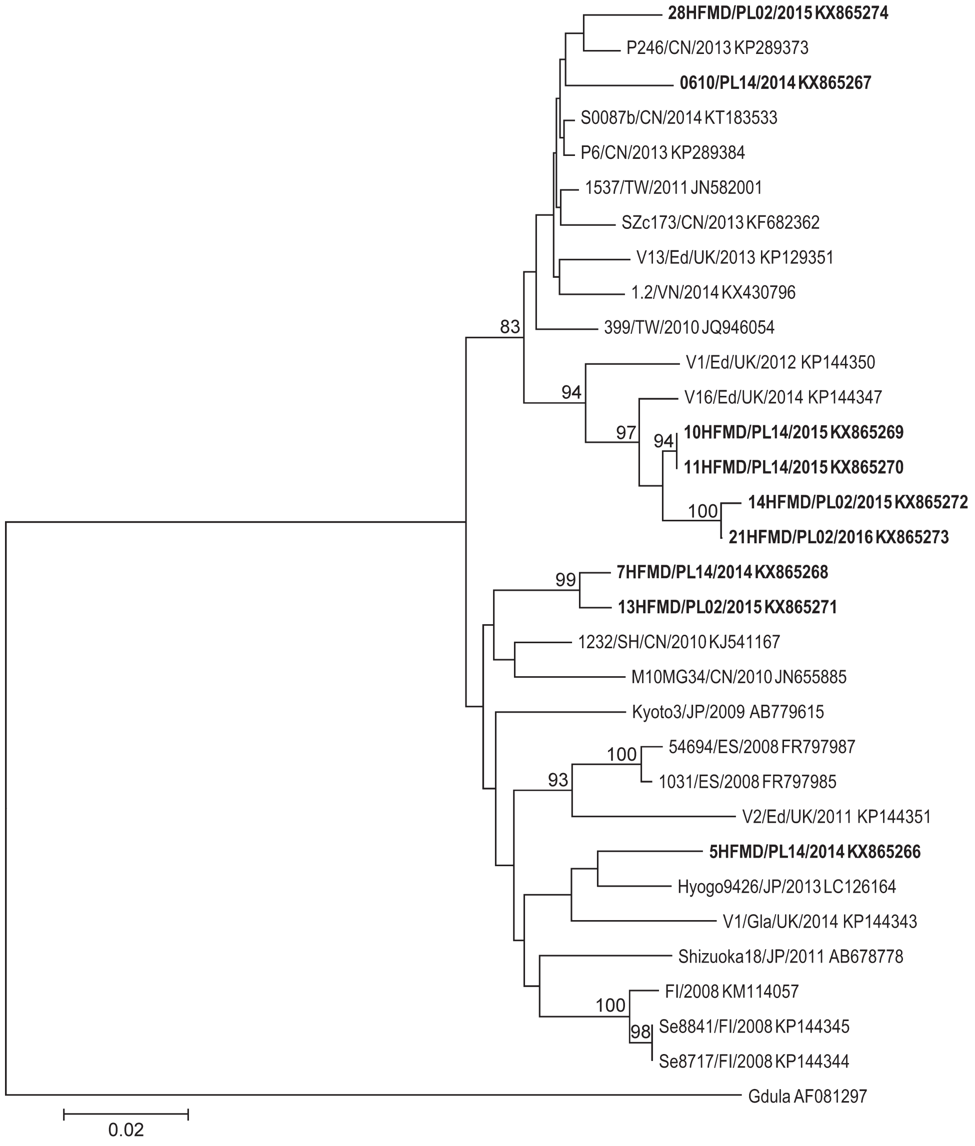

Fig. 2. Phylogenetic tree depicting the relationships between partial capsid gene sequences of 9 Polish CVA6 strains isolated from 2013 to 2016 and 23 sequences from GenBank (609 nt). Each strain is referenced by its geographical origin and its accession number.
The tree was constructed by neighbour-joining method and evaluated with 1000 bootstrap pseudoreplicates. Only bootstrap values ≥ 80% are indicated. In the analyses, genetic distances were calculated with Kimura 2-parameter algorithm. Analyses were conducted in MEGA 6.06 (Tamura *et al.*, 2013).

isolates and 7 strains primarily detected by RT-PCR. CVA6 was identified in 9 and CVA16 in 3 typed cases, respectively. One patient was positive for CVA10.

Genetic analysis of viral sequences showed that CVA6 strains were divided into two clusters (Fig. 1). In general, nucleotide sequence divergence in pairwise comparisons among isolates range from 0.0% to 7.5% (0.0–2.5% amino acid divergence). Compared with the prototype strain Gdula, the genetic divergence increased to 18.4–20.0%. Cluster I includes three sequences of CVA6 isolated in 2014–2015, presenting 1.0–5.6% nucleotide divergence (0.5–2.0% amino acid divergence). These strains had closest genetic relationship with isolates previously identified in various geographical origins (China 2010–2013, United Kingdom 2013, Japan 2013). Cluster II, comprised six strains from 2014–2016, presenting 0.0–5.6% VP1 nucleotide sequence divergence (0.0–2.0% amino acid divergence). Virus strains belonged to cluster II grouped together with strains isolated in the United Kingdom (2014) and China (2009–2014) (Fig. 2). Three Polish CVA16 strains from 2014–2015, were genetically homogeneous, presenting 0.0–2.0% nucleotide divergence and were closely related to Chinese strains isolated in 2011. One strain CVA10 grouped together with those from Russia isolated in 2009–2013 and Spain (2008).

In Poland, there is no the surveillance of HFMD. Therefore, little is known about the pathogenic roles of enteroviruses, their geographic distributions, and epidemiological data. The results demonstrated that CVA6, CVA10, and CVA16 emerged and co-circulated in Poland. To the best of our knowledge, this is the first report of CVA6 infections associated with HFMD in Poland. As an emerging pathogen, CVA6 increasingly became as common a causative agent of HFMD in Poland as was CVA16. CVA6 has been associated with more severe and extensive rash than HFMD caused by other enteroviruses (Wei *et al.*, 2011). Since 2008, international outbreaks of CVA6 HFMD in children and adults have been described (Osterback *et al.*, 2009; Blomqvist *et al.*, 2010; Wu *et al.*, 2010; Wei *et al.*, 2011; Fujimoto *et al.*, 2012; Mirand *et al.*, 2012; Puenpa *et al.*, 2013; Cabrerizo *et al.*, 2013; Sinclair *et al.*, 2014), but no outbreaks had been reported in Poland previously. Although all 9 of the CVA6 strains identified in the Polish cases were genetically closely related (based on partial VP1 gene sequences) to CVA6 strains identified in recent international outbreaks. Two genetically distinct CVA6 clusters were co-circulating in Poland suggesting two independent introductions of the virus to Poland. It may be noted that the analysis of data obtained in this study was limited due to the small size of samples available.

In mild cases of HFMD laboratory testing is not necessary. Testing is usually reserved for severe cases and public health investigation of outbreaks. Of the 20 samples identified as HEV on the basis of the 5'NCR, only 13 could be typed using primers specific to the VP1 region. The failure of amplification of typing regions in seven specimens may have been due to a low viral load. Genotyping was performed directly in clinical specimens for 7 samples and for 6 isolates. HEV identification was effective for 50% of the EV-positive clinical samples and for 100% of the isolates. During the 2008 Finnish outbreak, EV identification was effective for 55% of the EV-positive clinical samples (Blomqvist *et al.*, 2010). In most epidemiological studies of HFMD, enterovirus genotyping relies on virus isolates despite the difficulty of growing HEV-A serotypes in cell culture.

CVA are generally difficult to grow in culture (Nsaibia *et al.*, 2007). Because of this feature, isolation from clinical material is often unsuccessful. Most CVA could propagate in RD cells, but they generally require more than one passage before inducing a detectable cytopathic effect. In this study, all the isolates were obtained on RD cell in the second or third passage and only from stool specimen. All identified CVA10 and CVA16 strains were able to multiply in RD cells, whereas only 40% of identified CVA6 strains showed successful propagation in RD cells, suggesting that CVA6 is more difficult to isolate than CVA16 or CVA10.

As HFMD is not a notifiable disease in Poland, the actual number of HFMD cases is not known. Enteroviral infections are underdiagnosed as a result of the lack of routine surveillance and also frequently atypical presenting symptoms and signs. In addition, increasing awareness will help to improve laboratory diagnosis and management of infected children. Improved HFMD surveillance is required, with virus genotyping as a key element.

In summary, the present investigation highlights the co-circulation of CVA6, CVA10 and CVA16 types causing HFMD in Poland. These results emphasize the need for continuous monitoring of HFMD in Poland and facilitation of the diagnosis of the associated HEV infections using molecular approaches.

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KONFERENCJA POD PATRONATEM PTM

IV edycja konferencji
„Wektory i patogeny – w przeszłości i przyszłości”
Wrocław, 24 listopada 2017

Szanowni Państwo,

Instytut Genetyki i Mikrobiologii Uniwersytetu Wrocławskiego oraz Wrocławski Oddział Polskiego Towarzystwa Mikrobiologów i Wrocławski Oddział Polskiego Towarzystwa Parazytologicznego zapraszają na IV edycję konferencji pt. *„Wektory i patogeny – w przeszłości i przyszłości”*.

Konferencja ma na celu prezentację badań z zakresu mikrobiologii i parazytologii jakie są prowadzone aktualnie w krajowych jak i zagranicznych jednostkach naukowych. Pragniemy również udokumentować historyczny dorobek polskich naukowców w tych dziedzinach. W tym roku szczególną uwagą objęty będzie problem uwarunkowanych środowiskowo chorób infekcyjnych i inwazyjnych, których czynnikami etiologicznymi są patogeny transmitowane przez stawonogi (wektory), głównie hematofagiczne kleszcze i komary, a także ukazanie skutecznych sposobów zapobiegania i monitorowania tych zagrożeń.

Podczas konferencji planowana jest prezentacja praktycznych osiągnięć 20-letniej współpracy Instytutu Genetyki i Mikrobiologii UWr z Wydziałem Środowiska i Rolnictwa Urzędu Miasta Wrocławia w zakresie biologicznego (mikrobiologicznego) zwalczania komarów na terenie Aglomeracji Wrocławskiej. Ważnym celem konferencji jest także integracja środowiska naukowego oraz ukazanie osiągnięć naukowych młodych adeptów nauki.

Szczegółowe informacje zamieszczone są na stronie:
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INFORMACJA O PRACY PREZYDIUM ZARZĄDU GŁÓWNEGO PTM

Dnia 30 sierpnia i 12 września 2017 r. odbyły się elektroniczne głosowania szeregu Uchwał, które poniżej omówiono, przez członków Prezydium ZG PTM

1) Zgłosiło się szereg kandydatów, którzy chcieliby zapisać się do PTM. Jednomyślnie przyjęto **Uchwałę nr 23-2017 z dnia 30.08.2017 r. w sprawie przyjęcia 20 nowych członków zwyczajnych PTM.**

2) Dużo wysiłku wkładamy w porządkowanie przynależności osób do PTM i kwestii opłacania przez nich składek członkowskich. Dokładna liczba członków PTM jest trudna do ustalenia, ale określamy ją na około 1000 osób. Jednakże na dzień 28.08.2017 r. składki za dany rok zapłaciło: 2015 r. – 693, 2016 r. – 760, 2017 r. – 595 osób. Zgodnie ze Statutem PTM par.15 p. 3: „Członkostwo zwyczajne ustaje na skutek skreślenia z listy członków Towarzystwa z powodu nie zapłacenia składki członkowskiej przez dwa kolejne lata, skreślenie może nastąpić dopiero po pisemnym uprzedzeniu o zamiarze skreślenia, przez Oddział Terenowy PTM i upływie wyznaczonego terminu dodatkowego do uregulowania zaległych składek”. Do osób które w przewidzianym przez Statut PTM i Uchwałę ZG PTM z dnia 04.02.2014 nie opłaciły zaległych składek członkowskich i do których mieliśmy adresy wysłano w lipcu 2017 r. listy z zawiadomieniem o planowanym skreśleniu. Część z tych osób uzupełniła składki, część przysłała listy o rezygnacji z członkostwa w PTM. Pozostają osoby, do których nie możemy dotrzeć oraz osoby niepłacące składek przez wiele lat, ale nie skreślone z listy PTM z powodu niedopatrzenia tej sprawy. Jednomyślnie przyjęto **Uchwałę nr 24-2017 z dnia 30.08.2017 r. w sprawie uporządkowania listy członków zwyczajnych PTM i usunięcia z tej listy osób nie płacących składek członkowskich w statutowo przewidzianym terminie.** Na początku września br. wysłaliśmy listy do ponad 170 osób z informacją, że zostają wykreśleni z listy członków zwyczajnych PTM. Na zaktualizowanej liście pozostaną osoby, które regularnie opłacały składki do 2014 r. i zapłaciły składki za 2015 i/lub 2016 i/lub 2017. Nie dotyczy to osób, które przystąpiły do PTM w okresie 2015–2017.

3) Brak regularnego płacenia składek członkowskich jest niewątpliwie zaniedbaniem lub świadomą decyzją tych osób, jednakże wydaje się, że w minionym okresie Towarzystwo nie wykazało należytej aktywności w przypominaniu o konieczności zapłacenia zaległych składek i wysyłaniu do dłużników e-maili i listów w tej sprawie. Wiele osób zasłużonych dla mikrobiologii zaniedbało obowiązku regularnego płacenia składek. Opinie na temat utworzenia możliwość ponownego przystąpienia do PTM osobom, skreślonym z powodu niepłacenia składek, wśród członków Prezydium były podzielone. Większość członków Prezydium była jednak za przyjęciem **Uchwały nr 25-2017 z dnia 30.08.2017 r. w sprawie możliwości ponownego przystąpienia do PTM po rocznym okresie karencji osób skreślonych z listy członków PTM za niepłacenie składek członkowskich.** Tym samym wprowadza się możliwość ponownego przystąpienia do PTM osobom skreślonym z listy członków zwyczajnych z powodu niepłacenia składek członkowskich, po rocznym okresie karencji, od 01.10.2018 r., na zasadach nowo przystępujących do Towarzystwa członków zwyczajnych.

4) Dosyć długo zajmowaliśmy się kwestią przygotowania stanowiska PTM w sprawie przyznania absolwentom uniwersyteckich studiów magisterskich na kierunku Mikrobiologia prawa wykonywania zawodu Diagnosty Laboratoryjnego. Przy wsparciu 3-osobowego zespołu, którego prace koordynowała Pani prof. Beata Sadowska przygotowano propozycję tekstu, w którym staraliśmy się osiągnąć kompromis stanowisk. Większość członków Prezydium była za przyjęciem **Uchwały nr 26-2017 z dnia 30.08.2017 r. w sprawie stanowiska PTM odnośnie przyznania absolwentom uniwersyteckich studiów magisterskich na kierunku Mikrobiologia prawa wykonywania zawodu Diagnosty Laboratoryjnego.** Poniżej zamieszczony jest tekst, który będzie opublikowany w czasopismach PTM oraz wysłany do odpowiednich, ustalonych adresatów.

Stanowisko Polskiego Towarzystwa Mikrobiologów w sprawie przyznania absolwentom uniwersyteckich studiów magisterskich na kierunku Mikrobiologia prawa wykonywania zawodu Diagnosty Laboratoryjnego

W oparciu o Rozporządzenie Ministra Zdrowia z dnia 14 listopada 2008 r. (Dz. U. Nr 208, Poz. 1312), wpisujące mikrobiologię na listę dziedzin mających zastosowanie w ochronie zdrowia oraz ustawę Prawo o szkolnictwie wyższym z 2011 r. (Dz. U. Nr 84, poz. 455), utworzono niezależne kierunki Mikrobiologia na wydziałach pięciu Polskich uniwersytetów posiadających odpowiednie uprawnienia (Uniwersytetu Łódzkiego, Szczecińskiego, Warmińsko-Mazurskiego, Wrocławskiego oraz Zachodniopomorskiego Uniwersytetu Technologicznego). Opracowano standard kształcenia dla kierunku Mikrobiologia, dokonując przesunięcia profilu kształcenia z ogólnobiologicznego w kierunku ogólnomedycznym, co wydaje się, że powinno zapewnić zdobywanie przez studentów kierunku Mikrobiologia wiedzy i umiejętności upoważniających do podejmowania samodzielnych czynności w ramach szeroko rozumianej medycznej diagnostyki laboratoryjnej. Standard ten został opracowany przez mikrobiologów, immunologów i genetyków z uniwersytetów: Łódzkiego, Warszawskiego, Wrocławskiego i UMCS w Lublinie, przy współpracy z członkami Komitetu Mikrobiologii PAN oraz w porozumieniu z Krajową Radą Diagnostów Laboratoryjnych oraz kierownikami laboratoriów medycznych, i zatwierdzony przez Radę Główną Nauki i Szkolnictwa Wyższego. Na podstawie ww. znowelizowanej ustawy Prawo o Szkolnictwie Wyższym, standard ten został przepisany na odpowiednie efekty kształcenia w zakresie wiedzy, umiejętności i kompetencji społecznych.

Podczas pięcioletnich, dwustopniowych studiów (3+2), osoby uzyskujące najpierw licencjat na kierunku Mikrobiologia, a następnie tytuł zawodowy magistra Mikrobiologii powinni uzyskać efekty kształcenia wymagane do wykonywania zawodu Diagnosty Laboratoryjnego określone w Rozporządzeniu Ministra Nauki i Szkolnictwa Wyższego z dnia 24 sierpnia 2016 r. (Poz. 1434), które mają obowiązywać od roku akademickiego 2017/2018 studentów kierunku Analityka Medyczna/Medycyna Laboratoryjna. Studenci obu stopni studiów kierunku Mikrobiologia uzyskują wszystkie wymagane tym Rozporządzeniem i weryfikowane podczas egzaminów i kolokwiów zaliczeniowych, efekty kształcenia. Biorąc pod uwagę odpowiedzialność, jaka spoczywa na zawodzie Diagnosty Laboratoryjnego, wyrażamy przekonanie, iż tylko osiąganie określonych standardów / efektów kształcenia w ramach wybranych kierunków studiów wyższych, może być gwarancją przygotowania absolwentów do pracy w tym zawodzie, zapewniając jednocześnie bezpieczeństwo pacjentów i prawidłową współpracę diagnostów z lekarzami.

W związku z powyższym większość członków Polskiego Towarzystwa Mikrobiologów uważa, iż uprawnienia do tytułu Diagnosty Laboratoryjnego powinny być uzależnione od odpowiednich efektów kształcenia, które muszą być ustalone dla każdego kierunku, a nie od nazwy kierunku studiów. Absolwenci uniwersyteckich kierunków Mikrobiologia, z tytułem magistra mikrobiologii, powinni mieć prawo do starania się o wpis na Listę Diagnostów Laboratoryjnych, ponieważ podczas studiów uzyskują efekty kształcenia wymagane do wykonywania zawodu Diagnosty Laboratoryjnego, zgodne ze standardem przewidywanym dla kierunku Analityka Medyczna/Medycyna Laboratoryjna, dokumentowane dyplomem, dorobkiem (praca licencjacka, magisterska, doktorska, publikacje) oraz odbytymi praktykami i stażami zawodowymi. Prawo wpisu na Listę Diagnostów Laboratoryjnych dla absolwentów kierunku Mikrobiologia można by uzyskać po ukończeniu studiów i po rocznym stażu w wieloprofilowym laboratorium medycznym oraz zdaniu egzaminu państwowego.

5) Co roku odbywają się w różnych miastach Europy spotkania zarządu FEMS i przedstawicieli europejskich towarzystw mikrobiologicznych, tzw. FEMS Council. Zwykle jest to około 60 osób. Obrady trwają 2 dni piątek i sobotę w I połowie września. Poruszane są sprawy dotyczące FEMS, akceptowane sprawozdania, organizowane wybory członków zarządu, itp. sprawy organizacyjne. Na ubiegłorocznym spotkaniu w Atenach zapytano, czy nie można by zorganizować FEMS Council w Warszawie – nigdy nie było takiego spotkania w Polsce. Wydaje się, że wypadałoby po 43 latach takich spotkań zorganizować FEMS Council w Warszawie we wrześniu 2018 r. Przy okazji Konferencji krakowskiej zgłosiły się firmy: Global Congress sp. z o.o. oraz Warsaw Convention Bureau, które po rozmowach zaoferowały pomoc w organizacji FEMS Council oraz pewien wkład finansowy na pokrycie: cocktailu powitalnego, wycieczki po Warszawie i częściowe dofinansowanie wieczornej kolacji (to są stale punkty każdego FEMS Council). Federacja opłaca wynajem pomieszczeń na obrady, a koszty hotelu i podróży każdy z uczestników pokrywa we własnym zakresie. Pewne koszty, ale wydaje mi się do zaakceptowania, poniesie PTM. Niemniej z powodu braku do tej pory organizacji FEMS Council w Polsce i ze względów prestiżowych wskazane jest podjęcie tego wysiłku. Warto zaznaczyć, że FEMS przyznało grant w wysokości 3000 Euro na organizację Konferencji 90 lat PTM w Krakowie. Członkowie Prezydium jednomyślnie przyjęli **Uchwałę nr 27-2017 z dnia 30.08.2017 r. w sprawie zorganizowania we wrześniu 2018 r. spotkania FEMS Council w Warszawie.**

6) W związku ze zgłoszoną przez Panią prof. Izabelę Sitkiewicz na ostatnim posiedzeniu ZG PTM chęcią rezygnacji z funkcji Redaktora Naczelnego czasopisma Polish Journal of Microbiology, po rozeznaniu istniejącej sytuacji, postanowiono przychylić się do tej prośby i dokonać odpowiednich zmian kadrowych. Z dniem 01.09.2017 r. powierzono funkcję Redaktora Naczelnego PJM Pani prof. Jolancie Soleckiej z Narodowego Instytutu Zdrowia Publicznego – Państwowego Zakładu Higieny w Warszawie. Jednocześnie Pani prof. Elżbiecie A. Trafny zostaje powierzona funkcja Zastępcy Redaktora Naczelnego PJM (Deputy Editor). Zmiana składu zespołu redakcyjnego zostanie przeprowadzona przez Redaktor Naczelną PJM do dnia 01.11.2017 r. Jednomyślnie podjęto **Uchwałę nr 28-2017 z dnia 30.08.2017 r. w sprawie zmiany Redaktor Naczelnej i członków zespołu redakcyjnego czasopisma Polish Journal of Microbiology.**

7) Trudna sytuacja finansowa PTM, spowodowana przede wszystkim wycofaniem się MNiSW z dofinansowywania czasopism w 2016 r. (otrzymywaliśmy kwoty: 66 970 zł w 2013 i 2014, 51 000 zł w 2015 r.) wymusza podjęcie drastycznych kroków, aby ograniczyć koszty wydawania i dystrybucji czasopism PJM i PM. Roczny koszt to ponad 120 000 zł. Koszt samych znaczków do wysyłki czasopism to ponad 20 000 zł. PTM jako Towarzystwo którego przychody oparte są na składkach członkowskich nie jest w stanie finansować wydawanego w takiej formie czasopisma, należy w jak najszybszym czasie znaleźć możliwość znacznego zredukowania ponoszonych przez Towarzystwo, czyli nas wszystkich kosztów. Nowa Redaktor Naczelna PJM i zespół redakcyjny zaproponowali zrezygnować z wydawania papierowej wersji czasopisma Polish Journal of Microbiology od stycznia 2018 r. przy pozostawieniu tylko wersji internetowej – online. Większość członków Prezydium PTM podzieliło to stanowisko, przyjmując **Uchwałę nr 29-2017 z dnia 30.08.2017 r. w sprawie zaprzestania wydawania papierowej wersji czasopisma Polish Journal of Microbiology od stycznia 2018 r. i pozostawienia tylko wersji internetowej.**

8) Oszczędności muszą dotyczyć również procesu wydawniczego i dystrybucji Postępów Mikrobiologii. Względy finansowe zmuszają do zrezygnowania z bezpłatnego przekazywania zeszytów PM dla członków PTM. Czasopismo będzie się ukazywało nadal w wersji elektronicznej – online. Jeżeli ktoś z członków PTM chciałby otrzymywać papierową wersję PM, mógłby to uzyskać w 2018 r. w ramach płatnej prenumeraty. Musimy się jednak zorientować ile to by było osób i jaki byłby realny koszt takiej prenumeraty. Druk zostanie utrzymany jedynie dla prenumeratorów (osób i instytucji), a wersja papierowa PM rozsyłana będzie tylko do określonych adresatów w liczbie 100 egzemplarzy. Dokładna liczba prenumeratorów na rok 2018 zostanie określona w grudniu 2017 r. Członkowie Prezydium jednomyślnie przyjęli **Uchwałę nr 30-2017 z dnia 30.08.2017 r. w sprawie zaprzestania od stycznia 2018 r. nieodpłatnej wysyłki czasopisma Postępy Mikrobiologii do członków PTM.**

9) W zamian za zaprzestanie nieodpłatnej wysyłki czasopism PJM i PM członkowie PTM otrzymają od 2018 r. nowy przywilej, tj. możliwość, po zalogowaniu się na stronie PTM, dostępu do najnowszego, bieżącego numeru czasopism PJM i PM. Czyli tylko członkowie PTM, którzy opłacili składki na dany rok, prenumeratorzy, a także członkowie honorowi PTM oraz członkowie wspierający PTM będą mieli dostęp do internetowych wersji najnowszego bieżącego numeru PJM i PM. Pozostałe, starsze numery będą dostępne dla wszystkich osób, także nie należących do PTM. Przypominamy, że przywilejem jest również, iż autorzy korespondencyjni artykułów będący członkami PTM maja zniżkę na opłatę redakcyjną w czasopismach PM i PJM. Członkowie Prezydium jednomyślnie podjęli **Uchwałę nr 31-2017 z dnia 30.08.2017 r. w sprawie ograniczonego dostępu do bieżących najnowszych numerów PJM i PM w internecie, od stycznia 2018 r. Dostęp do bieżących numerów tych czasopism będzie jedynie dla członków PTM z opłaconą na danych rok składką członkowską dla prenumeratorów oraz dla członków honorowych i wspierających PTM. Pozostałe numery czasopism będą ogólnodostępne.**

10) Ponadto w zamian za zaprzestanie nieodpłatnej wysyłki czasopism PJM i PM członkowie PTM otrzymają drugi nowy przywilej, tj. członkowie PTM z opłaconą bieżącą składką członkowską biorący udział w konferencjach współorganizowanych przez PTM będą otrzymywać zniżki na opłatę konferencyjną. Tak było na Zjazdach PTM, na tegorocznej Konferencji 90-lat PTM i niedawnej konferencji Mikrobiologia Farmaceutyczna. Wysokość zniżek ustalana zostanie przez komitet organizacyjny danej konferencji w porozumieniu z ZG PTM. Członkowie Prezydium jednomyślnie przyjęli **Uchwałę nr 32-2017 z dnia 30.08.2017 r. w sprawie wprowadzenia zniżki na opłatę konferencyjna dla członków PTM biorących udział w konferencjach współorganizowanych przez PTM, od stycznia 2018 r.**

11) Na ostatnim zebraniu ZG PTM podnoszono sprawę dostępu do środków finansowych PTM przez Oddziały Terenowe. Jak wiadomo główne źródło przychodów PTM to składki członkowskie, z których finansowane jest wydawanie czasopism PM i PJM oraz ich dystrybucja do członków PTM (np. w przypadku dystrybucji PJM do członków PTM konieczna była ok. ponad 50% dopłata Towarzystwa), bieżąca działalność biura PTM i całego Towarzystwa. Składki płacone są nieregularnie i nie są w stanie pokryć kosztów Towarzystwa w dotychczas prowadzonej formie. Stąd poszukiwanie dróg redukcji wydatkowania funduszy poprzez obniżenie kosztów wydawania czasopism. Ponadto poszukujemy dodatkowych źródeł przychodów, jak np. Członków Wspierających PTM. Bardzo słaba jest aktywność Oddziałów Terenowych w pozyskiwaniu dodatkowych środków finansowych, natomiast zgłaszane są potrzeby, stad propozycja aktywizacji Oddziałów w omawianym obszarze i możliwość pozyskania przez nich środków na swoje cele statutowe. Członkowie Prezydium jednomyślnie przyjęli **Uchwałę nr 33-2017 z dnia 30.08.2017 r. w sprawie udostępnienia Oddziałom Terenowym PTM od stycznia 2018 r., 50% kwoty uzyskanej z tytułu pozyskania sponsora, Członka Wspierającego PTM, darowizny, lub innej dodatkowej kwoty, na rzecz PTM, przez dany Oddział, na pokrycie kosztów prowadzenia działalności statutowej przez ten Oddział.** Uzyskane kwoty rozliczane byłyby przez biuro i księgowość PTM.

12) Jednomyślnie przyjęto **Uchwałę nr 34-2017 z dnia 30.08.2017 r. w sprawie zmiany sekretarki ZG PTM od 01.10.2017 r.**

13) Aby zostać członkiem zwyczajnym PTM trzeba spełnić 2 warunki – a) wypełnić Deklarację Członka Zwyczajnego oraz b) wnieść składkę członkowską za dany rok. Decyzja ZG PTM o przyjęciu kandydata na członka PTM zapada przed lub po wniesieniu opłaty członkowskiej. Po podjęciu ww. decyzji z biura PTM wysyłany jest e-mail do danej osoby z informacją o przyjęciu w poczet członków PTM i prośbą o opłacenie składki rocznej, jeżeli nie została ona dokonana. Okazało się, że szereg osób wypełnia tylko Deklaracje członkowską i nie płaci pierwszej składki członkowskiej, ale uważa się za przyjętych do Towarzystwa, bo taką decyzję podjął ZG PTM. W związku z tą nieprawidłową sytuacją Członkowie Prezydium jednomyślnie podjęli **Uchwałę nr 35-2017 z dnia 12.09.2017 r. w sprawie wprowadzenia poprawki w tekście Deklaracji Członka Zwyczajnego PTM.** W deklaracji zaznaczono: **Pierwsza składka zostanie wpłacona w ciągu 30 dni od otrzymania drogą elektroniczną zawiadomienia o pozytywnej decyzji ZG PTM** oraz, że „ZG PTM **rekomenduje** na podstawie § 28 statutu Towarzystwa, przyjąć – odmówić przyjęcia – kandydata na członka zwyczajnego Oddziału .... Polskiego Towarzystwa Mikrobiologów. **Przyjęcie nastąpi po wpłaceniu pierwszej składki członkowskiej**”.

14) Przy okazji organizacji Konferencji 90-lat PTM udało nam się pozyskać 3 nowych Członków Wspierających PTM. Uchwałami Prezydium PTM (nr 36-2017, nr 37-2017 i nr 38-2017), jako Członka Wspierającego PTM – srebrnego, przyjęto firmę ECOLAB Sp. z o.o., jako Członka Wspierającego PTM – srebrnego, przyjęto firmę Miejskie Przedsiębiorstwo Wodociągów i kanalizacji S.A. w Krakowie WODOCIĄGI KRAKOWSKIE oraz Członka Wspierającego PTM – zwyczajnego przyjęto firmę MERCK Sp. z o.o.

15) Podjęliśmy współpracę z Kancelaria Prawną w celu uzyskania opinii odnoście powołania Pełnomocników Delegatów na Nadzwyczajne Walne Zgromadzenie Delegatów PTM podczas Konferencji 90 lat PTM, a także wprowadzania zmian w Statucie PTM i przygotowania wniosku do sądu (KRS), który musi zmiany zatwierdzić.

16) Bardzo dużo pracy i wysiłku poświęcamy w przygotowanie i organizację naszej Konferencji z okazji 90 lat PTM.

17) Dwaj członkowie PTM otrzymali Granty FEMS na wyjazdy do naukowych ośrodków zagranicznych. Złożone zostały 2 wnioski o granty FEMS dofinansowujące wyjazdy na konferencje naukowe.

SEKRETARZ
Polskiego Towarzystwa Mikrobiologów
A. Laudy
dr n. farm. Agnieszka E. Laudy

PREZES
Polskiego Towarzystwa Mikrobiologów
prof. dr hab. Stefan Tyski

CZŁONKOWIE WSPIERAJĄCY PTM

Członek Wspierający PTM – Złoty
od 27.03.2017 r.

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Aesculap Chifa Sp. z o.o. jest członkiem grupy B. Braun, jednej z wiodących na świecie firm medycznych, produkującej i dystrybuującej miedzy innymi preparaty do antyseptyki rąk, skóry, błon śluzowych, do mycia i dezynfekcji wyrobów medycznych oraz powierzchni.

Członek Wspierający PTM – Srebrny
od 12.09.2017 r.

Firma Ecolab Sp. z o.o. zapewnia: najlepszą ochronę środowiska pracy przed patogenami powodującymi zakażenia podczas leczenia pacjentów, bezpieczeństwo i wygodę personelu, funkcjonalność posiadanego sprzętu i urządzeń. Firma jest partnerem dla przemysłów farmaceutycznego, biotechnologicznego i kosmetycznego.

Członek Wspierający PTM – Srebrny
od 12.09.2017 r.

Od ponad 100 lat siedziba Wodociągów Krakowskich mieści się przy ul. Senatorskiej.
Budowę obiektu ukończono w 1913 roku.
W 2016 r. do sieci wodociągowej wtłoczono ponad 56 mln m^3 wody.
Szacuje się, że ponad 99,5% mieszkańców Gminy Miejskiej Kraków
posiada możliwość korzystania z istniejącej sieci wodociągowej.

Członek Wspierający PTM – Zwyczajny
od 12.09.2017 r.

Merck Sp. z o.o. jest częścią międzynarodowej grupy Merck KGaA z siedzibą w Darmstadt (Niemcy) i dostarcza na rynek polski od roku 1992 wysokiej jakości produkty farmaceutyczne i chemiczne, w tym podłoża mikrobiologiczne.