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EXPERIMENTAL ARTICLES

Biofilm of *Aureobasidium pullulans* var. *pullulans* on Winter Wheat Kernels and its Effect on Other Microorganisms[1]

U. Wachowska[a], *, K. Głowacka[b], W. Mikołajczyk[a], and K. Kucharska[a]

[a] *Department of Entomology, Phytopathology and Molecular Diagnostic, University of Warmia and Mazury in Olsztyn, Prawocheńskiego 17, 10-720 Olsztyn, Poland*

[b] *Department of Plant Physiology, Genetics and Biotechnology, University of Warmia and Mazury in Olsztyn, Oczapowskiego 1A, 10-720 Olsztyn, Poland*

**e-mail: urszula.wachowska@uwm.edu.pl*

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Abstract– Winter wheat, grown under greenhouse conditions, was protected four times with a cell suspension of *Aureobasidium pullulans* var. *pullulans* during the growing season. After harvest, the distribution and survival rates of the studied biocontrol agent were analyzed under a scanning electron microscope. The abundance of filamentous fungi, yeasts, pseudomonads and *Azotobacter* bacteria was determined by inoculation onto selective agar media. *A. pullulans* produced mostly unicellular chlamydospores on the surface and in the brush of kernels. Multicellular blastospore conglomerates secreted extracellular polymeric substances (EPS), and their biofilms were found in the brush and crease of kernels. The application of a cell suspension of *A. pullulans* with the density of 10^4 CFU to winter wheat spikes, repeated four times, inhibited the growth of pseudomonads, *Azotobacter* bacteria and filamentous fungi.

Keywords: biofilm, *Aureobasidium pullulans* var. *pullulans*, grain, wheat, biocontrol

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In the temperate climate zone, winter wheat grain is a staple food used to make flour (Cordier and Gram, 2005). Wheat grain is colonized by bacteria of the families *Pseudomonadaceae*, *Micrococcaceae*, *Lactobacillaceae* and *Bacillaceae* (Laca et al., 2006), yeasts of the genera *Cryptococcus*, *Aureobasidium*, *Rhodosporidium, Candida, Pichia, Saccharomyces, Debaryonomyces, Kazachstania* and *Sporobolomyces* (Olstrope et al., 2010), and filamentous fungi of the genera *Alternaria*, *Epicoccum*, *Cladosporium, Penicillium* and *Fusarium* (Cordier and Gram, 2005; Jackowiak et al., 2005; Laca et al., 2006). The species composition of microbial communities colonizing wheat kernels affects flour strength (Fleet, 2007). Enzyme-producing microorganisms contribute to the degradation of mycotoxins – harmful to human and animal health – produced by *Fusarium* species in wheat grains (Schisler et al., 2011; Ito et al., 2013).

Under natural conditions, microorganisms form cell aggregates on plant surfaces since they rarely survive as individual cells. The ability of microorganisms to form a biofilm is probably a trait that had developed very early in their evolution. Biofilm formation is a mechanism that enables microbes to survive in changing, nutrient-deficient environments such as the surface of crop plants (Danhorn and Fuqua 2007; Harding et al., 2009; Raulio et al., 2009; Olstrope et al. 2010). A biofilm is a multicellular aggregate in which cells adhere to each other on the surface. The microbial cells growing in a biofilm are embedded within a self-produced extracellular polymeric substance (EPS) (Gniewosz and Duszkiewicz-Reinhard, 2008; Donot et al., 2012). Biofilms exhibit a distinct phenotype with respect to gene transcription, therefore different groups of microorganisms may perform numerous specialized physiological functions that cannot be fulfilled by individual cells (Ippolito et al. 2000; Raacke et al. 2006). One of such functions is the production of enzymes which inhibit the growth of plant pathogens (Marquina et al., 2002; Wachowska et al., 2013). *Aureobasidium pullulans* (de Bary) G. Arnaud var. *pullulans* is a saprotrophic fungal species found in different environments and regions of the world. It is known for its morphological and phenotypic plasticity (Kachalkin, 2010; Schena et al., 2003; Zalar et al., 2008) as well as high enzymatic activity (Metzger et al., 2006; Fleet, 2007; Gaur et al., 2010; West, 2011). *A. pullulans* has been considered as a biocontrol agent for plant pathogens (Vero et al., 2002; Schena et al., 2003; Wachowska et al., 2013). It rarely occurs on the surface of cereal grains (Olstrope et al. 2010), and its exact mechanism of action has not been fully elucidated.

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The objectives of this study were to analyze the distribution and structure of an *A. pullulans* biofilm on the surface of winter wheat kernels, and to determine its effect on the development of other microbial groups.

MATERIALS AND METHODS

The Ap1 isolate of *A. pullulans* var. *pullulans* was obtained from own collection. It was isolated in 2007 from the surface of winter wheat grain cv. Tonacja (Polish National List... 2012), grown in a field experiment in north-eastern Poland, after six months' storage at 11°C. Microorganisms were washed off from the grain onto Martin's medium (Martin, 1950; Laca et al., 2006). The Ap1 isolate is characterized by a fast growth rate on potato-glucose agar, and it exerted a strong inhibitory effect on *F. culmorum in vitro* conditions.

The morphological characteristics of *A. pullulans* Ap1 colonies were described based on their appearance after four-day incubation on potato-glucose agar (Merck) in darkness, at 24°C. The size of blastospores and chlamydospores was determined under a light microscope (Nikon E200) and a scanning electron microscope (JSM – 5310LV, JEOL). The isolate's enzymatic activity was determined with the use of the API 20C AUX microtest system (bioMérieux), in accordance with the manufacturer's instructions. The isolate was identified to the species and variety level based on the ITS 1–5.8SrDNA–ITS 2 sequence regions. DNA was extracted as described by Irzykowski et al. (Irzykowski et al., 2010). The fragment containing the ITS 1–5.8SrDNA–ITS 2 sequence region was amplified using specific primers ITS5 (F) GTATCGGA CGGAGATCCAGC and ITS4 (R) TTGCTCAGTGCATTG TCGG (White et al., 1990) and the FailSafe PCR system (Epicentre). The reaction was carried out in a Mastercycler Ep Gradient (Eppendorf) with a profile of initial denaturation of 2 min at 94°C, followed by 34 cycles of 1 min at 95°C, 1 min at 58°C and 3 min at 72°C, with a final elongation of 10 min at 72°C. Ten microliters of each sample were analyzed by agarose (Prona) 1.2% gel electrophoresis and stained with ethidium bromide. Sequencing of the PCR product took place at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw, Poland. Sequence similarity searches were performed with the BLAST network service of the NCBI database http://blast.ncbi.nlm.nih.gov/Blast.cgi.

In fall, seeds of winter wheat cv. Bogatka (Polish National List... 2012) covered (by 1 hour immersion) with a water cell suspension of *A. pullulans* Ap1 with the density of 10^8 cells per mL (cell counting with a hemocytometer) were placed in pots with a diameter of 30 cm, filled with soil classified as proper brown soil developed from sandy-silty loam underlain by light loam, with pH 6.5. In winter, the pots were placed in soil, for vernalization in the field. In spring, at the beginning of the growing season, the plants were transferred to the greenhouse. The biocontrol agent was applied four times during the growing season, at the first node stage (BBCH 31), at the beginning of heading (BBCH 51), at the watery ripe stage (BBCH 71) and at the hard dough stage (BBCH 87). The plants were sprayed with a cell suspension of *A. pullulans* Ap1 isolate (10^8 cells per mL or 10^4 cells per mL). The control group comprised plants grown from seeds surface disinfected with a 1% sodium hypochlorite solution, that were not protected during the growing season. Over the entire growing season, the plants were watered and fertilized, to each pot were applied two grams of NPK fertilizer (Azofoska, INCO, Poland, 1 (N): 0.5 (P_2O_5) : 1.4 (K_2O)). The pot experiment was performed in three replications. After harvest, the spikes were threshed, and the obtained kernels were used for microbiological analyses.

Kernels were collected for analyses 6, 12, 24, 30 and 48 hours, and 6, 8, 15, 17 and 20 days after the last inoculation of spikes with a cell suspension of *A. pullulans* Ap1. After fixation (2.5% glutaraldehyde, overnight at 4°C) and dehydratation, wheat kernels were subjected to critical-point drying (CDP 030, BALTEC), mounted, and coated with gold (Fine Coater, JCF–1200, JEOL). The samples were analyzed by scanning electron microscopy (JSM – 5310LV, JEOL) at 10kV.

A microbiological analysis of grain from biologically protected and control wheat plants was performed at harvest. In order to wash off epiphytes from kernels, random grain samples of 10 g each were placed in 250 mL flasks containing 90 mL sterile water, and were shaken for 30 min (180 rpm) using a laboratory shaker (358-S, Poland). 0.1 mL dilute microbial suspensions were transferred onto Petri dishes (9 cm diameter). Filamentous fungi and yeasts were counted on Martin's medium (Martin, 1950), pseudomonads on King's B medium (King et al., 1954) and *Azotobacter* bacteria on nitrogen-free agar medium (Martyniuk and Martyniuk, 2003). The experiment was performed in four replications.

The data regarding the abundance of microbial communities were log transformed (number of cells or cells aggregates + 1). The significance of differences between mean values was estimated by analysis of variance, and the mean values were compared by the Student–Newman–Keuls (SNK) test at $p < 0.01$ using Statistica 10 program (StatSoft 2011).

RESULTS

When cultivated on potato-glucose agar, the Ap1 isolate of *A. pullulans* var. *pullulans* produced tiny, fast-growing, cream-colored colonies with characteristic ragged edges, which did not turn black. Elongated blastospores and pseudohyphae were viewed under a

Table 1. Abundance and distribution of *A. pullulans* cells and cell aggregates (± SE, in brackets) on winter wheat kernels (number of cells or aggregates per one kernel)

Cells/aggregates	Kernel surface	Kernel crease	Kernel brush
Cells	25.94[c] (±4.51)	15.48[b] (±2.97)	22.53[c] (±2.29)
Aggregates 2÷10 cells	1.58[a] (±0.63)	1.76[a] (±0.72)	0.58[a] (±0.11)
Aggregates 10÷100 cells	0.09[a] (±0.05)	0.17[a] (±0.07)	0.11[a] (±0.04)
Aggregates > 100 cells	0.08[a] (±0.04)	0.13[a] (±0.06)	0.06[a] (±0.03)

a, b, c – Values followed by the same letter differ not significantly according to SNK test at $p < 0.01$.

light microscope. The blastospores had an average length of 6.3 μm, and often produced secondary blastospores by budding at the apex of a cell. The Ap1 isolate of *A. pullulans* produced also chlamydospores with an average length of 17.3 μm. Hyphae were observed in older cultures. The amplified r-DNA product was 698 bp long. The analyzed strain was identified as *Aureobasidium pullulans* var. *pullulans*. It differed with respect to the insertion of one nucleotide and the substitution of two nucleotides in the ITS 1–5.8rDNA–ITS2 regions from *A. pullulans* var. *pullulans* strain CBS 100280. The Ap1 isolate of *A. pullulans* var. *pullulans* assimilated D-glucose, L-arabinose, D-galactose, D-sorbitol, D-maltose, D-sucrose, D-trehalose and D-melezitose. The carbon utilization profiles corresponded to those of several *A. pullulans* strains isolated from Thailand (Prasongsuk et al., 2005).

Individual cells of the isolate were unevenly distributed on the surface, in the crease and brush of kernels (Table 1). They were observed on the surface and brush hairs of kernels, where they accounted for 37.86 and 32.88% of the total fungal counts, respectively. Cell aggregates (more than 100 cells) were sporadically found in the crease, in the brush and on the surface of kernels, where they had a 0.39% share of the total colonies forming units (CFU).

Individual cells of *A. pullulans* were found in greater abundance on the grain surface after the application of the cell suspension of *A. pullulans* Ap1 at a concentration of 10^8 CFU to wheat spikes, compared with kernels treated with the cell suspension with the density of 10^4 CFU only to the fifth evaluation date (Table 2). On day 20, the abundance of individual cells was 2.29 times lower in the crease of kernels treated with the suspension of 10^8 CFU *A. pullulans* Ap1, in comparison with kernels sprayed with the suspension with the density of 10^4 CFU. The abundance of individual cells of *A. pullulans* was most stable in the brush of wheat kernels – after 20 days of observation, it was only 18.44% lower (10^8 CFU) or 32% higher (10^4 CFU) than on the first sampling day. It was interesting that generally the cell suspension density of *A. pullulans* treatments did not have a significant influence to the single cell number observed on a scanning electron microscope wheat kernels.

The survival of multicellular aggregates on the grain surface showed a rising trend (Table 3). On the kernel surface the peak abundance of multicellular aggregates was noted eight and 15 days after spike inoculation with the cell suspension of *A. pullulans* with the density of 10^8 CFU and 10^4 CFU, respectively. In the following days their abundance was substantially lower in both treatments. Multicellular aggregates were sporadically found in the crease of kernels, and their presence was confirmed on four sampling days only. Few cell aggregates of *A. pullulans* Ap1 were found in the brush of kernels. They were observed the most numerous on days 15 and 17, on kernels treated with the cell suspension at a lower concentration.

Elongated chlamydospores, with a smooth surface and an average length of 17.3 μm, were observed on wheat kernels in all analyzed batches, usually as individual cells (Fig. 1A – arrow) or aggregates of 2–10 cells (Fig. 1 F). The outer tissues of wheat grain were colonized by patches of yeasts. Biofilms of 10 and more cells of *A. pullulans* blastospores were observed mostly in the brush and crease of kernels (Fig. 1 A, B, C, E). The structure of cell aggregates was similar. Several microscopic images showed probably extracellular polymeric substances (EPS) secreted by tiny blastospores. *A. pullulans* cells adhered to each other (Fig. 1 B – arrow) and were embedded within a matrix of EPS (Fig. 1 D – arrow).

Colonies of *A. pullulans* proliferated in Petri dishes containing Martin's medium. They dominated in samples of wheat grain treated with the cell suspension of *A. pullulans* (Table 4). The abundance of the studied microbial community was 1.9-fold higher, compared with the control treatment, after the application of the cell suspension of *A. pullulans* at a higher concentration.

Table 2. Mean survival rates (Log (number of cells+1) on 1 kernel) of *A. pullulans* cells on the surface, in the crease and in the brush of winter wheat kernels (± SE, in brackets) after spraying of plants with the suspensions of 10^4 and 10^8 cells per 1 mL

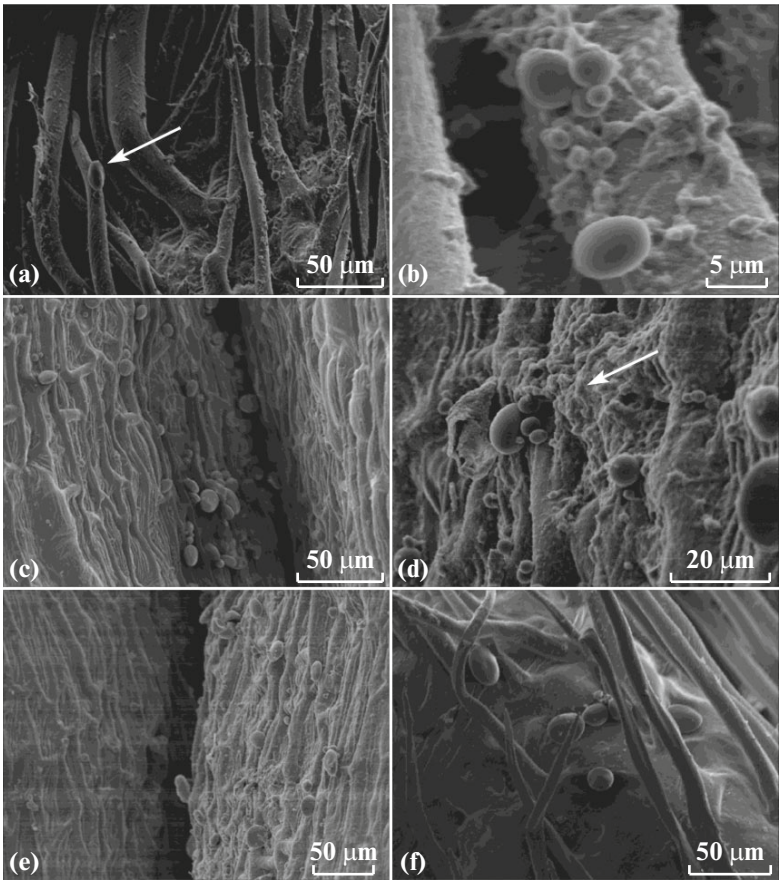
Suspension (cells per 1 mL)	6h	12h	24h	30h	48h	Mean (6 ÷ 48 h)	6 days	8 days	15 days	17 days	20 days	Mean (6 ÷ 20 days)
Surface of the kernel												
10^4	1.06^{ab} (±0.20)	0.82^{ab} (±0.21)	1.29^{b} (±0.12)	0.99^{ab} (±0.09)	1.26^{b} (±0.17)	1.08* (±0.08)	1.53^{b} (±0.00)	0.52^{a} (±0.05)	1.36^{b} (±0.10)	1.56^{b} (±0.07)	0.84^{ab} (±0.06)	1.16 (±0.06)
10^8	1.47^{b} (±0.08)	1.10^{b} (±0.10	1.37^{b} (±0.05)	1.09^{ab} (±0.05)	1.34^{b} (±0.02)	1.27* (±0.04)	1.23^{b} (±0.05)	1.32^{b} (±0.11)	1.32^{b} (±0.04)	1.33^{b} (±0.07)	0.50^{a} (±0.15)	1.14 (±0.06)
Crease of the kernel												
10^4	0.75^{abc} (±0.20)	0.35^{a} (±0.13)	1.18^{c} (±0.14)	0.45^{abc} (±0.21)	0.75^{bc} (±0.20)	0.70 (±0.19)	0.24^{abc} (±0.24)	0.95^{c} (±0.05)	0.63^{abc} (±0.05)	0.99^{c} (±0.09)	1.03^{c} (±0.25)	0.77 (±0.07)
10^8	1.21^{c} (±0.13)	0.50^{abc} (±0.13)	0.84^{bc} (±0.06)	1.09^{c} (±0.03)	0.60^{abc} (±0.00)	0.85 (±0.21)	0.59^{abc} (±0.11)	0.54^{abc} (±0.06)	0.95^{c} (±0.05)	0.93^{bc} (±0.10)	0.45^{abc} (±0.15)	0.69 (±0.02)
Brush of the kernel												
10^4	1.03^{d} (±0.07)	0.85^{abc} (±0.13)	1.41^{e} (±0.14)	1.17^{ab} (±0.45)	1.03^{d} (±0.07)	1.10 (±0.11)	1.04^{d} (±0.24)	0.24^{a} (±0.05)	0.65^{abc} (±0.15)	1.68^{f} (±0.09)	1.36^{e} (±0.55)	0.99 (±0.371
10^8	1.41^{e} (±0.16)	0.15^{a} (±0.20)	1.33^{e} (±0.06)	1.58^{f} (±0.21)	1.33^{e} (±0.06)	1.16 (±0.09)	1.41^{e} (±0.00)	0.89^{abc} (±0.11)	0.45^{ab} (±0.06)	0.45^{ab} (±0.05)	1.15^{abc} (±0.15)	0.87 (±0.65)

a–f – Values followed by the same letter(for each part of the kernel separately) differ not significantly according to SNK test at $p < 0.01$.
A.p. 10^4, *A.p.* 10^8 – spikes treated with a cell suspension of *A. pullulans* at a concentration of 10^4 and 10^8 cells per mL, respectively.
* Difference between mean values significant at $p<0.05$ according to SNK test

Table 3. Mean survival rates (Log (number of cells+1) on 1 kernel) of *A. pullulans* aggregates on the surface, in the crease and in the brush of winter wheat kernels (± SE, in brackets) after spraying of plants with the suspensions of 10^4 and 10^8 cells per 1 mL

Suspension (cells per 1 mL)	6h	12h	24h	30h	48h	6 days	8 days	15 days	17 days	20 days
Surface of the kernel										
10^4	0.10^{ab} (±0.12)	0.12^{ab} (±0.14)	0.24^{ab} (±0.14)	0.30^{abc} (±0.13)	0.21^{ab} (±0.09)	0.39^{abc} (±0.12)	0.24^{ab} (±0.14)	0.48^{bc} (±0.00)	0.45^{abc} (±0.11)	0.00^{a}
10^8	0.25^{ab} (±0.13)	0.00^{a}	0.00^{a}	0.00^{a}	0.48^{bc} (±0.01)	0.15^{ab} (±0.11)	0.59^{bc} (±0.16)	0.15^{ab} (±0.11)	0.00^{a}	0.00^{a}
Crease of the kernel										
10^4	0.00^{a}	0.00^{a}	0.39^{b} (±0.13)	0.00^{a}	0.00^{a}	0.00^{a}	0.30^{b} (±0.09)	0.00^{a}	0.00^{a}	0.00^{a}
10^8	0.18^{b} (±0.32)	0.00^{a}	0.53^{b} (±0.54)	0.00^{a}	0.00^{a}	0.00^{a}	0.40^{b} (±0.39)	0.00^{a}	0.00^{a}	0.15^{ab} (±0.00)
Brush of the kernel										
10^4	0.00^{a}	0.15^{a} (±0.00)	0.15^{a} (±0.24)	0.30^{ab} (±0.00)	0.00^{a}	0.00^{a}	0.15^{a} (±0.15)	0.60^{b} (±0.00)	0.65^{b} (±0.15)	0.15^{a} (±0.15)
10^8	0.53^{b} (±0.21)	0.00^{a}	0.24^{ab} (±0.15)	0.30^{ab} (±0.13)	0.24^{ab} (±0.12)	0.15^{a} (±0.15)	0.15^{a} (±0.15)	0.00^{a}	0.15^{a} (±0.05)	0.00^{a}

All designations as in Table 2.



The *Aureobasidium pullulans* cells visible in the brush (a, b, f) and crease (c, d, e) of wheat kernels after application of Ap1 isolate. Arrows indicate: Elongated chlamydospore (a), *A. pullulans* cells adhered to each other (b), *A. pullulans* cells embedded within a matrix of EPS (d).

The abundance of pseudomonads was 2.7-fold lower on wheat kernels treated with the cell suspension of *A. pullulans* with the density of 10^4 CFU, relative to control samples (Table 4). Bacteria of the genus *Azotobacter* were not isolated from those kernels, and the population size of filamentous fungi was reduced by 45.64%, compared with the control treatment. The abundance of filamentous fungi was 35.9% higher, and the counts of pseudomonads were 22.7% lower on kernels sprayed with *A. pullulans* at a concentration of 10^8 CFU, in comparison with the control treatment.

DISCUSSION

The colonizing of *A. pullulans* var. *pullulans* Ap 1 on wheat kernels was determined with the use of scanning electron microscopy after the application of cell suspension cultures to wheat spikes. Yeast *A. pullulans* produced mostly unicellular chlamydospores on the surface and in the brush of wheat kernels, whose survival rates were lower on the grain surface than in the brush. Multicellular blastospore conglomerates secreted EPS and produced short filaments to anchor themselves to the surface of kernels. *A. pullulans* biofilms occurred rarely and were found in the brush and crease of kernels. In most SEM images, there were single, elongated chlamydospores on the grain surface. The surface of kernels is exposed to adverse environmental conditions, and thick-walled chlamydospores are well adapted to water deficits (Kocková-Kratochvílová et al., 1980). The size and morphological characteristics of *A. pullulans* chlamydospores match published descriptions (Gniewosz and Duszkiewicz-Reinhard, 2008; Zalar et al., 2008). Gniewosz and Duszkiewicz-Reinhard (2008) described chlamydospores in *A. pullulans* as oval thick-walled cells covered by slimy, undulating capsules, larger than blastospores.

In our study, several microscopic images showed budding blastospores at the apex of a cell. The blastospores frequently formed clusters, were embedded in amorphous polymers and produced short filaments to anchor themselves to the surface of kernels. As demonstrated by Gniewosz and Duszkiewicz-Reinhard (2008), under conditions of high water and nutrient availability, *A. pullulans* formed a vast network of filaments connecting chlamydospores and blastospores. No such connections between blastospores were observed in the presented study. Most probably, water and nutrient availability on the grain surface is much lower than in mineral culture media. In our study, amorphous EPS was found in the brush and crease of wheat kernels, which were probably characterized by the highest moisture content. Raulio et al (2009) reported that exopolymeric matrix formed only in barley kernels steeped with water, which were heavily colonized with microbes. EPS secretion by *A. pullulans* has also been observed on abiotic surfaces (Srivastava et al., 2006; Gniewosz and Duszkiewicz-Reinhard, 2008). The EPS secreted by *A. pullulans* has been described by many authors (Srivastava et al., 2006; Gniewosz and Duszkiewicz-Reinhard, 2008) as a heterogeneous matrix of polymers, containing the polysaccharide pullulan (Donot et al., 2012), proteins (Metzger et al., 2006) and melanin (Gniewosz and Duszkiewicz-Reinhard, 2008). The obtained results indicate that typical *A. pullulans* Ap 1 biofilms with cell aggregates and EPS occurred rarely. Probably they could not develop due to insufficient moisture, but the counts of aggregated cells remained stable throughout the experiment. Biofilm plays an important role in protecting microorganisms against adverse environmental conditions (Sailer and Knol 2010).

A microbiological analysis of barley grain revealed that *A. pullulans* was the predominant yeast species immediately after harvest (Olstrop et al., 2010), and its application to the spikes of cereal plants may contribute to suppressing pathogen growth (Ippolito et al., 2000, Vero et al., 2002, Gaur et al., 2010; Wachowska et al. 2013). In our experiment, *A. pullulans* Ap 1 exerted an inhibitory effect on autochthonous bacteria colonizing wheat kernels. According to Fleet (2007), the reason for reducing yeast-bacteria interactions is ethanol production by yeasts. The Ap 1 isolate applied

Table 4. Abundance of selected microbial groups (± SE, in brackets) isolated from winter wheat grain treated with a cell suspension of *A. pullulans* at harvest

Treatments	*A. pullulans*	Filamentous fungi	Bacteria of the genus *Azotobacter*	Bacteria of the pseudomonad group
Log (CFU+1) on the area of 1 g kernel				
Control	1.40^{bc} (±0.10)	1.49^{b} (±0.03)	2.43^{cde} (±0.35)	3.47^{e} (±0.17)
A. pullulans 10^4	2.28^{cd} (±0.08)	0.81^{ab} (±0.07)	0.00^{a}	1.30^{c} (±1.30)
A. pullulans 10^8	2.72^{de} (±0.04)	2.01^{cd} (±0.06)	2.42^{cd} (±0.03)	3.03^{de} (±1.03)

a–e – Values followed by the same letter differ not significantly according to SNK test at $p < 0.01$.

to wheat plants at a concentration of 10^4 cells per mL significantly inhibited the growth of filamentous fungi. It seems that the *A. pullulans* isolate produced siderophores or competed for space and nutrients with other microorganisms (Vero et al. 2002). Interestingly, the suspension of *A. pullulans* at lower cell density more effectively reduced the counts of autochthonous bacteria and fungi that colonized wheat kernels. Our findings indicate that *A. pullulans* applied at a lower concentration developed rapidly over longer time intervals.

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