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Biochar-Rhizosphere Interactions – a Review

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Abstract

Biochar is a solid material of biological origin obtained from biomass carbonization, designed as a mean to reduce greenhouse gases emission and carbon sequestration in soils for a long time. Biochar has a wide spectrum of practical utilization and is applied as a promising soil improver or fertilizer in agriculture, or as a medium for soil or water remediation. Preparations of biochar increase plant growth and yielding when applied into soil and also improve plant growth conditions, mainly bio, physical and chemical properties of soil. Its physical and chemical properties have an influence on bacteria, fungi and invertebrates, both in field and laboratory conditions. Such effects on rhizosphere organisms are positive or negative depending on biochar raw material origin, charring conditions, frequency of applications, applications method and doses, but long term effects are generally positive and are associated mainly with increased soil biota activity. However, a risk assessment of biochar applications is necessary to protect food production and the soil environment. This should be accomplished by biochar production and characterization, land use implementation, economic analysis, including life cycle assessment, and environmental impact assessment.

Key words: biochar, rhizosphere, soil bacteria, soil microbiota, soil quality

Introduction

The rhizosphere is defined as the layer of soil around the roots that is influenced by the roots (Hiltner, 1904). It supports the development and activity of many diversified microbial communities, which can be up to 1000 times richer in microorganisms than bulk soil, because plant roots secrete organic compounds utilized by microbes as nutrients. Plant roots can modify the rhizosphere chemistry in a number of ways: (I) by the release and uptake of organic compounds, (II) by the gaseous exchange (CO_2/O_2) associated with the respiration of roots and rhizosphere microorganisms, and (III) by water and nutrient uptake or release, which is associated with the uptake or extrusion of protons and modification of the redox potential (Neuman and Römheld, 2012). As they grow through the soil, the roots also modify the physical properties of the rhizosphere soil, such as aggregate stability, hydrophobicity and the number and size of micropores, which are also modified by the presence of polymeric substances (Neuman and Römheld, 2012). About 5–20% of the carbon fixed by plants is secreted, mainly as root exudates (Marschner, 1995). Microorganisms living in the rhizosphere have different trophic, or living, habitats, and varied inter- and intra-relationships: saprotrophic, symbiotic or antagonistic (Kobayashi and Crouch, 2009). Benefits of mycorrhizal colonization include direct access to P and organic N and their better uptake, increased heavy metal and Al tolerance, decreased disease susceptibility, and in some cases improved water uptake. However, mycorrhizal colonization comes at a cost to plants, which have to supply the fungi with carbon (Marschner, 2012).

Biochar is a material originating from organic matter, produced by pyrolysis at high temperature in the absence of oxygen. Charred materials include: wood chips, crop residues, food industry wastes, animal manure, sewage sludge, microalgae biomass (Chan *et al.*, 2007; Sohi *et al.*, 2010; Farrell *et al.*, 2013; Marks *et al.*, 2014a; 2014b; Hosseini Bai *et al.*, 2015) and chemical co-products such as bio-oil and syngas (Bridgwater and Peacocke, 2000). The chemical and physical characteristics of biochars vary depending on the conditions of the thermochemical conversion applied to the biomass (Table I). Biochars produced from the same biomass under similar pyrolysis conditions, but in different plants can result in various properties of the final product (Spokas *et al.*, 2012a). The final product can constitute a material that contains no residual structures of the original feedstock material or can have relic structures (Spokas *et al.*, 2010). The physical structure of biochars affects the organic and inorganic composition: the pH can range from 5.6 to 13.0, the C content from 33.0% to

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Table I
Physicochemical parameters of selected types of biochar in comparison with compost

Feedstock material and temperature (if given)	pH (H_2O)	C (%)	N (%)	Ash (%)	Cd	Cu	Zn	P	Conductivity (mS cm^{-1})	Density (kg L^{-1})	CEC	References
					(mg kg^{-1})							
Compost (for comparison)	7.1*	27.0	2.2	–	0.14	86.0	321.0	–	1.4	0.77	–	Akhter *et al.*, 2015
Biochar derived from:												
Beech wood	8.78	80.3	0.4	15.2	< 2.00	16.0	93.0	–	0.54	0.36	9.83**	Akhter *et al.,* 2015
Garden waste residues	9.03	79.8	0.7	19.3	< 2.00	21.0	95.0	–	1.67	0.34	12.85**	
Oak wood (350°C)	4.80	74.9	–	1.1	–	–	–	12	–	–	294.2***	Lehmann *et al.*, 2011
Oak wood (600°C)	6.38	87.5	–	1.3	–	–	–	29	–	–	75.7***	
Corn stover (350°C)	9.39	60.4	–	11.4	–	–	–	1889	–	–	419.3***	
Corn stover (600°C)	9.42	70.6	–	16.7	–	–	–	2114	–	–	252.1***	
Poultry litter (350°C)	9.65	29.3	–	51.2	–	–	–	21256	–	–	121.3***	
Poultry litter (600°C)	10.33	23.6	–	55.8	–	–	–	23596	–	–	58.7***	
Glucose	–	64,6	0,0	–	–	–	–	–	–	–	–	Steinbeiss *et al.,* 2009
Yeast	–	67.4	5.5	–	–	–	–	–	–	–	–	
Eucalyptus wood (350°C)	7.0	82.4	0.6	–	–	–	–	0.6	–	–	–	Atkinson *et al.*, 2010

Norte: (–) Parameter not measured, * measured in $CaCl_2$, ** (mmol 100 mL^{-1}), *** (mmolc kg^{-1})

82.7%, N content from 0.1% to 6.0%, and the C: N ratio can range from 19 to 221 (Jha *et al.*, 2010; Spokas *et al.*, 2012b). Biochar can also contain appreciable quantities of P, K, Ca, Mg and micronutrients (Cu, Zn, Fe, Mn) with ashes accounting for 5–60% of the weight, depending on the source of the biomass and pyrolysis conditions (Cheng *et al.*, 2008b; Enders *et al.*, 2012).

The main goal of biochar applications in previous years was carbon sequestration in soil deposits (Jha *et al.*, 2010). Now the goal is also to increase crop yields (Jeffery *et al.*, 2011) and to nowadays the main focus is also to increase soil fertility (Atkinson *et al.*, 2010) using biochar-induced specific properties of soil (Blackwell *et al.*, 2010; Anderson *et al.*, 2011; Parvage *et al.*, 2013).

Biochar as a soil amendment exhibits some mechanisms that could explain its influence on soil organisms. They include:

- changing in the availability of soil nutrients and shifts soil nutrients ratios: N, P and others (Gundale and DeLuca, 2006; DeLuca *et al.*, 2006; Prendergast-Miller *et al.*, 2014; Ojeda *et al.*, 2015),
- stimulating soil microbial processes by absorbing/detoxifying inhibitory compounds (DeLuca *et al.*, 2006; Elad *et al.*, 2010),
- altering signalling dynamics between plants and their symbionts by binding both signalling or stimulatory molecules produced by soil microorganisms or plant roots and can serve as a secondary source of signal molecules (Akiyama *et al.*, 2005; Spokas *et al.*, 2010; Ni *et al.*, 2011; Masiello *et al.*, 2013).
- biochar also can serve as a refuge for soil microorganisms, which colonize biochar particles and can be protected from soil predators like large protozoans, nematodes, mites and collembola (Ezawa *et al.*, 2002; Thies and Rillig, 2009).
- biochar is also an effective sorbent of heavy metals and organic pollutants (Jiang *et al.*, 2012), which can have an influence not only on soil microbiota, but also on plants (Cao *et al.*, 2009) and soil fauna (Denyes *et al.*, 2012).

Plant growth regulation by biochar

Biochar addition to soil has a great impact on plant development and root colonization by microorganisms (*e.g.* mycorrhizal fungi) and nematodes (Table II). Experiments have shown that biochar additions to soil can increase the biomass of the roots of maize (Yamato *et al.*, 2006) and barley (Prendergast-Miller *et al.*, 2014), but low doses (2.5–10 t/ha) of charred plant biomass didn't impact corn seed germination on sand or fine sandy loam soils, in comparison to non-treated control soil group (Free *et al.*, 2005). Biochar addition influenced root growth of *Satsuma mandarin* (*Citrus unshiu* Marc.) trees on trifoliate orange (*Poncirus trifoliata* Raf.) rootstocks, which were reported to be 1.5 times longer and

Table II
Comparison of biochar effects on different horticultural crops and root colonization by AMF and nematodes

Crop plant	Type of biochar/ raw material	Effect on plants and soil	Reference
Apple	acacia hardwood	Better plant growth	Eyles *et al.*, 2015
Apple	woody residues	Higher soil microorganisms activity, increased root growth	Ventura *et al.*, 2014
Apple rootstock	Green waste	Increased nutrient content and higher dry mass	Street *et al.*, 2014
Apple seedlings	rice husk at 450 °C biochar	enhanced the plant height, fresh weight, and photosynthetic parameters	Wang *et al.*, 2016
Peach	Pinewood	Higher biomass and better nutrient content in plants	Atucha and Litus, 2015
Strawberry	Citrus wood or greenhouse wastes char	Fungal disease suppress	Meller Harel *et al.*, 2012
Tomato	Wood chips biochar (WB) mixed with compost or	According to control: WB decrease root and shoot dry weight, decreased AMF colonization	Akhter *et al.*, 2015
	green waste biochar (GWB) mixed with compost	GWB increased root and shoot dry weight, decreased AMF colonization	
Tomato	Powdered wood charcoal	Better plant growth and higher yield	Yilangai *et al.*, 2014
Tomato	rice husk and shell of cotton seed at 400°C	Better water use efficiency in reduced irrigation regimes and yield similar as in full irrigation	Akhtar *et al.*, 2014
Carrot	Spelt husk biochar and wood residues biochar	Bigger biomass of tap roots and fine roots of nematode *Pratylenchus penetrans* treated plants in comparison to control	George *et al.*, 2016
Lettuce	-sewage sludge, slow pyrolysis char gasification	-Stimulation of plant growth	Marks *et al.*, 2014a; 2014b
	-fast-pyrolysis pine and poplar wood char	-Strong inhibition of plant growth	
Lettuce Chinese cabbage	rice-husk char	increased final biomass, root biomass, plant height and number of leaves	Carter *et al.*, 2013
Beans	grass with horse dung at 300°C	No influence on height, higher number and longer leaves	William and Qureshi, 2015

had a bigger mass in biochar-treated trees, in comparison with the control (Ishii and Kadoya, 1994). There is also evidence that roots prefer to grow towards biochar particles (Prendergast-Miller *et al.*, 2014). Biochar's influence on root growth is visible as clusters of biochar particles bound to plant roots, root hairs and hyphae of mycorrhizal fungi (Joseph *et al.*, 2010; Lehmann *et al.*, 2011). The mechanisms of root-biochar interactions coincide with biochar impact on soil: pH, bulk density, aeration and water holding capacity, nutrient content and availability (Jones *et al.*, 2012; Prendergast-Miller *et al.*, 2014). Biochar has also an influence on microbial communities in soil (Rutigliano *et al.*, 2014) and on particles signalling molecules in the soil, by absorbing or releasing them (Akiyama *et al.*, 2005; Spokas *et al.*, 2010). Results of experiments show that biochar has greater impact on plants grown in nutrient-rich soils, in comparison with poor soils or poor, but fertilized soils (Noguera *et al.*, 2010). Following the application of biochar, the levels of some available nutrients in soil gradually increase in subsequent years, but this effect is not obvious immediately after raw biochar has been introduced into soil (Dong *et al.*, 2013).

The formation and growth of root hairs, which are essential for normal root growth (Gilroy and Jones, 2000), is also regulated by soil microbiota. Arbuscular mycorrhizal fungi and other soil microorganisms can inhibit the formation of root hairs and limit their length in maize roots (*Zea mays*) (Kothari *et al.*, 1990). On the other hand, application of *Azospirillum brasilense* strain ATCC 29710 onto young wheat plants increases the total number of root hairs, like applications of IAA (Martin *et al.*, 1989). Biochar addition can inhibit the development of root hairs, in comparison with the control without biochar. This observation can be explained by a higher phosphorus content of biochar-enriched soil (Prendergast-Miller *et al.*, 2014).

Biochar influence on rhizosphere microorganisms

The kind of influence of biochar on the number and biomass of microorganisms, and their effectiveness in colonizing plant roots is most likely associated with the type of the soil into which it has been introduced

Table III
Biochar influence on soil microorganisms in different soils

Type of biochar/raw material	Effect on soil microorganisms	Soil type	Reference
Willow wood and swine manure digestate feedstock:	Increased microbial biomass in both cases and:	Sandy loam	Ameloot *et al*., 2013
slowly pyrolyzed at 350°C biochar	-Increased dehydrogenase activity		
slowly pyrolyzed at 700°C biochar	-Decreased dehydrogenase activity		
Biochars: poultry litter (PL) and pine chips (P) at 400 or 500°C	Increased SOM and microbial biomass, higher N mineralization in (PL)	Silt loam	Ameloot *et al*., 2015
Mixed leafy tree chipped trunks and branches biochar	increased soil respiration, fungal and bacterial growth rate	Eutric cambisol	Jones *et al*., 2012
Fast-pyrolysis wood-derived biochar	increased microbial abundance with	Sandy loam/ clay/clay loam Gram-negative bacteria-domination	Gomez *et al*., 2014
Wheat straw pyrolysis between 350°C and 550°C	-increased in bacterial 16S rRNA gene copy -decreased fungal 18S rRNA gene copy	Hydragric anthrosol	Chen *et al*., 2013
Compost inoculated or not with AMF as a control and:		Sterilized soil-sand-clay mixture inoculated or not with *F. oxysporum* f.sp. *lycopersici* (Fol+ or Fol-)	Akhter *et al*., 2015
Wood biochar + compost	-Increased root colonization by AMF in Fol+ treatment in comparison to Fol-		
Green waste biochar + compost	-Decreased root colonization by AMF in Fol+ treatment in comparison to Fol-		
Empetrum nigrum L. twigs charcoal (EmpCh) forest humus charcoa (HuCh), both prepared at 450°C for 30 min	Imcreased microbial biomass carbon and number of cell in both biochar treatments in comparison to control	Scots pine and Norway spruce forest humus	Pietikäinen *et al*., 2000

(Table III). Biochar can increase the biomass of microorganisms and their activity in soils. Kolb *et al*. (2009) observed that increased doses of charcoal increase the populations of soil microbes as measured by their respiration activity. Opposite effect of different kinds of biochar added into soil on microbial activity was observed by Chintala *et al.* (2014). Corn stover biochar (CS), switchgrass biochar (SG), and Ponderosa pine wood residue biochar (WC) decrease of microorganisms activity measures as activity of dehydrogenase and esterase. *Miscanthus* biochar addition increase abundances of genera of phosphorus and sulphur mobilizing bacteria like *Acidothermus*, *Bacillus*, *Isosphaera*, *Planctomyces*, *Bradyrhizobium*, *Rhodobium*, *Pseudolabrys* and *Rhodanobacter* (Fox *et al.*, 2016). Rice stem biochar (3% in soil) increased the abundance of living cells of *Neorhizobium huautlense* T1-17 strain in soil in a pot experiment (Wang *et al.*, 2016).

Microorganisms can also change the properties of biochar, especially when causing it to oxidize the surface of particles, which increases the oxygen content (from 7% to 24%) and decreases the carbon content (from 91% to 71%) in biochar particles (Cheng *et al.*, 2008a). These results in the formation of oxygen-containing groups, which form negatively-charged surfaces, leading to a greater cation exchange capacity (CEC) of biochar (Glaser *et al.*, 2002) and nutrient retention in soil (Liang *et al.*, 2006), in comparison to new, non-oxidized biochar. Microbial oxidation of biochar is more effective when it is conducted in the presence of organic matter, whereas in the absence of organic matter it does not produce oxidation effects as measured by CEC (Cheng *et al.*, 2006).

Biochar activity in the rhizosphere and bulk soil

Various mechanisms, such as water holding, changes in soil pH, mineral nutrient content, shifts in soil nutrient ratios, absorption or detoxification of inhibitory compounds, altering signalling dynamics between plants and their symbionts, have great impact on soil microbiota (Table IV). Biochar modifies water infiltration and soil water retention (Ajayi *et al.*, 2009; Ojeda *et al.*, 2015). Biochar affects soil pH (liming effect) (Chan *et al.*, 2007; Beesley *et al.*, 2010; van Zwieten *et al.*, 2010), has positive impact on cation exchange capacity and electrical conductivity (DeLuca *et al.*, 2009). Nutritional properties of biochar are associated with nutrients as nitrogen, phosphorus or sulphur content of biochars (DeLuca *et al.*, 2009; Atkinson *et al.*, 2010; Sohi *et al.*, 2010). Biochar addition modifies nitrogen flux in soil and reduces gaseous N emission (Rondon *et al.*,

Table IV
Summary of possible mechanisms by which microbial abundance is affected by biochar additions to soil

Mode of action	Rhizobia or other N assimilators	Other bacteria	Mycorrhizal fungi	Other fungi	References
Better soil hydration	rnk	+	rnk	+	Pietikäinen *et al.*, 2000; Thies and Rilling, 2009
Increased N availability	+ or –	rnk	rnk	rnk	Laird *et al.*, 2010; Wang *et al.*, 2013; Güereña *et al.*, 2015; Wang *et al.*, 2015,;
Improved other macronutrient availability	rnk	+	+	rnk	Laird *et al.*, 2010; Yao *et al.*, 2012; Postma *et al.*, 2010; Hammer *et al.*, 2014
Increased pH	+	+	rnk	rnk	Beesley *et al.*, 2010
Habitat formation and/or protection from grazers	rnk	+	+ or -	rnk	Ishii and Kadoya, 1994; Pietikäinen *et al.*, 2000; Gryndler *et al.*, 2006, Birk *et al.*, 2009, Rillig *et al.*, 2010; Warnock *et al.*, 2010, Jaafar, 2014
Sorption/transformation of inhibitory compounds	rnk	+	rnk	+	Kim *et al.*, 2013; Mitchell *et al.*, 2015
Sorption of signalling compounds	rnk or -	rnk	rnk	rnk	Ni *et al.*, 2011, Masiello *et al.*, 2013
Biofilm formation	+	+	rnk	rnk	Piscitelli *et al.*, 2015
Sorption of dissolved OM as an energy source for microorganisms	rnk	+	nc	rnk	Pietikäinen *et al.*, 2000; Steiner *et al.*, 2008

Note: (+) indicates that relative abundance may increase (not necessarily better growth conditions); (–) indicates that relative abundance decreases; (nc) – no change; (rnk) – reaction not known.

2006). As another positive factor biochar increases nitrification activity (nitrification potential, net nitrification, gross nitrification) in forest soils, whereas such changes are not observed in grassland soils. This effect may be caused by the absorbing properties of biochar, which can alleviate the factors inhibiting soil microbes (DeLuca *et al.*, 2006). Biochar also contributes to the reduction in N_2O emissions from soil (Rondon *et al.*, 2006), but this is not associated with the liming properties of biochar (Yanai *et al.*, 2007). In a laboratory experiment, biochar addition resulted in a decrease in low weight aromatic acids (cinnamic and coumaric acids), which are important allelochemicals (Ni *et al.*, 2011). Additionally biochar modifies nitrogenase dynamics in soil. Mia *et al.* (2014) showed that application of biochar had an influence on nodules formation and N fixation (lower rate at high biochar doses). On the other hand, biochar can decrease soil enzymes activity by blocking or absorption of substrates (Bailey *et al.*, 2011). Biochar has been also found to decrease nutrient leaching on its own (Downie *et al.*, 2007; Dünisch *et al.*, 2007), as well as after incorporation within soil (Lehmann *et al.*, 2003). Soil phosphorus seems to be more available for plants in biochar-enriched soils (Edelstein and Tonjes, 2011) due to less binding to non-soluble forms (Cui *et al.*, 2011). Animal bone char (ABC) is a suitable material as a source of phosphate for plants and as a carrier for beneficial soil bacteria, meanwhile reusing P from wastes of the food chain (Postma *et al.*, 2010). ABC, as fertilizer, should be supported by microorganisms which can solubilize phosphorus from char. These beneficial microorganisms belong, for example, to the genera: *Arthrobacter*, *Bacillus*, *Burkholderia*, *Collimonas*, *Paenibacillus*, *Pseudomonas*, *Serratia, Streptomyces* and *Aspergillus* (Postma *et al.*, 2010; Vassilev *et al.*, 2013).

Biochar particles as microbial habitats

Thanks to its physicochemical properties, biochar can be utilized as a habitat by soil microorganisms. Biochar's properties of absorbing organic compounds from the environment may help form new habitats for soil microbiota, different from those formed in *e.g.* soil humus (Pietikäinen *et al.*, 2000). Biochar as a refuge for soil microorganisms can reduce the extent of predation caused by predatory soil micro- and mesofauna like large protozoas, nematodes, mites or collembola (Ezawa *et al.*, 2002; Thies and Rillig, 2009).

Biochar changes the physical and chemical properties of the soil. One of the biochar's modes of action on rhizosphere microbes is to shift soil microbial populations into PGPRs or soil beneficial fungi (Graber *et al.*, 2010). On the other hand, biochar additions can also decrease the abundance of mycorrhizal fungi by reduced mycorrhizal symbiosis requirements due to increased nutrient and water availability, changes in soil physical or chemical properties and direct negative effect on mycorrhiza formation, including high levels of nutrients or heavy metals (Gryndler *et al.*, 2006; Birk

et al., 2009; Warnock *et al.*, 2010). However, Ishii and Kadoya (1994) observed higher rates of root colonization by arbuscular mycorrhizal fungi in the roots of *S. mandarin* (*C. unshiu* Marc.) trees grafted on rootstocks of trifoliate orange (*P. trifoliata* Raf.) planted in soil with biochar. Rillig *et al.* (2010) have shown that hydrochar (biochar obtained from hydropyrolysis process) produced from beet root chips had positive effects on AM fungal root colonization up to an addition rate of 20% (by volume), and demonstrated that the material could stimulate germination of spores of an AM fungus.

Upon improving soil water capacity, biochar additions can also favour some zoospore-forming pathogens like *Pythium* or *Phytophtora*, in comparison with bulk soil (Thies and Rilling, 2009).

The role of biochar in plant disease reduction

Biochar addition to soil or other growing media can reduce the susceptibility of plants to diseases. There are a few mechanisms of this action.

One of them is a modification of metabolic pathways. It has been observed that biochar is capable of mediating plant systemic resistance against diseases, for example, of greenhouse pepper and tomato, in which the severity of the disease caused by *Botrytis cinerea* was reduced in biochar-amended treatments (Mehari *et al.*, 2015). A similar effect of increased systemic resistance against *B. cinerea*, *Colletotrichum acutatum* and *Podosphaera apahanis* was also observed in strawberry plants, which was confirmed by the results of a qPCR study of defence-related gene expression (Meller Harel *et al.*, 2012). Biochar reduced the susceptibility of *Asparagus officinalis* to *Fusarium* root rot so that the extent of root infection is 50% in biochar-containing soils, in comparison with 93% in soil-only treatment. Supplementing of biochar also increased the colonization of *Asparagus* roots by AM fungi (Matsubara *et al.*, 2002). The effect of improved root colonization by AMF in biochar-enriched soils has also been observed in other trials (Elmer and Pignatello, 2011). In addition, it has also been observed that biochar can increase the resistance of plants to leaf mites, *e.g.* in pepper (Elad *et al.*, 2010).

Another mechanism of biochar protective properties is absorption and detoxification of xenobiotics, like for example phenolic compounds, noted by Wang *et al.* (2014). Besides direct absorption of allelochemicals, biochar also offers other mechanisms, which enhance soil microorganisms and plant growth and their resistance to biotic and abiotic stresses. Biochar is initially a sterile material and its compounds in residual tars may have direct toxic properties on soil pathogens. There is a number of identified biochar compounds that are known to adversely affect microbial growth and survival. These include ethylene glycol and propylene glycol, hydroxy-propionic and butyric acids, benzoic acid and o-cresol, quinones and 2 phenoxyethanol (Schnitzer *et al.*, 2007; Graber *et al.*, 2010). When used in low doses, these compounds could suppress the sensitive species of soil microbiota, thereby resulting in the proliferation of resistant communities and inducing resistance mechanisms in plants (Graber *et al.*, 2010).

Biochar influence on soil meso- and macrofauna

There has been so far only a minor number of studies on the influence of charcoal/biochar on soil fauna, but there have been a lot of studies on wildfire charcoals in forest soils, not on biochar in agricultural soils, so the results of those studies are not applicable to research on synthesized biochars (McCormack *et al.*, 2013). Dry biochar introduced into the soil causes desiccation of the soil environment. Earthworms, for example, avoided the soil freshly enriched with biochar but wetted biochar reduced this problem (Li *et al.*, 2011). Addition of biochar to the soil also has a direct influence on soil fauna. Liesch *et al.* (2010) observed that the addition of a biochar derived from poultry manure caused higher mortality and weight loss in earthworms *Eisenia fetida* than the biochar obtained from pine chips. The toxic effect of the poultry manure biochars can be explained by high Na and Mg content, which led to high salinity and subtoxic levels of some metals ions (Fe, Cu, Zn, Al, As). Weight loss in *E. fetida* was also observed by Gomez-Eyles *et al.* (2011) in treatments with hardwood-derived biochar.

On the other hand, biochar reduces the adverse effects of organic pollutants on soil fauna. For example, the reduction in PCB (polychlorinated biphenyl) in the tissues of *E. fetida* earthworm was from 52% (2.8% of biochar) up to 88% (11.1% of biochar) (Denyes *et al.*, 2012). Biochar also decreased the amounts of 4-ring and heavier polycyclic aromatic hydrocarbons (PAH) and heavy metal ions in earthworm tissues, but increased the amount of 2-ring PAH (Gomez-Eyles *et al.*, 2011). Biochar addition positively influenced the growth and reproduction of *E. fetida* used for vermicomposting of the mixture of sewage sludge and wheat straw (Malińska *et al.*, 2016). Preference for biochar in soil by *E. fetida* was also evident in another study, in which another earthworm species, *Aporectodea caliginosa*, was susceptible to biochar amendments (Hale *et al.*, 2013). Combining together biochars and earthworms in different types of soils increases the biomass (total, shoot and root), yield and grain number, and weight of rice plants grown in three different types of soils, and the

effects are clearly visible on both nutrient-rich and poor soils, but mineral fertilization decreases the benefits of using biochar and earthworms (Noguera *et al.*, 2010). Other soil macroorganisms, for example collembolas also respond positively to biochar-amended soil (Hale *et al.*, 2013; Marks *et al.*, 2014a; 2014b).

Biochar as an ingredient for fertilizers and biofertilizers

Biochar, thanks to its physico-chemical properties is promising carrier for beneficial microorganisms and can replace current carriers like peat, lignite, vermiculite or perlite (Saranya *et al.*, 2012; Hale *et al.*, 2015; Głodowska *et al.*, 2016) (Table V). Of course not all types of biochar are applicable for this purposes (Hale *et al.*, 2015). Conditions of feedstock material pyrolysis, additives and biochar particles surface treatment with chemical reagents or dissolving agents also has big influence on survival rate of bacterial inoculum (Vanek *et al.*, 2016). The improvements of biochar based biofertilizers are focused on shelf life and inoculum potential of different strains of microorganisms (Sun *et al.*, 2016). Another trials are focused on selection of the most efficient strains of microorganisms for the best utilization of nutrients contained in biochar (Postma *et al.*, 2010; Zwetsloot *et al.*, 2016). These works will allow to develop new microbiologically enriched biochar preparations for wide range of crops.

Biochars of different origins are also tested as a support material for slow release mineral fertilizers (Steiner *et al.*, 2009; González *et al.*, 2015) or as a fertilizer itself. This is conducted with another properties of biochar, especially cation exchange capacity (an important parameter in retaining inorganic nutrients in soil) and water holding capacity (Lee *et al.*, 2013; Glaser *et al.*, 2015). Biochar made from *Miscanthus* was tested as a slow release silicon fertilizer (Houben *et al.*, 2014) whereas animal bone biochar seems to be good phosphorus fertilizer (Vassilev *et al.*, 2013; Siebers *et al.*, 2014).

Biochar alone is widely offered as a soil conditioner but there are only some biochar based bioorganic fertilizers on the market containing except biochar also beneficial microorganisms and another ingredients. For example SEEK Organic BamBoo Power BBP No. 2 is bio-organic fertilizer, which consists bamboo biochar, bamboo vinegar, humic acids, amino acids, beneficial soil organisms (not specified in product description) ($20 \times 10^6 \cdot g^{-1}$) and other amendments. This granular preparation is recommended for organic horticulture, including vegetables, fruits and flowers, especially for berries, such as blueberry, strawberry, raspberry, grapes, *etc.* (www.seekfertilizer.com). Another biopreparation enriched in biochar are available on local and global markets like: Biochar Organic Bio Fertilizer Bacteria Fertilizer made by Hebei Woxin Bio-Technology Co., Ltd but they are not characterized and described as good as biochar based fertilizer of SEEK company.

Conclusions

Biochar, when incorporated into soil, has a great impact on dynamics and modification of rhizosphere processes. It has direct effect on soil pH, nutrients content and moisture of treated soils. On the one hand biochar increase content of some nutrients like K or P in soil, but contrarily absorbs nitrogen ions and causes possible deficiencies of this nutrient for soil microorganisms and plants. Effects of biochar applications will include not only changes to the chemical and physical soil properties, but also its impact on the composition of the soil biological community and

Table V
Selected utilization of biochar as a microorganism carrier or ingredient of fertilizers

Type of biochar/raw material	Utilization	Effects	Reference
Hardwood or softwood biochar fast pyrolyzed at 700°C	Carrier for bacteria *Pseudomonas libanensis* for seed coating	Life cells of bacteria viable more than twenty weeks	Głodowska *et al.*, 2016
Softwood slow pyrolyzed at 450°C		Life cells of bacteria viable not more than two weeks	
Pinewood biochar slow pyrolyzed at 600°C, supplemented with LB broth	Carrier for bacteria *Pseudomonas putida* UW4	Life cells of bacteria detectable up to five months	Sun *et al.*, 2016
Miscanthus, draff pyrolyzed at 650°C mixed with mineral fertilizer or organic residues	Ingredient of fertilizer	Increased yield according to mineral fertilizer	Glaser *et al.*, 2015
Miscanthus straw pyrolyzed at 600°C	Slow release silicon fertilizer	Increased Si amount in com parison to bulk soil	Houben *et al.*, 2014
Pig bone char pyrolized 1 h at 850°C	Bacteria carrier and slow release phosphorus fertilizer	Lifespan of majority of bacterial strains longer than 100 days	Postma *et al.*, 2010

plant-soil-microbial interactions. Understanding these complex interactions is crucial for developing on-farm soil management and conservation practices to improve soil properties and agricultural productivity in environmentally sustainable ways.

Adsorbing properties of biochars are applicable in contaminated soils improvement, when toxic for microorganisms and plants compounds like heavy metal ions or organic xenobiotics are inactivated on biochar particles. On the other hand biochar itself can be source of compounds which negatively affects soil microorganisms and plant roots. It is especially related to poly aromatic hydrocarbons (PAH) and heavy metals ions.

The wide spectrum of possible consequences of biochar use in soil is still unknown and requires further scientific inquiry, especially in natural landscapes, to avoid the negative consequences of these works.

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ORIGINAL PAPER

Genetic Characterization of a Novel Composite Transposon Carrying *arm*A and *aac*(*6*)-*Ib* Genes in an *Escherichia coli* Isolate from Egypt

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Abstract

Aminoglycosides are used in treating a wide range of infections caused by Gram-positive and Gram-negative bacteria; however, aminoglycoside resistance is common and occurs by several mechanisms. Among these mechanisms is bacterial rRNA methylation by the 16S rRNA methyl transferase (16S-RMTase) enzymes; but data about the spread of this mechanism in Egypt are scarce. Cephalosporins are the most commonly used antimicrobial agents in Egypt; therefore, this study was conducted to determine the frequency of 16S-RMTase among third generation cephalosporin-resistant clinical isolates in Egypt. One hundred and twenty three cephalosporin resistant Gram-negative clinical isolates were screened for aminoglycosides resistance by the Kirby Bauer disk diffusion method and tested for possible production of 16S-RMTase. PCR testing and sequencing were used to confirm the presence of 16S-RMTase and the associated antimicrobial resistance determinants, as well as the genetic region surrounding the *arm*A gene. Out of 123 isolates, 66 (53.66%) were resistant to at least one aminoglycoside antibiotic. Only one *Escherichia coli* isolate (E9ECMO) which was totally resistant to all tested aminoglycosides, was confirmed to have the *arm*A gene in association with $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$ and *aac(6)-Ib* genes. The *arm*A gene was found to be carried on a large A/C plasmid. Genetic mapping of the *arm*A surrounding region revealed, for the first time, the association of *arm*A with *aac(6)-Ib* on the same transposon. In conclusion, the isolation frequency of 16S-RMTase was low among the tested aminoglycoside-resistant clinical samples. However, a novel composite transposon has been detected conferring high-level aminoglycosides resistance.

Key words: 16S rRNA methyl transferases, aminoglycosides, *arm*A, β-lactmase

Introduction

Aminoglycosides are used in treating a wide range of infections caused by both Gram-negative and Gram-positive bacteria. They have been classified by the World Health Organization as critically important antimicrobial drugs in human medicine (WHO, 2011). They bind specifically to the aminoacyl site (A-site) of 16S rRNA within the prokaryotic 30S ribosomal subunits and interfere with protein synthesis. The most commonly encountered resistance mechanism to aminoglycosides is enzymatic inactivation. Other known mechanisms of aminoglycoside resistance include defect of cellular permeability, active efflux and rarely, nucleotide substitution of the target molecule (Magnet and Blanchard, 2005). Since 2003, a new mechanism of aminoglycoside resistance has emerged (Galimand *et al*., 2003; Yokoyama *et al*., 2003) which is mediated by a newly recognized group of 16S rRNA methyl transferases (16S-RMTase), with modest similarity to those produced by aminoglycoside-producing actinomycetes. Their presence confers an extraordinarily level of resistance to clinically useful aminoglycosides, such as amikacin, tobramycin and gentamicin (Doi and Arakawa, 2007).

Seven types of plasmid-mediated 16S-RMTase (*arm*A, *rmt*A, *rmt*B, *rmt*C, *rmt*D, *rmt*E, *rmt*F and *npm*A) have so far been identified (Davis *et al*., 2010; Doi *et al*., 2004; 2007; Doi and Arakawa, 2007; Galimand *et al*., 2003; 2012; Wachino *et al*., 2006; Yokoyama *et al*., 2003). Published data concerning this new group of enzymes indicate that 16S-RMTase genes have already disseminated globally among pathogenic Gram-negative bacilli, although their overall prevalence appears to remain low (Doi and Arakawa, 2007).

Genes encoding 16S-RMTases are often carried by mobile genetic elements like transposons. They are frequently associated with other antimicrobial resistance genes such as $bla_{\text{CTX-M}}$, bla_{DHA}, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-10}}$, $bla_{\text{TEM-1}}$, $bla_{\text{VIM-1}}$ as well as plasmid-mediated quinolone resistance determinants, thus conferring multidrug resistance phenotypes. In addition, some pathogenic microbes such as *Salmonella* species have already acquired these genes. Therefore, this genetic apparatus

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may contribute to the rapid worldwide dissemination of this resistance mechanism among different pathogenic microbes (Yamane *et al*., 2008; Yang *et al*., 2011; Galani *et al*., 2012; Poirel *et al*., 2012; Wachinoa and Arakawa, 2012).

Egypt has a high population density (15th world) and a central location at the crossroad of major transportation routes, thus it is important to monitor the status of antibiotic resistance in this country. However, little data about the incidence of 16S-RMTase producing bacteria in Egypt are available. Since cephalosporins are the most commonly used antibiotics in Egypt (Hassan *et al*., 2011), this investigation was conducted to determine the isolation frequency of 16S-RMTase producers among third-generation cephalosporin-resistant Gram-negative pathogenic bacteria in Egyptian medical facilities. In addition, this study also determined the plasmid origin of *arm*A, the replicon type of plasmid, and the 16S-RMTase genetic neighborhood. Other associated antimicrobial resistance determinants have been studied.

Experimental

Materials and Methods

Bacterial strains. One hundred and twenty three isolates, previously characterized as being resistant to at least one of the third-generation cephalosporins, were included in the study. All isolates were collected between 2009 and 2010 from outpatients with suspected infections, in Abu El-Rish Children's Hospital, Cairo. Isolates were identified by API 20E identification system (Kashif *et al*., 2012). *Escherichia coli* TOP10 was used as a recipient strain for transformation experiments.

Antimicrobial susceptibility testing. Antibiotic susceptibility to gentamicin (10 μg), tobramycin (10 μg) and amikacin (30 μg) was determined by the Kirby-Bauer disk diffusion method as proposed by Doi and Arakawa (2007).

Isolates with 16S-RMTase genes were tested by the Kirby-Bauer disc diffusion method for susceptibility to other antimicrobials, such as: ciprofloxacin (5 μg), cefoxitin (30 μg), cefotaxime (30 μg), piperacillin (100 μg), piperacillin/tazobactam (100/10 μg), trimethprim/sulfamethoxazole (1.25/23.75 μg), imipenem (10 μg). The results were interpreted following the guidelines of the Clinical and Laboratory Standards Institute (2012a).

Characterization of 16S-RMTase genes and the associated antimicrobial resistance genes. Genomic DNA was extracted from isolates suspected to contain 16S-RMTase genes by the boiling method. The isolates were tested for *arm*A, *rmt*A, *rmt*B, *rmt*C, *rmt*D and *npm*A genes by PCR amplification, which was performed as previously described (Doi and Arakawa, 2007; Fritsche *et al*., 2008). PCR identification of other resistance genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ group 1 and group 9 and quinolone resistance genes: *aac(6)-Ib-cr*, *qep*A, *qnr*A, *qnr*B and *qnr*S) was also performed as previously described (Park *et al*., 2005; Cattoir *et al*., 2007; Yamane *et al*., 2008; Dallenne *et al*., 2010). PCR products were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific, Lithuania) and sequenced by ABI 3730 xl DNA sequencer (Applied Biosystems, Foster City, CA). Similarity searches for the nucleotide sequences were performed with BLASTN program (http://www.ncbi.nlm.nih.gov/blast) with default settings.

Transformation of 16S-RMTase genes. Plasmid DNA was extracted with GeneJET Plasmid Miniprep Kit (Thermo Scientific, Lithuania) from 16S-RMTase positive strain (E9ECMO). Two microliters of plasmid extract from E9ECMO was electroporated into 50 μl of electrocompetent *E. coli* TOP10 cells using a Gene Pulser electroporator (Bio-Rad, Hemel Hempstead, UK), according to the manufacturer instructions. Transformants were selected on LB agar medium supplemented with either 100 μg/ml cefotaxime, or 100 μg/ml gentamicin. The Kirby-Bauer disc diffusion method was used to determine the antibiogram of the obtained transformants. The MICs of cefotaxime, ciprofloxacin, gentamicin and amikacin for isolate E9ECMO, the obtained transformants and *E. coli* TOP10 were determined by broth microdilution method (CLSI, 2012b).

Plasmid analysis. Plasmid DNA was extracted from E9ECMO and the obtained transformants, as previously described. The size of *arm*A carrying plasmid was determined by restriction digestion using *BsaBI* (Thermo Scientific, Lithuania). Electrophoresis of the extracted and digested plasmid was performed on 0.5% agarose gels in 1 × TAE buffer and visualized by staining with ethidium bromide. Fragment size was determined by comparison with Gene-Ruler 1 kb DNA ladder (Thermo Scientific, Lithuania). Plasmid replicon typing was performed using plasmid DNA extracted from E9ECMO as well as from the transformants. This was done using PCR-based replicon typing as previously described by Johnson *et al.* (2007).

Determination of *arm*A genetic context. Tn*1548* was identified as the genetic platform that mobilizes *arm*A between plasmids in most isolates. PCR mapping experiments were performed to investigate the genetic context of the *arm*A using the method of Granier *et al.* (2011) with slight modifications. Some primer pairs used didn't produce any amplicons (Tn1-F/Tn1-R, Tn2-F/Tn2-R, Tn3-F/Tn3-R and Tn5-F/Tn5-R), so modification of the used primer pairs combinations was done (Tn1-F/Tn2-R, Tn2-F/ Tn3-R and Tn4-F/ Tn5-R). All PCR products were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific,

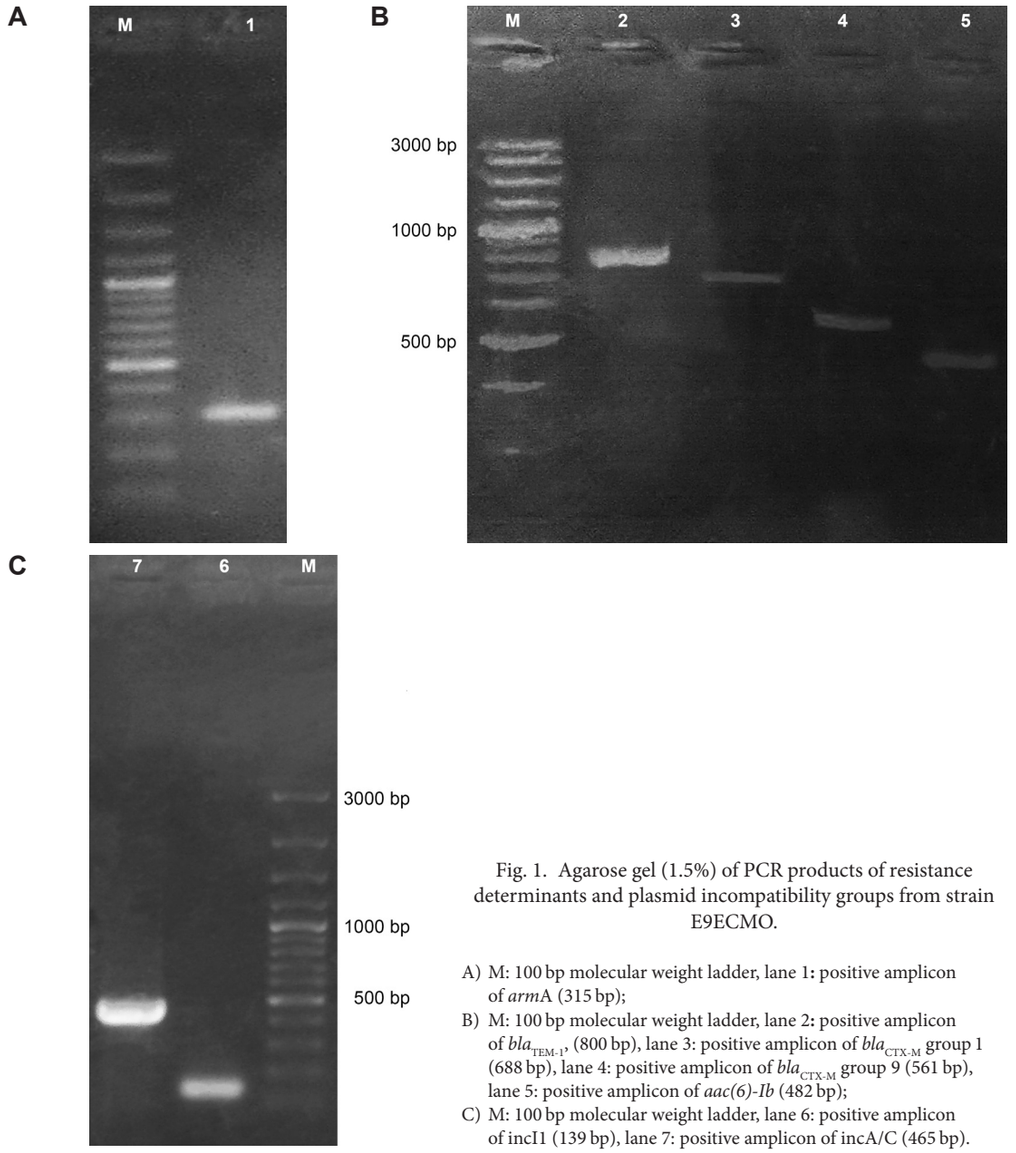

Fig. 1. Agarose gel (1.5%) of PCR products of resistance determinants and plasmid incompatibility groups from strain E9ECMO.

A) M: 100 bp molecular weight ladder, lane 1**:** positive amplicon of *arm*A (315 bp);
B) M: 100 bp molecular weight ladder, lane 2**:** positive amplicon of $bla_{\text{TEM-1}}$, (800 bp), lane 3: positive amplicon of $bla_{\text{CTX-M}}$ group 1 (688 bp), lane 4: positive amplicon of $bla_{\text{CTX-M}}$ group 9 (561 bp), lane 5: positive amplicon of *aac(6)-Ib* (482 bp);
C) M: 100 bp molecular weight ladder, lane 6: positive amplicon of incI1 (139 bp), lane 7: positive amplicon of incA/C (465 bp).

Lithuania) and sequenced using ABI 3730 xl DNA sequencer (Applied Biosystems, Foster City, CA). Similarity searches for the nucleotide sequences were performed with the BLASTN program (http://www.ncbi.nlm.nih.gov) using default settings.

Results and Discussion

As stated above, the aim of this study was to assess the spread of 16S-RMTase resistance genes among cephalosporin-resistant bacterial isolates. For this purpose, one hundred and twenty three isolates, obtained from Abu El-Rish Children Hospital in Cairo and previously determined as being resistant to third-generation cephalosporins, were screened for aminoglycoside resistance by the Kirby Bauer disk diffusion method.

Detection of 16S-RMTase producing isolates. Production of 16S-RMTase was suspected when no or little inhibitory zone was observed with any of the aminoglycoside disks (Doi and Arakawa, 2007). Out of 123 isolates tested, 66 were resistant to aminoglycosides and were thus candidates for production of 16S-RMTase. However, only one out of those 66 isolates was confirmed to possess *arm*A gene by PCR (Fig. 1A) followed by sequencing (GenBank accession number KM357401 and KM357402). The positive isolate (E9ECMO) was identified as *E. coli*. Higher rates were recorded in areas such as Korea (32–45%) and China (90%) (Lee *et al.*, 2006; Xia *et al.*, 2011; Yang *et al.*, 2011). On the other hand, less than 1% prevalence of *arm*A elements was recorded in some countries such as Taiwan (0.7%), Belgium (0.11%) and Japan (0.03%) (Yamane *et al.*, 2007; Bogaerts *et al.*, 2010). The low rate, recorded

Table I
Minimum Inhibitory Concentration (MIC) of selected antimicrobials against E9ECMO, *E. coli* TOP 10, transformant T1 and transformant T2.

Anti-microbials	MIC (μg/ml)			
	E9ECMO	*E. coli* TOP 10	T1	T2
Amikacin	< 256	2	< 256	> 256
Gentamicin	> 256	0.5	> 256	> 256
Ciprofloxacin	> 256	> 0.25	256	4
Cefotaxime	> 256	0.5	> 256	> 256

in this study, may be because most isolates collected in this study were from low socioeconomic children (2–16 years old) attending outpatient clinics in a governmental hospital in Cairo. Those Children are usually not in close contact with animals where 16S-RMTase were detected in several instances and thus can spread by horizontal dissemination of plasmids (Xia *et al*., 2011).

E9ECMO is multidrug resistant. E9ECMO was also resistant to ciprofloxacin, cefoxitin, cefotaxime, piperacillin, piperacillin/ tazobactam and trimethoprim/sulfamethoxazole. The resistance genes $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M}}$ group 1 ($bla_{\text{CTX-M-15}}$), $bla_{\text{CTX-M}}$ group 9 ($bla_{\text{CTX-M-14}}$) and *aac(6)-Ib* were also detected by PCR and sequencing in E9ECMO (Fig. 1B). This is the same resistance tendency as that reported by Bogaerts *et al.* (2010). Confirmed sequences of the resistance genes observed in the present study have been deposited in GenBank under accession numbers KM357388, KM357389, KM357390, KM357391, KM357403, KM357404, KM357405 and KM357406.

Transformation of 16S-RMTase genes. Analysis of transformants revealed the presence of two types of transformants with two corresponding phenotypes: i) Transformant, T1, was resistant to gentamicin, tobramycin, amikacin, ciprofloxacin, cefoxitin, cefotaxime, piperacillin, piperacillin/tazobactam and trimethoprim/sulfamethoxazole. ii) Transformant, T2, showing only high-level resistance to cefotaxime and the tested aminoglycosides (gentamicin, amikacin and tobramycin) as shown in Table I. PCR testing confirmed the presence of *arm*A, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M}}$ group 1 and group 9 and *aac*(*6*)-*Ib* in transformant T1 while only *arm*A, $bla_{\text{CTX-M}}$ group 9 and *aac(6)-Ib* were present in transformant T2.

Plasmid analysis. Plasmid DNA extracted from E9ECMO and each of the obtained transformants, was separated using gel electrophoresis. The plasmid profile of E9ECMO and transformants T1 showed the same number and pattern of plasmids. However, the plasmid profile of transformant T2 confirmed the presence of only one large plasmid in T2 (Fig. 2). Thus, *arm*A is carried on a plasmid pMO2; conferring a multidrug resistance phenotype.

Only one linear fragment was obtained after restriction of pMO2 with *BsaBI* of approximate size 13500 bp. Thus, the size of pMO2 was approximately 13500 bp. PCR-based replicon typing of plasmids showed that plasmids extracted from both E9ECMO and transformant T1 belonged to incompatibility groups A/C and I1 while pMO2 belonged to incompatibility group A/C (Fig. 1C). This indicates that *arm*A, $bla_{\text{CTX-M}}$ group 9 and *aac(6)-Ib* are borne on A/C type plasmid which is one

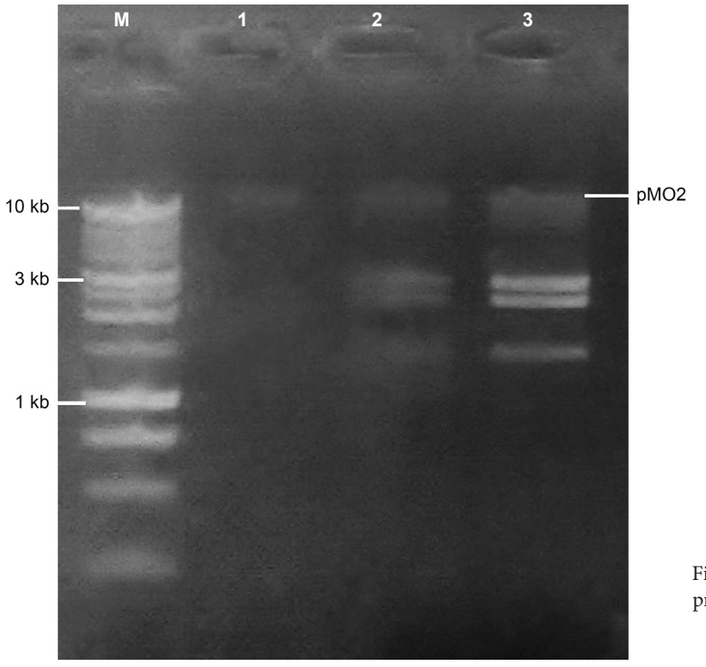

Fig. 2. Agarose gel (0.5%) showing the plasmid profile of lane M: 1 kb molecular weight ladder, 1: transformants T2, 2: transformants T1, 3: Isolate E9ECMO.

of the prevalent plasmid families known of carrying *arm*A (Carattoli, 2009). The antimicrobial resistance pattern of E9ECMO was consistent with the phenotype suggested by Kang *et al.* (2008) for isolates having the *arm*A gene carried on A/C type plasmid. This confirmed the presence of linkage between *arm*A to other associated antimicrobial resistance genes as well as to the specific plasmid backbone.

Genetic context of *arm*A in pMO2. Some primer pairs used didn't produce any amplicons, so modification of the used primer pairs combinations was done. PCR mapping and sequencing experiments revealed variation in the sequences upstream of the *arm*A gene in transformant T2 from that of the genetic context of *arm*A genes reported in *E. coli* AY522431 and *Citrobacter freundii* AF550415 (Gołebiewski *et al*., 2007; González-Zorn *et al*., 2005a). This is the first time to record a deletion of *ant3"9* (aminoglycoside resistance gene) and *qac*E*Δ1* (quaternary ammonium compounds resistance gene), which were commonly recorded upstream from *arm*A in Tn*1548*. They were replaced by another aminoglycoside resistance gene, *aac*(*6*)-*Ib*, and this explains the failure to obtain amplicons from some previously used primer pair combinations (Galimand *et al*., 2005; González-Zorn *et al*., 2005b; Johnson *et al*., 2007; Zhang *et al*., 2008). This coinicides with González-Zorn *et al.* (2005b) conclusion about the spread of *arm*A within a unique transposon composite. We reveal, for the first time, the presence of *aac-6-lb* gene together with *arm*A on the same transposon. The association of *arm*A together with other aminoglycosides resistance gene (*aac-6-lb*) accounts for the high level aminoglycosides resistance conferred by this transposon. This genetic combination is worrisome; mutation in *aac(6)-lb* gene can produce its *cr* allele which confers resistance to floroquinolones limiting the available choices for treatment of *arm*A associated infections (Park *et al*., 2006).

In addition, other antimicrobial resistance genes were detected on the same transposon. The gene *sul1* was detected upstream from *arm*A and confer resistance to sulphonamides. The genes coding for the macrolide resistance efflux-pump *mef*E/*mel*, and a macrolide phosphotransferase gene, *mph*, were also identified downstream of *arm*A gene (Fig. 3A, B). This association necessitates reinforcing the control measures

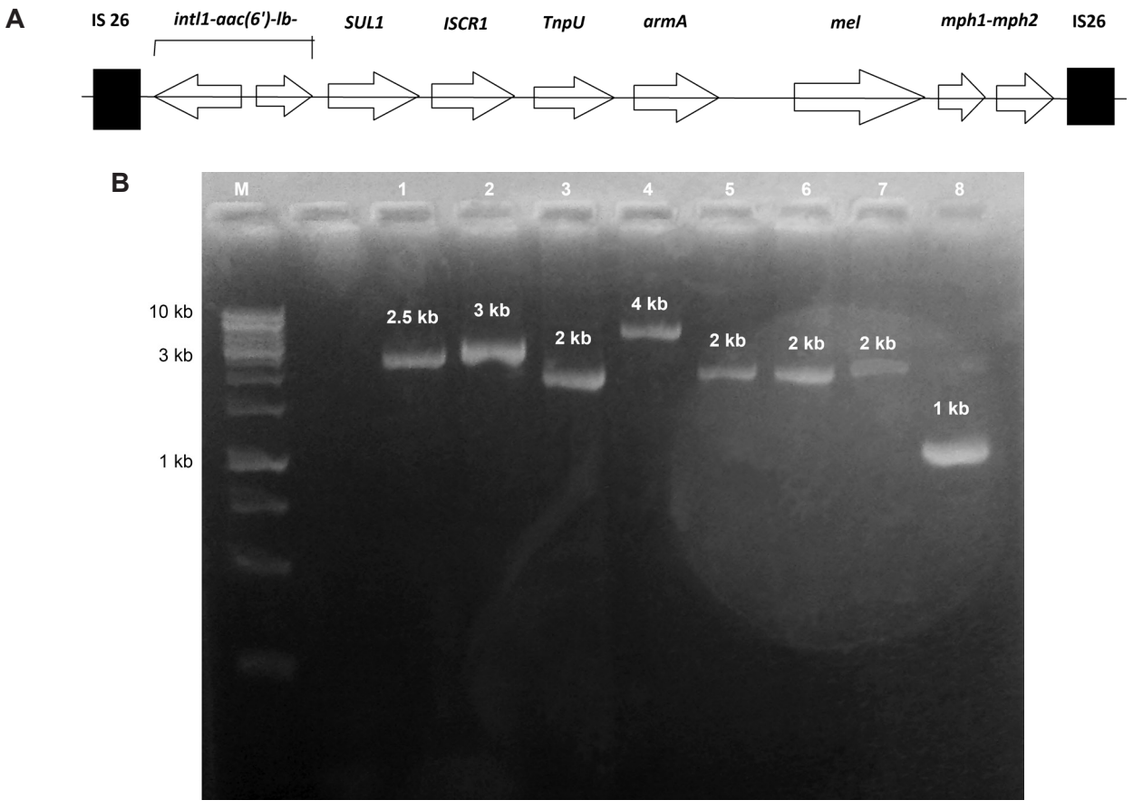

Fig. 3.

A) Genetic structure of transposon 1548 in plasmid pMO2 from strain E9ECMO;

B) Agarose gel (1%) of PCR amplicons obtained from transposon 1548 mapping in plasmid pMO2 from strain E9ECMO. M: 1 kb molecular weight ladder; lanes 1 to 8: represent amplicons from the following primer pairs combination: (Tn1-F & Tn2-R, Tn 2-F & Tn3-R, Tn4-F & Tn4-R, Tn4-F & Tn5R, Tn6-F & Tn6-R, Tn7-F & Tn7-R, Tn8-F & Tn8-R, Tn9-F & Tn9-R).

in both human and animal environment to avoid the spread of this aminoglycosides resistance mechanism that could be selected by sulphonamides and macrolides consumption.

To the best of our knowledge this is the first article discussing the genetic context of 16S-RMTase genes in a clinical isolate from Egypt. A partial sequence Tn*1548* variant observed in the present study has been deposited in GenBank under accession numbers KM357386, KM357387, KM357392-KM357400 and KM357407.

In conclusion, a novel mobile genetic element carried on a plasmid has been detected in this study, combining *arm*A and *aac-6-lb*. The pattern and sequence of the genetic context suggests a composite transposon. The dissemination of this transposon may lead to pan aminoglycosides resistance with an impact on morbidity, mortality, and costs of healthcare in both clinical and livestock-breeding environments. Organisms possessing 16S-RMTase genes are usually more likely to develop multidrug resistance, because of associated β-lactamase genes. Therefore, despite the low rate of prevalence of *arm*A in Abu El-Rish children hosptial, strict surveillance should be implemented to limit its local spread.

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ORIGINAL PAPER

Trends of Bloodstream Infections in a University Greek Hospital during a Three-Year Period: Incidence of Multidrug-Resistant Bacteria and Seasonality in Gram-negative Predominance

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Abstract

The aim of the study was to assess the epidemiology, the incidence of multidrug-resistant bacteria and bloodstream infections' (BSIs) seasonality in a university hospital. This retrospective study was carried out in the University General Hospital of Patras, Greece, during 2011–13 y. Blood cultures from patients with clinical presentation suggestive of bloodstream infection were performed by the BacT/ALERT System. Isolates were identified by Vitek 2 Advanced Expert System. Antibiotic susceptibility testing was performed by the disk diffusion method and E-test. Resistance genes (*mec*A in staphylococci; *van*A/*van*B/*van*C in enterococci; bla_{KPC}/bla_{VIM}/bla_{NDM} in *Klebsiella* spp.) were detected by PCR. In total, 4607 (9.7%) blood cultures were positive from 47451 sets sent to Department of Microbiology, representing 1732 BSIs. Gram-negative bacteria (52.3%) were the most commonly isolated, followed by Gram-positive (39.5%), fungi (6.6%) and anaerobes bacteria (1.8%). The highest contamination rate was observed among Gram-positive bacteria (42.3%). Among 330 CNS and 150 *Staphylococcus aureus*, 281 (85.2%) and 60 (40.0%) were *mec*A-positive, respectively. From 113 enterococci, eight were *van*A, two *van*B and two *van*C-positives. Of the total 207 carbapenem-resistant *Klebsiella pneumoniae* (73.4%), 202 carried bla_{KPC}, four bla_{KPC} and bla_{VIM} and one bla_{VIM}. A significant increase in monthly BSIs' incidence was shown (R^2: 0.449), which may be attributed to a rise of Gram-positive BSIs (R^2: 0.337). Gram-positive BSIs were less frequent in spring ($P<0.001$), summer ($P<0.001$), and autumn ($P<0.001$), as compared to winter months, while Gram-negative bacteria ($P<0.001$) and fungi ($P<0.001$) were more frequent in summer months. BSIs due to methicillin resistant *S. aureus* and carbapenem-resistant Gram-negative bacteria increased during the study period. The increasing incidence of BSIs can be attributed to an increase of Gram-positive BSI incidence, even though Gram-negative bacteria remained the predominant ones. Seasonality may play a role in the predominance of Gram-negative's BSI.

Key words: bacteremia, *Candida* non-*albicans*, *Staphylococcus aureus*, carbapenem-resistance, methicillin resistant vancomycin resistant enterococci (VRE)

Introduction

Bloodstream infections (BSIs) remain a crucial public health problem of increasing incidence and importance in the modern world, whereas, they are characterized by high morbidity and mortality (Wisplinghoff *et al*., 2004; Bouza *et al*., 2014; Koupetori *et al*., 2014). The reported raising incidence can be explained by the ageing of hospitalized patients, the increasing number of immunosuppressed patients and the acquisition of virulence factors by common pathogens (Goto and Al-Hasan, 2013; Papadimitriou-Olivgeri *et al*., 2015). The wide use of invasive devices, such as central venous catheters, is another important factor leading to the rise of the number of catheter-related bloodstream infections (CR-BSIs) (Rodriguez-Creixems *et al*., 2013).

Resistance to antimicrobial agents played a determining role in the epidemiology of bloodstream infections during the last decades (Koupetori *et al*., 2014). While Gram-negatives were the most common cause of BSIs before 90s (Karchmer, 2000), this changed after the dissemination of resistant Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* and vancomycin resistant enterococci (VRE) (de Kraker *et al*.,

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2013). Thus, the last two decades Gram-positive bacteria were the most prominent worldwide (Wisplinghoff *et al*., 2004; de Kraker *et al*., 2013; Gubbels *et al*., 2015). The dissemination of carbapenem-resistant bacteria has changed the situation and currently Gram-negative ones have become the most common cause of BSIs in countries where these bacteria have invaded (Koupetori *et al*., 2014). Besides the role of resistance, studies have shown that environmental factors, such as, higher temperatures, can influence the epidemiology (Eber *et al*., 2011; Paul, 2012). It has been reported that *Escherichia coli* is more prominent in higher temperatures (Eber *et al*., 2011; Paul, 2012). In a multicenter study, it was shown that countries with low distance from the equator have higher proportion of Gram-negative bacteremias as compared to Gram-positive ones (Fisman *et al*., 2014).

The aim of the present study was to assess the evolution of bloodstream infections, especially, those related with the use of intravenous catheters, to determine the causative agents and spread of multidrug resistant (MDR) isolates, as well as, to evaluate the role of seasonality in trends of microorganisms isolation during a three-year period in a tertiary university Greek hospital.

Experimental

Materials and Methods

This retrospective observational study has taken place in the University General Hospital of Patras (UGHP), Greece, a 770 bed tertiary hospital. The UGHP is the only tertiary hospital in Southwestern Greece, receiving patients from eight prefectures with 921 852 total population served. The study was carried out under the Hospital Surveillance Programme for multi-drug resistant infections of hospitalized patients, and was approved by the University Hospital Ethics Committee (HEC No: 571).

During a three-year period (from January 2011 till December 2013), two blood sets (consisted of an aerobic and an anaerobic blood culture bottle) from peripheral sites and one from the central venous line (if present) were obtained and sent to the Department of Microbiology, whenever a patient developed fever ($\geq 38.0°C$) or the clinical presentation was suggestive of bloodstream infection. The BacT/ALERT System (bioMerieux, Marcy l'Etoile, France) for blood culture incubation is used in our setting. Prolonged incubation (until 28 days) of blood culture bottles was applied in cases of suspicion for *Brucella* spp. or HACEK infection (*Haemophilus* spp., *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella* spp.). Catheter tips from central venous lines of septic patients were processed and cultured by the rolling plate method onto blood agar plates (bioMerieux).

Isolates recovered from positive blood cultures and catheter tips were identified by Gram stain, catalase and coagulase production and by the Vitek 2 Advanced Expert System (bioMerieux). Antibiotic susceptibility testing was performed by the agar disk diffusion method against antimicrobials suggested by EUCAST according to bacterial species, whereas the E-test was applied as follows: for Enterobacteriacae and *Acinetobacter* spp. imipenem, meropenem, colistin and tigecycline were tested; for *Pseudomonas* spp. imipenem, meropenem and imipenem/EDTA; for staphylococci, enterococci and streptococci vancomycin, teicoplanin, linezolid and daptomycin; for *Streptococcus pneumoniae* penicillin, ampicillin, cefepime, cefotaxime and ceftriaxone; for anaerobic bacteria penicillin, ampicillin, amoxicillin-clavulanic acid, piperacillin, piperacillin-tazobactam, ticarcillin, imipenem, meropenem and clindamycin, whereas Gram-positive anaerobic bacteria were additionally tested for vancomycin (EUCAST, 2015). All results were interpreted according to EUCAST guidelines (EUCAST, 2015).

BSI was defined according to CDC definition (Horan *et al*., 2008). Isolation of a common commensal organism from blood cultures, such as *Aerococcus* spp., *Bacillus* spp., coagulase-negative staphylococci (CNS), *Corynebacterium* spp., *Micrococcus* spp., *Propionibacterium* spp., and viridans group streptococci, was characterized as true BSI if the pathogen was isolated from at least two blood culture sets, as described by CDC guidelines (Horan *et al*., 2008). For all other pathogens, only one positive blood culture associated with clinical signs of infection were needed in order to be defined as BSI. CR-BSI was defined when the central venous catheter tip grew over 15 colony-forming units of the phenotypically same strain as the blood culture isolate. Positive blood cultures that were not associated with BSI were characterized as contamination.

Phenotypic identification of carbapenemase production among imipenem-resistant Enterobacteriaceae was performed by the EDTA synergy test (MER/MER-EDTA) and the boronic acid synergy test (MER/MER-boronic acid) by the disk diffusion method, which distinguishes the production of MBL and serine carbapenemase (Tsakris *et al*., 2010). All imipenem-non-susceptible (IMP-NS) *Pseudomonas aeruginosa* isolates (MICs ≤ 1 mg/l) were examined for metallo-beta-lactamase (MBL) production using the E-test MBL assay (bioMerieux).

PCR was used to identify *mec*A gene in phenotypically cefoxitin-resistant staphylococci, *van*A, *van*B and *van*C genes in phenotypically vancomycin-resistant enterococci and bla_{KPC}, bla_{VIM} and bla_{NDM} in phenotypically carbapenem-resistant *Klebsiella pneumo-*

niae isolates (Queenan and Bush, 2007; Papadimitriou-Olivgeris *et al*., 2015).

SPSS version 19.0 (SPSS, Chicago, IL) software was used for all analyses. Bacterial BSI trends that were assessed by Spearman's correlation analysis. Incidence was defined as the number of BSI of a pathogen per 10 000 patient-days for all pathogens, while for *Brucella* spp. BSI yearly incidence was defined per 100 000 habitants of western Greece. Poisson regression distribution with general log-linear analysis was used to estimate the adjusted incidence rate ratios (aIRR) with 95% confidence intervals (CI) by comparing the BSI incidence according to the season of occurrence. $P < 0.05$ was considered statistically significant.

Results

During the study period, in total 47457 blood cultures sets were processed in the Microbiology Department, corresponding to 15292, 16335 and 15824 the years 2011, 2012 and 2013, respectively. Among them, 1464 (9.6%), 1619 (9.9%) and 1524 (9.6%) were positive collected from 903, 1010 and 1024 patients, accordingly. Gram-positive bacteria represented the majority of isolates (2653, 57.6%), followed by Gram-negative ones (1693, 36.7%), fungi (197, 4.3%) and anaerobes (64, 1.4%) (Table I). More specifically, CNS (n = 1808), especially *Staphylococcus epidermidis* (n = 1115) was the most common species among Gram-positive bacteria followed by *S. aureus* (n = 309), enterococci (n = 167), streptococci (n = 204), and others Gram-positive (n = 142). *Klebsiella* spp. was isolated from 632 blood cultures, while *E. coli*, *P. aeruginosa*, *Acinetobacter* spp., other Enterobacteriaceae, *Brucella melitensis* and other Gram-negative bacteria were isolated from 632, 320, 232, 231, 151, 59 and 68, respectively.

During the study period, 2115 catheter tips were evaluated (761 in 2011, 686 in 2012 and 668 in 2013). Among them, 267 (35.1%), 272 (39.7%) and 240 (35.9%) were culture-positive, recovered from 230, 253 and 224 patients, respectively. The isolated pathogens in declining order were: CNS (358), *Klebsiella* spp. (145), *Acinetobacter* spp. (110), fungi (43), *P. aeruginosa* (37), *S. aureus* (23), enterococci (17), Enterobacteriaceae other than *Klebsiella* spp. and *E. coli* (16), streptococci (eight), *E. coli* (eight), other Gram-positive (seven), other Gram-negative (seven).

Aforementioned positive blood cultures represented 1732 BSIs (486, 606 and 640 in 2011, 2012 and 2013, respectively), of which 312 (18.0%) where catheter-related. Gram-negative bacteria were the main cause of BSI (901, 52.3%), followed by Gram-positive (685, 39.5%), fungi (115, 6.6%) and anaerobes (31, 1.8%).

The number of positive blood cultures associated with true BSI or contamination is shown in Table I. The highest contamination rate was observed for Gram-positive bacteria (42.3%). No blood culture positive for Gram-negative bacteria or fungi was considered as contamination. Table II and Fig. 1 depict the rates of isolates from BSIs according to pathogen isolated and the Department of patients' hospitalization.

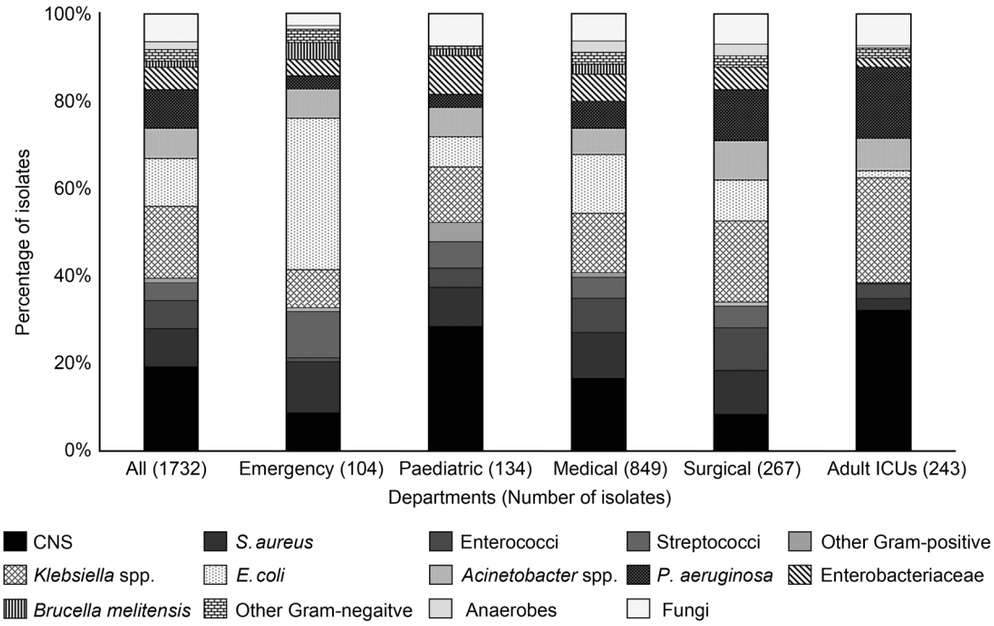

Fig. 1. Bloodstream infection rates according to pathogen isolated and the Department of patient's hospitalization. ICU, Intensive Care Unit; CNS, coagulase negative staphylococci.

Table I
Number of positive blood cultures associated with true bloodstream infections or contamination

Isolates	Positive blood cultures					
	All (4607)	Associated with contamination (1140)	Contamination rate (24.7%)	Associated with BSI (3467)	Number per BSI episode (2.0)	BSI episodes (1732)
Gram-positive	2653 (57.6%)	1121 (98.3%)	42.3%	1532 (44.2%)	2.2	685 (39.5%)
CNS	1808 (39.2%)	944 (82.8%)	52.2%	864 (2.5%)	2.6	330 (19.1%)
S. epidermidis	1115 (24.2%)	436 (38.2%)	39.1%	679 (19.6%)	2.6	265 (14.7%)
Non-*S. epidermidis*	693 (15.0%)	508 (44.6%)	73.3%	185 (5.3%)	2.8	65 (3.8%)
S. aureus	309 (6.7%)	0 (0.0%)	0.0%	309 (8.9%)	2.0	151 (8.7%)
Enterococci	167 (3.6%)	0 (0.0%)	0.0%	167 (4.8%)	1.5	113 (6.5%)
Streptococci	204 (4.4%)	52 (4.6%)	25.5%	152 (4.4%)	2.1	73 (4.2%)
Other	142[a] (3.1%)	102 (8.9%)	71.8%	40 (1.2%)	2.2	18[b] (1.0%)
Gram-negative	1693 (36.7%)	0 (0.0%)	0.0%	1693 (48.8%)	1.9	901 (52.3%)
Klebsiella spp.	632 (13.7%)	0 (0.0%)	0.0%	632 (18.2%)	2.2	282 (16.3%)
E. coli	320 (6.9%)	0 (0.0%)	0.0%	320 (9.2%)	1.7	189 (10.9%)
Acinetobacter spp.	231 (5.0%)	0 (0.0%)	0.0%	231 (6.7%)	1.5	151 (8.7%)
P. aeruginosa	232 (5.0%)	0 (0.0%)	0.0%	232 (6.7%)	1.9	120 (6.9%)
Enterobacteriaceae	151 (3.3%)	0 (0.0%)	0.0%	151 (4.4%)	1.7	90 (5.2%)
B. melitensis	59 (1.3%)	0 (0.0%)	0.0%	59 (1.7%)	2.4	25 (1.4%)
Other	68[c] (1.5%)	0 (0.0%)	0.0%	68 (2.0%)	1.5	44[d] (2.5%)
Anaerobes	64[e] (1.4%)	19 (1.7%)	29.7%	45 (1.3%)	1.5	31[f] (1.8%)
Fungi	197[g] (4.3%)	0 (0.0%)	0.0%	197 (5.7%)	1.7	115[h] (6.6%)

CNS, coagulase negative staphylococci; BSI, bloodstream infections
[a] *Corynebacterium* spp. (72), *Micrococcus* spp. (30), *Bacillus* spp. (27), *Listeria monocytogenes* (eight), *Aerococcus* spp. (three), *Leuconostoc* spp. (two)
[b] *Listeria monocytogenes* (seven), *Corynebacterium* spp. (five), *Aerococcus* spp. (two), *Bacillus* spp. (two), *Micrococcus* spp. (two)
[c] *Strenotrophomonas maltophilia* (20), *Sphingomonas* spp. (eight), *Burkholderhia cepacia* (four), *Morganella morgannii* (four), *Alcaligenes* spp. (three), *Achromobacter putrefasciens* (two), *Moraxella* spp. (two), *Neisseria meningitidis* (one), *Rhizobium radiobacter* (one), *Haemophilus influenzae* (one)
[d] *Strenotrophomonas maltophilia* (14), *Sphingomonas* spp. (13), *Morganella morgannii* (four), *Alcaligenes* spp. (three), *Burkholderhia cepacia* (three), *Achromobacter putrefasciens* (two), *Moraxella* spp (two), *Neisseria meningitidis* (one), *Rhizobium radiobacter* (one), *Haemophilus influenzae* (one)
[e] *Propionibacterium* spp. (25), *Bacteroides* spp. (22), *Peptococcus* spp. (seven), *Clostridium* spp. (three), *Peptostreptococcus* spp. (three), *Eubacterium* spp. (two), *Fusobacterium* spp. (two)
[f] *Bacteroides* spp. (14), *Peptococcus* spp. (five), *Clostridium* spp. (three), *Propionibacterium* spp. (three), *Eubacterium* spp. (two), *Fusobacterium* spp. (two), *Peptostreptococcus* spp. (two)
[g] *Candida parapsilosis* (94), *C. albicans* (70), *C. glabrata* (22), *C. tropicalis* (eight), *Cryptococcus neoformans* (two), *C. kruzei* (one)
[h] *C. parapsilosis* (50), *C. albicans* (44), *C. glabrata* (15), *C. tropicalis* (three), *Cryptococcus neoformans* (two), *C. kruzei* (one)

In the Paediatric Departments, including the neonatal ICU, Gram-positive bacteria predominate, whereas, in all adult Departments Gram-negatives prevailed (Table II, Fig. 1).

Figure 2 shows the monthly variation of BSIs' incidence per 10000 patient-days during the study period. A significant rise was observed (R^2: 0.449; P 0.006) that may be attributed to the rise in Gram-positive BSIs (R^2: 0.337; P 0.044). No significant variation was observed for Gram-negatives, anaerobes or fungi. No pathogen had a significant variation in BSI's incidence during the study period, with the exception of BSIs caused by *S. aureus* which increased from 2.0 per 10 000 patient-days in 2011 to 3.4 in 2013 (R^2: 0.995; P 0.033). When *B. melitensis*' BSI yearly incidence was calculated according to the population of Southwestern Greece a significant rise was found, from 0.4 per 100 000 habitants in 2011 to 1.5 in 2013 (R^2: 0.974; $P < 0.001$).

Figure 3 depicts the seasonal adjusted incidence rate ratio of BSIs according to isolated pathogen category (Gram-positive, Gram-negative, anaerobes, fungi), as compared to winter. The incidence of BSIs was 19% higher in summer months (P 0.024; aIRR 1.19; 95% CI 1.02–1.39) and 25% less frequent in autumn (P 0.001; aIRR 0.75; 95% CI 0.63–0.89) as compared to winter. BSIs due to Gram-positive bacteria was less frequent in spring ($P < 0.001$; aIRR 0.052; 95% CI 0.40–0.68), summer ($P < 0.001$; aIRR 0.58; 95% CI 10.45–0.74) and autumn ($P < 0.001$; aIRR 0.61; 95% CI 0.47–0.77), while Gram-negative bacteria ($P < 0.001$; aIRR 1.84; 95% CI 1.47–2.29) and fungi ($P < 0.001$; aIRR 3.20; 95% CI 1.57–6.51) were more frequent in summer months. No significant difference was observed in BSIs' incidence due to anaerobes.

Among the 330 CNS and the 150 *S. aureus* BSIs, 281 (85.2%) and 60 (40.0%) were methicillin-resistant

Table II
Bloodstream infection isolates according to the Department of patients' hospitalization.

Isolates	Emergency Department		Paediatric Departments		Medical Departments		Surgical Departments		Adult ICUs		All Departments	
	All (104)	CR-BSI (0)	All (134)	CR-BSI (23)	All (849)	CR-BSI (102)	All (267)	CR-BSI (22)	All (378)	CR-BSI (165)	All (1732)	CR-BSI (312)
Gram-positive	34 (32.7%)	0	70 (52.2%)	22	345 (40.6%)	92	91 (34.1%)	7	145 (38.4%)	84	685 (39.5%)	205
CNS	9 (8.7%)	0	38 (28.4%)	22	140 (16.5%)	91	22 (8.2%)	3	121 (32.0%)	83	330 (19.1%)	199
S. aureus	12 (11.5%)	0	12 (9.0%)	0	89 (10.5%)	1	27 (10.1%)	3	11 (2.9%)	0	151 (8.7%)	4
Enterococci	1 (1.0%)	0	6 (4.5%)	0	68 (8.0%)	0	26 (9.7%)	0	12 (3.2%)	1	113 (6.5%)	1
Streptococci	11 (10.6%)	0	8 (6.0%)	0	40 (4.7%)	0	13 (4.9%)	0	1 (0.3%)	0	73 (4.2%)	0
Other	1 (1.0%)	0	6 (4.5%)	0	8 (0.9%)	0	3 (1.1%)	1	0 (0.0%)	0	18 (1.0%)	1
Gram-negative	66 (63.5%)	0	54 (40.3%)	0	428 (50.4%)	9	150 (56.2%)	15	203 (53.7%)	73	901 (52.3%)	97
Klebsiella spp.	9 (8.7%)	0	17 (12.7%)	0	116 (21.1%)	3	49 (18.4%)	9	91 (24.1%)	40	282 (16.3%)	52
E. coli	36 (34.6%)	0	9 (6.7%)	0	113 (13.3%)	0	25 (9.4%)	1	6 (1.6%)	0	189 (10.9%)	1
Acinetobacter spp.	3 (2.9%)	0	4 (3.0%)	0	52 (6.1%)	3	31 (11.6%)	4	61 (16.1%)	21	151 (8.7%)	28
P. aeruginosa	7 (6.7%)	0	9 (6.7%)	0	52 (6.1%)	1	24 (9.0%)	1	28 (7.4%)	11	120 (6.9%)	13
Enterobacteriaceae	4 (3.8%)	0	12 (9.0%)	0	52 (6.1%)	2	14 (5.2%)	0	8 (2.1%)	0	90 (5.2%)	2
B. melitensis	4 (3.8%)	0	2 (1.5%)	0	19 (2.2%)	0	0 (0.0%)	0	0 (0.0%)	0	25 (1.4%)	0
Other	3 (2.9%)	0	1 (0.7%)	0	24 (2.8%)	0	7 (2.6%)	0	9 (2.4%)	1	44 (2.5%)	1
Anaerobes	1 (1.0%)	0	0 (0.0%)	0	21 (2.5%)	0	7 (2.6%)	0	2 (0.5%)	0	31 (1.8%)	0
Fungi	3 (2.9%)	0	9 (6.7%)	1	55 (6.5%)	1	19 (7.1%)	0	28 (7.4%)	8	115 (6.6%)	10

ICU, Intensive Care Unit; CR-BSI, Catheter-related bloodstream infection; CNS, coagulase negative staphylococci

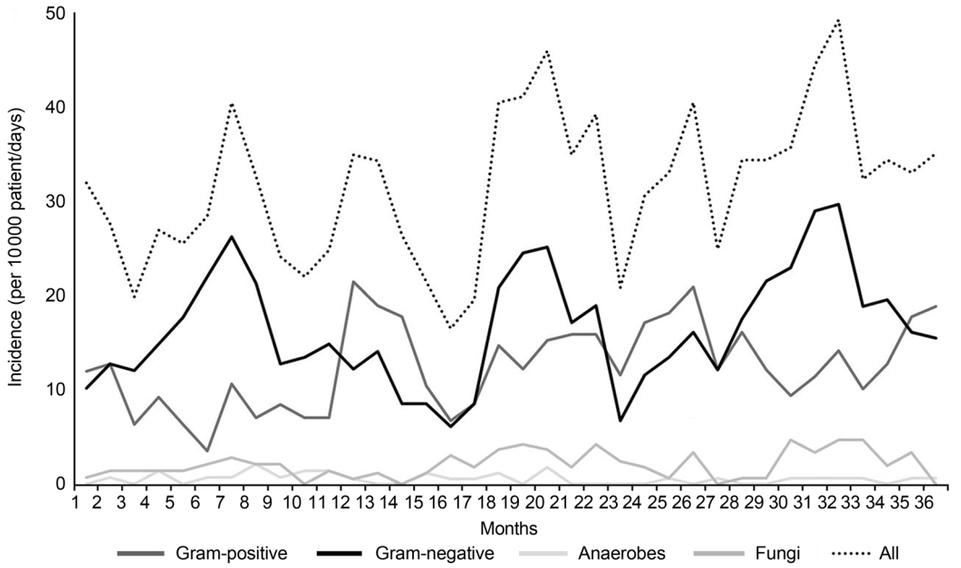

Fig. 2. Monthly variation of bloodstream infection incidence per 10000 patient-days during the study period

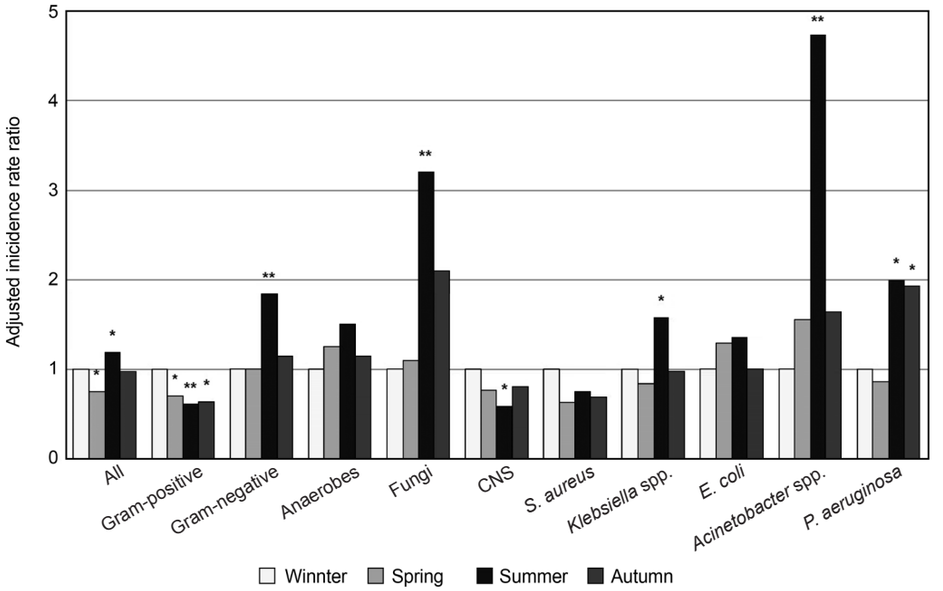

Fig. 3. Seasonal adjusted incidence of BSIs rate ratio according to isolated pathogen Winter months were defined as reference.
* $P < 0.05$; ** $P < 0.001$

(*mec*A-positive; MR-CNS and MRSA), respectively. Among the 113 enterococci, 12 (10.6%) were VRE (eight *van*A, two *van*B and two *van*C-positive). Carbapenem-resistance was observed in 207 *K. pneumoniae* (73.4%); 202 bla_{KPC}, four bla_{KPC} and bla_{VIM}, one were bla_{VIM}-positive, whereas, none carried bla_{NDM}. Moreover, 96 (63.6%) *Acinetobacter* spp., 43 (35.8%) *P. aeruginosa* and 12 (13.3%) Enterobacteriaceae were MBL-positive. No *E. coli* was carbapenem-resistant. An increase in MRSA BSIs incidence was observed during the study period (0.9 per 10000 patient-days in 2011 to 1.3 in 2013; R^2: 0.987; *P* 0.014), while no significant variation was observed in MR-CNS and VRE incidence. In total, BSIs due to carbapenem-resistant Gram-negative bacteria increased from 5.3 to 7.4 per 10000 patient-days; R^2: 0.818; *P* 0.042).

Among the 115 patients with fungemia, 113 episodes (98.2%) were caused by *Candida* spp. (50 *Candida parapsilosis*, 44 *Candida albicans*, 15 *Candida glabrata*, three *Candida tropicalis*, one *Candida kruzei*). Among patients with candidemia, 38 (33.6%) had a previous episode of bacteremia (21 due to KPC-producing

K. pneumoniae, six *Staphylococcus* spp. four *Acinetobacter* spp., three enterococci, two *P. aeruginosa* and two *E. coli*). Previous KPC-producing *K. pneumoniae* BSI was significantly higher among *Candida* non-*albicans* BSIs as compared to those caused by *C. albicans* (19 of 69, 27.5% *vs* two of 44, 4.5%; *P* 0.002).

Discussion

BSIs comprise one of the commonest types of infections in hospitalized patients, associated with high mortality (12.0–43.0%) (Goto and Al-Hasan, 2013; Bouza *et al*., 2014). The incidence of BSIs (community and healthcare-associated) in our study was 13.6 per 1000 admissions. A point prevalence study of healthcare-associated infections in European hospitals in 2011–12 found that 1.7% of patients from Greek hospitals developed a BSI, percentage comparable to ours (1.4%) (ECDC, 2013). BSIs rate in Greece was the highest (18.9%) among the European countries (10.7%) (ECDC, 2013). The incidence of BSIs was higher in our study as compared to those reported in a review from healthcare-associated BSIs from European countries and USA (2.7–8.4 per 1000 admissions), but was significantly lower to that reported from 'Laikon' General Hospital, a tertiary hospital in Athens, Greece, from 1995 to 2002 (26.9 per 1000 admissions) (Hadziyannis *et al*., 2004; Goto and Al-Hasan, 2013). Overall, Southern European countries, including Greece, show higher prevalence of healthcare associated infections as compared to those of Central or North Europe (ECDC, 2013). A significant rise in BSIs incidence was observed during the study period, a finding similar to that in other studies, probably due to ageing of hospitalized population, growing number of immunocompromised patients and increased use of invasive devices (Hadziyannis *et al*., 2004; de Kraker *et al*., 2013; Bouza *et al*., 2014).

The pathogens responsible for BSIs vary widely between continents and countries. (Wisplinghoff *et al*., 2004; ECDC, 2013; Anderson *et al*., 2014). In our study, Gram-negative bacteria were the predominant pathogens (52.3%), followed by Gram-positive (39.5%), fungi (6.6%) and anaerobes (1.8%). Even though, our results are close to those reported in related studies from Greece and Spain (Bouza *et al*., 2014; Koupetori *et al*., 2014), these findings differ from those of other European countries and North America reporting that during the last two decades Gram-positive bacteria have become the most common cause of sepsis (Hadziyannis *et al*., 2004; de Kraker *et al*., 2013; ECDC, 2013; Anderson *et al*., 2014; Gubbels *et al*., 2015). On the other hand, countries with closer proximity to equator exhibit higher percentages of Gram-negative BSIs (Fisman *et al*. 2014). Despite the predominance of Gram negatives in our and other Greek studies, a significant increase of Gram-positive incidence was detected in our setting, mainly attributed mainly to an increase of *S. aureus* BSIs. The same rising trend in BSIs due to Gram-positive bacteria was reported in a study from 31 Greek hospitals due to an increase in CNS and *S. aureus* incidence (Koupetori *et al*., 2014). Taking into account results from aforementioned study and the present one, we may hypothesize that in the future we will witness in Greece a shift in BSIs etiology from Gram-negative towards Gram-positive bacteria.

In most studies, *E. coli*, *S. aureus*, enterococci and streptococci constitute the most commonly isolated pathogens from BSIs, findings that contradict our results, where CNS and *Klebsiella* spp. were the main pathogens, followed by *E. coli*, *Acinetobacter* spp. and *S. aureus* (de Kraker *et al*., 2013; ECDC, 2013; Anderson *et al*., 2014; Gubbels *et al*., 2015; Khatib *et al*., 2015). A great disparity of isolated pathogens rates among the different Departments of our Hospital was found. In the Emergency Department, *E. coli*, *S. aureus* and streptococci were the most common cause of BSIs, reflecting the epidemiology of community-associated bacteremic infections (*E. coli* from urinary tract and abdominal infections, streptococci from respiratory tract infections, and *S. aureus* from skin and soft tissue infections) (ECDC, 2013). Similarly to previous reports, he highest rate of CNS was reported in the adult ICUs (32.0%) and Paediatric Departments (28.4%) due to the high usage of central venous catheters in adult critically ill patients and premature neonates (Wisplinghoff *et al*., 2004; Pereira *et al*., 2013).

Contamination rate among positive blood cultures (24.7%) was comparable to that reported in previous studies (16.5–19.5%) (Kitaura *et al*., 2014; Chang *et al*., 2015; Khatib *et al*., 2015). This finding may be explained by the low adherence to antiseptic procedures due to low nurse-to-patient ratio in Greek hospitals (Papadimitriou-Olivgeri *et al*., 2015). The probability of contamination and true bloodstream infection depends on the identity of the isolated pathogen, therefore, no blood culture positive for Gram-negative bacteria or fungi was judged to be contaminated in our Setting. (Kitaura *et al*., 2014; Chang *et al*., 2015; Khatib *et al*., 2015). On the contrary, among 72 *Corynebacterium* spp., 30 *Micrococcus* spp., 27 *Bacillus* spp. and 25 *Propionibacterium* spp. isolated from positive blood cultures, only five (6.9%), two (6.7%), two (7.4%) and three (12.0%) true BSIs were verified, respectively, results comparable to those previously reported (Urban, 2012; Kitaura *et al*., 2014; Chang *et al*., 2015). Despite the fact that CNS were the most commonly isolated pathogen from positive blood cultures (39.2%), they accounted for only 19.1% of BSIs. Since CNS are normal skin commensals, the

contamination rate of blood cultures was high (52.2%). Moreover, among CNS, *S. epidermidis* isolates were associated with a significantly lower contamination rate as compared to non-*S. epidermidis* (39.1% *vs* 73.3%; $P < 0.001$), in accordance with other investigators. (Uyanik *et al*., 2014; Papadimitriou-Olivgeri *et al*., 2015) Thus, it is imperative to distinguish true BSIs, since contamination may lead to unnecessary antibiotic consumption. (Papadimitriou-Olivgeri *et al*., 2015)

A low percentage of BSIs were catheter-related (18.0%), comparable to that reported from other investigators (8.2–24.0%), but lower to that from the point prevalence study in Europe (39.5%) (Wisplinghoff *et al*., 2004; Rodriguez-Creixems *et al*., 2013; ECDC, 2013). Gram-positive bacteria were the most commonly isolated pathogens (65.7%) from CR-BSIs, as previously shown, whereas, CNS predominated (Rodriguez-Creixems *et al*., 2013; Papadimitriou-Olivgeri *et al*., 2015). In a study from Israel, Gram-negative bacteria accounted for 76.4% of CR-BSIs, whereas, the low percentage of Gram-positive bacteria was due to the low percentage of CNS CR-BSIs (0.8%), which in our setting accounted for 63.8% of cases (Braun *et al*., 2014).

Another important finding of the present study was the high incidence of bacteremic brucellosis in Southwestern Greece. While brucellosis remains a rare infection in Europe, high notification rates were reported from Mediterranean countries that were not officially brucellosis-free in animal populations (cattle, sheep or goats) (EFSA and ECDC, 2015). The highest rate of human brucellosis was reported from Greece which increased from 0.9 per 100 000 habitants in 2011 to 1.4 in 2013, comparable to that found in the present study (EFSA and ECDC, 2015). Even though, no significant variation in brucellosis trends was found in Europe during 2011–3, we shown a significant increase in *B. melitensis* bacteremia, which may be attributed to lack of animal vaccination and consumption of raw non-heat treated cheese that remains the main risk factor for brucellosis (EFSA and ECDC, 2015; Karagiannis *et al*., 2012). As previously shown, in addition to population health education, the complete and proper animal vaccination can lead to reduction of human brucellosis (Jelastopulu *et al*., 2008).

Resistance among tested pathogens was high in the present study and coincided to the rates reported for Greece from the ECDC (ECDC, 2015). MRSA rates among *S. aureus* were 40.0%, a fact that may be attributed to the dissemination of the highly successful ST80 clone not only in the community, but also in the hospital (Drougka *et al*., 2014; Papadimitriou-Olivgeris *et al*., 2015). Methicillin-resistance among CNS was even higher, as previously shown, while VRE rates were lower (Papadimitriou-Olivgeri *et al*., 2015; Papadimitriou-Olivgeris *et al*., 2015; ECDC, 2015).

The most important issue in our study was the presence of carbapenem-resistance among Gram-negative bacteria. The rates were higher among *K. pneumoniae*, which in their majority produced the carbapenemase KPC. This is in accordance with a previous Greek study from 31 hospitals (Koupetori *et al*., 2014). As compared to other Gram-negative nosocomial bacteria, *Klebsiella* spp. were associated with persistent BSI (2.2 positive blood culture bottles per BSI as compared to 1.9). In a previous study in our adult general ICU during 2010–12, persistence of *K. pneumoniae* BSIs was due ,to inappropriate empiric therapy, which may also explain the findings of the present study, since only 67.9% of patients with KPC-producing *K. pneumoniae* BSIs received appropriate antibiotic therapy (Kang *et al*., 2013; Papadimitriou-Olivgeris *et al*., 2014a). Carbapenem-resistance rates among *P. aeruginosa* and *Acinetobacter* spp. were lower than that reported from ECDC (ECDC, 2015). Even though Gram-negative BSI incidence remained stable over time, a significant increase was observed in carbapenem-resistant BSI incidence. This was propagated by the low nurse-to-patient ratio, the absence of isolation rooms (< 5%) and the high antibiotic consumption in Greek hospitals (rate 54.7%) (ECDC, 2013).

Seasonality is an important issue for healthcare-associated infections (Eber *et al*., 2011; Paul, 2012). In our study, total BSI incidence was higher during summer months due to higher incidence of *Candida* spp. and Gram-negative bacteria during these months, especially *Klebsiella* spp., *Acinetobacter* spp. and *P. aeruginosa*. It was previously proven that Gram-negative BSI incidence increases proportionately to temperature's increase (Paul, 2012; Schwab *et al*., 2014). Even though, this was shown for *E. coli*, we found no difference in seasonal distribution of *E. coli* (de Kraker *et al*., 2013; Schwab *et al*., 2014). This is the first study to demonstrate higher CNS BSIs incidence in winter months, which cannot be explained by temperature-dependence of CNS. Seasonality was previously found among *S. aureus* infections, with higher incidence during summer months and autumn (Paul, 2012; Schwab *et al*., 2014). No such association was found in the present study.

Candidemia comprises an important cause of BSIs worldwide, associated with high mortality rates (Wisplinghoff *et al*., 2004; Falagas *et al*., 2010). In our study, BSIs due to *Candida* spp. accounted for 6.5% of all BSI, with a predominance of *C.* non-*albicans* species (61.1%). A great variability in distribution of different *Candida* spp., with *C. albicans* being the dominant species in North and Central Europe and *C.* non-*albicans* species in South America, South Europe, and Asia has been reported in a systematic review (Falagas *et al*., 2010). It is also shown that the percentage of *C.* non-

albicans falls steadily during the last decade worldwide (Falagas *et al*., 2010). During an eleven-year period (1998–2008), candidemias accounted for 3.3% of BSIs in UGHP with *C. albicans* being the predominant species (64.0%) (Spiliopoulou *et al*., 2010). This abrupt change in *Candida* spp. epidemiology in our Institution can be explained by the dissemination of KPC-producing *K. pneumoniae* BSIs. In a previous study among ICU patients we showed that KPC-producing *K. pneumoniae* BSIs predispose to candidemia and selection of *C.* non-*albicans* species (Papadimitriou-Olivgeris *et al*., 2014b). Similarly, same results are shown in the present study, since previous episodes of KPC-producing *K. pneumoniae* was significantly higher among candidemia due to *C.* non-*albicans* species as compared to *C. albicans*. Candidemia showed seasonality in the present study, being significantly higher in summer months as compared to winter ones, a finding that reinforces previously published results of a previous study, where hospitalization during summer months was an independent risk factor for *Candida* spp. isolation. (Papadimitriou-Olivgeris *et al*., 2014b)

The present study has several limitations. No clinical data were included rendering the calculation of BSIs mortality or the role of appropriate treatment in survival impossible. Second, this is a single center study; however, it reflects not only the local epidemiology but also the national one (ECDC, 2013; Koupetori *et al*., 2014; Papadimitriou-Olivgeris *et al*., 2015). Despite the evaluation of seasonality, no temperature data were included.

In conclusion, BSIs remain an important cause of healthcare-associated infections. The increasing incidence of BSIs was due to similar increase of Gram-positive BSI incidence, even though Gram-negative bacteria were the predominant ones. CNS was the most commonly isolated pathogen from bacteremic patients and was more common during winter months, in contrast to summer occurrence of Gram-negative bacteria especially *Klebsiella* spp. Antibiotic resistance of both Gram-positive cocci and Gram-negative bacilli was high, contributing in their dissemination in the hospital environment. Candida epidemiology changed abruptly the last years, with *C.* non-*albicans* species being the predominant ones, probably due to the high incidence of KPC-producing *K. pneumoniae* since it is shown to be a prelude of *C.* non-*albicans* candidemia. In the context of budgetary crisis, absence of isolation rooms, low nurse-to-patient ratio and high antibiotic consumption, even though intensive effort in infection control measures is made, they remain inefficacious.

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The authors declare no conflicts of interest.

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ORIGINAL PAPER

Effect of Lyophilization on Survivability and Growth Kinetic of *Trichoderma* Strains Preserved on Various Agriculture By-Products

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Abstract

Growth kinetics of four *Trichoderma* strains was tested on lignocellulosic by-products in solid state fermentation (SSF). The strains were also analyzed for their survival rate and growth after lyophilization on these carriers. All applied monocomponent and bicomponent media were substrates for the production and preservation of *Trichoderma* biomass. However, the maximum number of colony forming units (CFU/g dm) was acquired on bicomponent media based on dried grass and beet pulp or grass with corn cobs, when compared to monocomponent media. Although the process of lyophilization reduced the survival rate by 50–60%, the actual number of viable cells in obtained biopreparations remained relatively high ($0.58 \times 10^8 - 1.68 \times 10^8$ CFU/g dm). The studied strains in the preserved biopreparations were characterized by a high growth rate, as evaluated in microcultures using the Bioscreen C system.

K e y w o r d s: *Trichoderma* sp., Bioscreen C system, lignocellulosic carriers, lsurvivability after lyophilization

Introduction

Due to the growing awareness of the society with respect to environmental protection and increased concern about health, alternatives to chemical methods of plant protection are sought. Hope is seen in biological methods based on natural agents that inhibit the development of undesirable microflora (Gerhardson, 2002; Manso *et al.*, 2010). The search for isolates with effective protective properties, mainly against phytopathogens, is based on testing their antagonistic action, identification of promising strains, and their subsequent use in the production of biologicals. Fungi of the *Trichoderma* genus are often used as biological agents in biocontrol products. Fungi of this genus exhibit many advantageous properties, that allow their application in plant protection (Błaszczyk *et al.*, 2014; Smolińska *et al.*, 2014). The most desirable features include: widespread occurrence in the soil environment, dynamic growth, abundant sporulation, rapid colonization of the roots of plants as well as the ability to utilize a wide variety of nutrients from the soil (Jash and Pan, 2007). In addition, these fungi are producers of various hydrolytic enzymes, including cell wall degrading enzymes (CWDEs), which damage cell walls of phytopathogens, such as chitinases, beta-glucanases or proteases. They also synthesize numerous fungitoxic compounds, such as peptaibole, sesquiterpenoids, polyketides, isonitriles and certain octaketide antibiotics, and demonstrate the ability of mycoparasitism and induction of systemic plant resistance (Viterbo *et al.*, 2002; Howell, 2003; Daniel and Filho, 2007; Degenkolb *et al.*, 2008). The result of complex action of antagonistic agents of *Trichoderma* fungi is growth inhibition of phytopathogens from genera *Rhizoctonia*, *Phytium*, *Sclerotium*, *Fusarium*, *Botrytis* and *Verticillum* (Witkowska and Maj, 2002; Monte and Llobell, 2003; Matroudi *et al.*, 2009; Piegza *et al.*, 2009; John *et al.*, 2010; Monteiro *et al.*, 2010). Furthermore, these fungi are resistant to their own metabolites, metabolites produced by other microorganisms, terpenoid phytoalexines secreted by plants, as well as fungicides and heavy metals present in the soil (Khan and Shahzad, 2007).

A suitable method for the preparation of products based on *Trichoderma* fungal cultures are cultures in solid medium, such as solid state fermentation (SSF)

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with the use of lignocellulosic waste materials. These compounds are inexpensive, readily available in large amount sand, generally do not require nutrient supplementation. Moreover, these materials are rich sources of C, N and P, which contributes to rapid growth and sporulation of fungi. One of the main advantages of the SSF culture techniques is low water consumption, and thus the limited production of wastewater and minimization of energy consumption (Thomas *et al*., 2014; Mondala, 2015).

Industrial biopreparations containing microorganisms should demonstrate a high rate of survival, growth and maintenance of important biological properties after preservation of biomass, as well as the stability of these characteristics during storage. One of the methods for the preservation of microbial biomass is lyophilization. However, the application of the large difference of temperatures in the process of lyophilization can lead either to the destruction or deformation of the cell membrane, as well as denaturation of cellular proteins. Therefore, protective agents are typically used, which should be selected individually for each microorganism. The preservation of biomass in the process of lyophilization allows for the long-term storage of microbial cells, but also draws attention to a large variation in survival rate that depends on factors, such as the preserved strain, the stage of development, biomass density or protective agent concentration (Hubalek, 2003; Morgan *et al*., 2006; Prakash *et al*., 2013).

The aim of this study was to evaluate the biomass production of four strains, *i.e.*, *Trichoderma atroviride* TRS14, *T. atroviride* TRS7, *Trichoderma simmonsii* TRS75 and *Trichoderma virens* TRS109, in the SSF cultures on lignocellulosic by-products used as media, and its subsequent preservation on these carriers in the process of lyophilization, in order to retain high survival and growth rates of strains in the obtained biopreparations.

Experimental

Materials and Methods

Microrganisms. Microbial material consisted of the following strains: *T. atroviride* TRS7, *T. atroviride* TRS14, *T. simmonsii* TRS75, and *T. virens* TRS109 (Culture Collection of Microbiology Lab, Research Institute of Horticulture, Skierniewice). Molecular identification of these strains, based on sequences of *ITS1* and *ITS2*, *tef1alpha*, *rpb2* and *chi18–5*, was described in the previous work (Skoneczny *et al*., 2015; Oskiera *et al*., 2015). The tested strains were stored on PDA (Potato Dextrose Agar) slants at 4°C.

Lignocellulosic by-products. The study was conducted with three lignocellulosic by-products (beet pulp, corn cobs and dried grass), in monocomponent and bicomponent cultures in the proportion of 1:1 of dried grass with beet pulp or dried grass with corn cobs.

Biomass production of *Trichoderma* fungi on lignocellulosic by-products in solid state fermentation (SSF). The agriculture by-products, initially dried and ground in a mill, were put into 1000 ml Roux flasks in the following amounts: cultures with a single component (beet pulp or corn cobs) – 80.0 g + 160 ml of distilled water, and (dried grass) – 40.0 g + 80 ml of distilled water; whereas in cultures with two components (dried grass with beet pulp and corn cobs, respectively) – 40.0 g + 80 ml of distilled water. The media were left for 60 minutes at room temperature, then moisture content was determined and sterilization was carried out at 121°C for 60 minutes. After sterilization, the media were incubated for 24 hours in the incubator at 25°C in order to control for sterility. The prepared media with mean moisture content in the range of 59.9–69.4% and a mean pH of 5.3–5.7 were inoculated with a standardized suspension of conidia in 0.1% Tween 80, obtained from 10-day fungal culture on PDA medium to the initial density of $1.3 \times 10^6 - 2.3 \times 10^6$ conidia/g dm of media and incubated for 10 days in the phytotron chamber at constant humidity of 75% and temperature 25°C. All cultures were performed in duplicate.

Preservation of the *Trichoderma* fungal biomass and spores on lignocellulosic carriers in the process of lyophilization. The lignocellulosic by-products overgrown with the tested strains (10.0 g) were introduced into 500 ml round bottom flasks together with 10 ml of 20% sterile solution of maltodextrin, used as the protective agent or an equivalent amount of sterile distilled water (control), and mixed thoroughly. The lyophilization process was preceded by pre-freezing to a temperature of –24°C, followed by freeze-drying for 20 hours at the external manifold of the Labconco Triad freeze dryer, at the pressure of 0.2 mbar. Once the process was complete, the lyophilizates were put into plastic bags and vacuum-sealed.

Evaluation of selected parameters of the growth kinetics of *Trichoderma* strains (in lyophilized biopreparations) using a Bioscreen C system. The parameters of the growth kinetics of *Trichoderma* strains in lyophilized biopreparations were analyzed using Bioscreen C system. Microcultures (five replicates each) were carried out in potato dextrose broth (PDB) under the following conditions: temperature of incubation 25°C, time of incubation – 3 days, continuous shaking, absorbance read at 540 nm, reading intervals every 20 minutes. Strains in SSF cultures prior to lyophilization were used as controls. Growth of the strains was recorded in the form of curves showing the dependence

of optical density against time. The selected parameters of growth kinetics (lag phase duration in h, the maximum rate of specific growth increase μ_{max} in h^{-1} and the maximum biomass yield ΔOD_{max} expressed as the difference between the maximum and minimum optical density) were determined based on the analysis of growth curves.

The length of the lag-phase was determined directly from the growth curves, whereas the other parameters – using the following formulas:

$$\mu_{max} = (\ln OD_2 - \ln OD_1)/(t_2 - t_1)$$

where:

OD_1 – optical density at the onset of the logarithmic phase

OD_2 – optical density at the end of the logarithmic phase

t_1 – onset of logarithmic phase

t_2 – end of logarithmic phase

$$\Delta OD_{max} = OD_{max} - OD_{min}$$

where:

OD_{max} – maximal value of optical density

OD_{min} – minimal value of optical density

Analytical methods. The number of viable cells, expressed as the number of CFU/g dm, in SSF cultures and lyophilizates (after rehydration in 10% maltodextrin solution or distilled water, respectively) was determined by the Koch plate method in potato dextrose agar medium (PDA) containing rose bengal (0.035 g/l) from three consecutive dilutions in 0.1% Tween 80 in triplicate.

The percentage of viable cells (% survivability) was expressed as a percentage of surviving cells compared to live cells prior to preservation.

Statistical calculations. Results pertaining to the number of CFU/g dm and growth rate of the tested strains in lyophilizates were statistically analyzed by three-way analysis of variance (type of medium, strain, biological product). The differences between means were determined by Duncan's test with a significance level of $p = 0.05$. The calculations were carried out using the Statistica 10 software (StatSoft).

Results

Biomass production of *Trichoderma* fungi on lignocellulosic by-products in solid state fermentation (SSF). In this study, four strains were used for the production of lyophilized biopreparations, including two strains of *T. atroviride* species (TRS14 and TRS7), one of *T. simmonsii* (TRS75) species and one of *T. virens* (TRS109) species, cultured on solid medium to obtain large amount of biomass. It was shown that the applied materials constituted suitable and inexpensive source of energy and nutrients for growing fungi. On monocomponent media (dried grass, beet pulp or corn cobs), the tested strains produced biomass in the amount of $8.6 \times 10^7 - 1.7 \times 10^8$ CFU/g dm. The exception was the strain *T. atroviride* TRS14, which grew poorly on corn cobs (7.4×10^6 CFU/g dm). For all the strains tested, the bicomponent substrates proved to be more efficient for biomass production, expressed as the number of CFU (either dried grass and beet pulp or dried grass with corn cobs). In this case, biomass production ranged from 1.7×10^8 to 5.3×10^8 CFU/g dm, depending on the strain. Strain *T. simmonsii* TRS75, was the most effective biomass producer on both, monoand bicomponent, substrates among the strains investigated ($1.1 \times 10^8 - 5.3 \times 10^8$ CFU/g dm). Three other strains, *i.e.*, *T. atroviride* TRS7, *T. atroviride* TRS17 and *T. virens* TRS109, produced comparable level of biomass ($9.3 \times 10^7 - 3.5 \times 10^8$ CFU/g dm) (Table I).

There was a slight increase in mean moisture content recorded after 10 days of SSF cultures within the range of 66.9–73.5% in comparison with mean initial values of this parameter (59.9–69.4%) (Fig. 1). A slight shift in the pH of medium was also observed after 10 days of culture – mean pH increased from the range 5.3–5.7 to 6.7–6.8 for mono- and bicomponent media, respectively (Fig. 2).

Preservation of the *Trichoderma* fungal biomass and spores on lignocellulosic carriers in the process of lyophilization. The biomass of tested strains obtained on lignocellulosic substrates was preserved by lyophilization, with the use of maltodextrin as the protective agent or with an equivalent amount of distilled

Table I
Biomass expressed as the number of cfu/g dm in 10-day SSF cultures of *Trichoderma* strains on lignocellulosic materials.

Strain	Monocomponent medium			Bicomponent medium	
	Dried grass	Beet pulp	Corn cobs	Dried grass + beet pulp	Dried grass + Corn cobs
T. atroviride TRS7	9.3×10^7	1.2×10^8	8.6×10^7	1.7×10^8	2.0×10^8
T. atroviride TRS14	1.1×10^8	1.3×10^8	7.4×10^6	2.2×10^8	3.5×10^8
T. simmonsii TRS75	1.7×10^8	1.1×10^8	1.2×10^8	5.3×10^8	2.8×10^8
T. virens TRS109	1.5×10^8	1.2×10^8	1.3×10^8	2.0×10^8	2.4×10^8

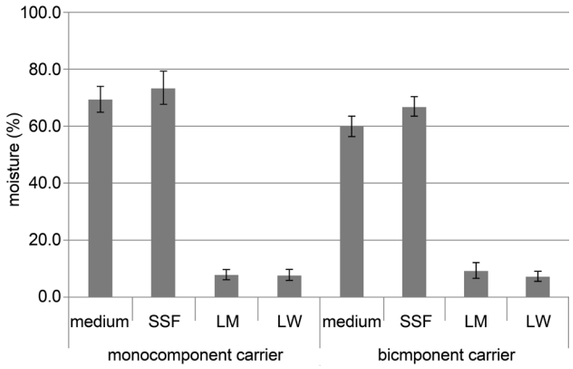

Fig. 1. Mean moisture content (%) of medium, after SSF cultures and of lyophilizates produced on the lignocellulosic materials with *Trichoderma* strains.

SSF – solid state fermentation (culture);
LM – lyophilizates with maltodextrin;
LW – lyophilizates with distilled water (control)

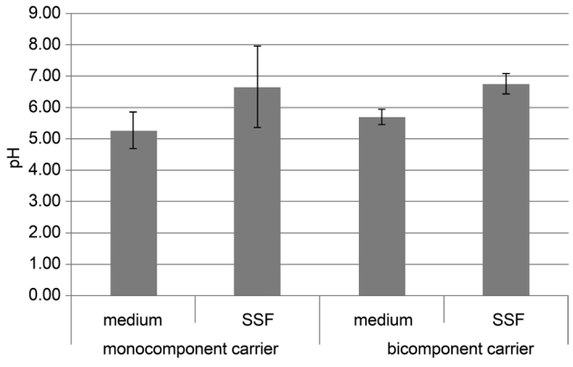

Fig. 2. Mean pH value of medium and after SSF cultures on the lignocellulosic materials with *Trichoderma* strains.

SSF – solid state fermentation (culture)

water (control), by reducing the level of moisture of lyophilizates to a range of 7.9–9.4% (LM, lyophilizates with maltodextrin) and to range 7.3–7.8% for lyophilizates with water (LW, control) (Fig. 1). The lyophilized products demonstrated a significant reduction in the number of viable cells as compared to their amount prior to the preservation procedure. The biopreparations demonstrated survivability in the range from 40.89% to 48.43% of the CFU/g dm. The addition of a protective medium had no significant effect on the survivability of the tested strains. In lyophilizates with maltodextrin (0.84×10^8 CFU/g dm) or without it (0.62×10^8 CFU/g dm), similar count of viable cells was detected (Fig. 3). There were no significant differences in the survival of fungi in lyophilizates in relation to the test strain (54.99–70.27%) as well as the biomass carrier (56.88–75.45%). However, when considering the number of viable cells (CFU) in the preparations, but not their percentage compared with their initial number before lyophilization, a significant correlation was found between survival rate and both the test strain as well as the material used in the medium. A higher count of viable cells was observed in the biopreparation of the strain *T. simmonsii* TRS75 (1.48×10^8 CFU/g dm) as compared with preparations of other tested strains ($0.90 \times 10^8 - 0.97 \times 10^8$ CFU/g dm). Although the survival rate of strains on the monocomponent lignocellulosic carriers was higher (56.88–75.45%) than on the bicomponent compositions (58.57– 62.32%), the actual number of viable cells was higher on bicomponent carriers ($1.55 \times 10^8 - 1.68 \times 10^8$ CFU/g dm) compared with monocomponent ($0.59 \times 10^8 - 0.83 \times 10^8$ CFU/g dm), which was due to the higher initial CFU number of the tested strains in SSF cultures on mixed compositions

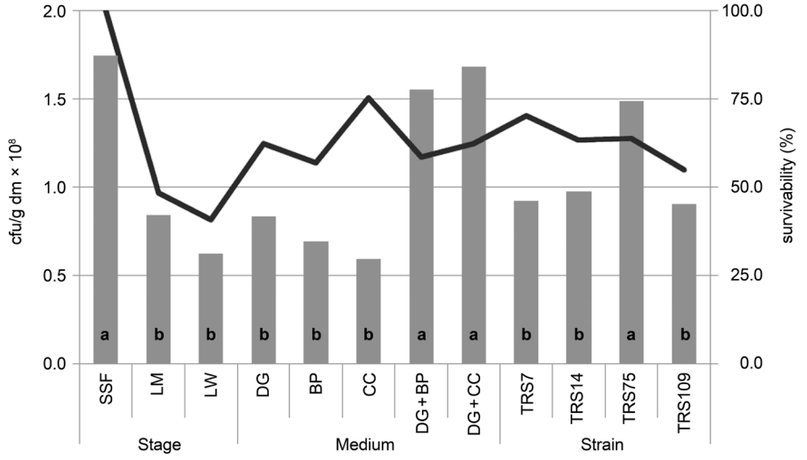

Fig. 3. The average values of cfu/g dm (bars) and survivability (line) of *Trichoderma* strains in SSF cultures and after lyophilization depending on the stage, medium and tested strain (three-way analysis of variance).

a, b – homogeneous groups according to the Duncan's test at $p = 0.05$
SSF – solid state fermentation (culture), LM – lyophilizates with maltodextrin, LW – lyophilizates with distilled water (control);
DG – dried grass, BP – beet pulp, CC – corn cobs

Table II
Mean values of selected parameters of the growth kinetics of *Trichoderma* strains depending on the stage, medium and tested strain (three-way analysis of variance).

Variation factor		Dependent variables		
		Lag phase [h]	μ_{max} [h^{-1}]	ΔOD_{max}
Stage	SSF	15.16 a	0.1599 a	1.715 a
	LM	14.33 a	0.1528 a	1.548 a
	LW	14.56 a	0.1132 a	1.518 a
Medium	Dried grass	16.58 a b	0.2212 a	1.616 a
	Corn cobs	14.64 b c	0.1175 a	1.587 a
	Beet pulp	17.88 a	0.1277 a	1.497 a
	Dried grass + beet pulp	12.33 c	0.1156 a	1.675 a
	Dried grass + corn cobs	12.00 c	0.1279 a	1.594 a
Strain	*T. atroviride* TRS7	15.33 a	0.1179 a	1.573 a
	T. atroviride TRS14	13.31 a	0.1175 a	1.655 a
	T. simmonsii TRS75	13.66 a	0.1357 a	1.582 a
	T. virens TRS109	16.44 a	0.1969 a	1.566 a

a, b, c – homogeneous groups according to the Duncan's test at p = 0.05
SSF – solid state fermentation (culture)
LM – lyophilizates with maltodextrin
LW – lyophilizates with distilled water (control)

of lignocellulosic by-products. In summary, the most effective monocomponent carrier that contributed to high survivability of the investigated strains (75.45%) was corn cobs, while the combination of this component with dried grass resulted in a slightly lower survival rate of the strains, *i.e.*, 62.32% (Fig. 3).

Evaluation of selected parameters of the growth kinetics of *Trichoderma* strains (in lyophilized biopreparations) using a Bioscreen C system. No significant influence was shown for the lyophilization process and the protective agent used in this procedure (maltodextrin) on the growth kinetics of selected parameters of the tested strains in the lyophilizates. The mean values of the lag phase length (14.33–14.56 h), the maximum specific growth rate (μ_{max} 0.1132 – 0.1528 h^{-1}) and the biomass yield expressed as ΔOD_{max} (1.518 – 1.548) for the strains lyophilized on lignocellulosic carriers, did not differ significantly from the values of the growth kinetics of strains in SSF control cultures prior to lyophilization (lag phase – 15.16 h, μ_{max} – 0.1599 h^{-1}, ΔOD_{max} – 1.715) (Table II).

Evaluating the growth kinetics parameters in relation to the composition of lignocellulosic by-products used as substrates during the production of biomass, and after lyophilization as its carriers as well as to the test strain, a significant effect of the preservation process was demonstrated only on the length of the lag phase, whereas there were no significant differences in the growth rates μ_{max} (0.1156 h^{-1} – 0.2212 h^{-1}) and biomass yield ΔOD_{max} (1.497 – 1.675). The shortest lag phases in cultures of tested strains were recorded in lyophilizates on bicomponent media of corn cobs and beet pulp with dried grass (lag phase 12.00 – 12.33 h) and significantly longer in lyophilizates on beet pulp (17.88 h), dried grass (16.58 h) and corn cobs (14.64 h). Among the studied fungi, *T. virens* TRS109 had a longer lag phase (16.44 h) than the other strains analyzed (lag phase 13.31–15.33 h), but the differences between the strains were not statistically significant (Table II).

Discussion

Saprophytic fungi, mainly of *T. virens* species and *Trichoderma harzianum* species complex including *T. simmonsii*, have been successfully introduced as components of biopesticides in many countries, including Sweden (Binab T WG), Belgium (Trichodex, Biofungus), the USA (RootShield, PlantSheld), or India (Bioderma Bioderma-H) (Kaewchai *et al*., 2009; Chaverri *et al*., 2015). In contrast, there are very few biocontrol products of domestic origin (*e.g.*, Vital Plus based on *Trichoderma viride*). Therefore, it is important to search for new native species, which could be used as a base for the production of biopreparations adapted to Polish conditions of agrarian cultivation. An important aspect of the production of such biopreparations, in addition to the selection of strains, is the use of appropriate methods of culture and inexpensive, readily available substrates, which would ensure a high biomass yield. Solid media are more effective in obtaining large amounts of microbiologically pure conidia (Lewis and

Papavizas, 1983), and their main advantage is the limited risk of contamination due to the low water activity.

This study used SSF culture technology applying lignocellulosic by-products, which constituted suitable culture environment for the production of biomass of filamentous fungi. The strains utilized biopolymers present in the media owing to numerous hydrolytic enzymes synthesized by them, including cellulases, xylanases polygalacturonases, laminarinasis and proteases that allow them to use hardly assimilable sources of carbon and nitrogen (Mitchell *et al*., 2002). It was also found that more favorable for biomass production of *Trichoderma* strains were bicomponent compositions of dried grass with corn cobs and beet pulp ($1.7 \times 10^8 - 5.3 \times 10^8$ CFU/g dm) than the monocomponent media: beet pulp, corn cobs and dried grass ($7.4 \times 10^6 - 1.7 \times 10^8$ CFU/g dm), which was presumably associated with a more varied and diverse chemical composition of these substrates. This was more advantageous for obtaining higher biomass yield than in monocomponent substrates. Tewari and Bhanu (2004) cultured various species of *Trichoderma* fungi, including *T. harzianum*, on similar substrates as in the present study, *i.e.*, corn cobs and sugarcane, and obtained biomass yield of this species at 2.24×10^8 CFU/g dm and 2.79×10^8 CFU/g dm, respectively. Corresponding results were obtained in the previous study, where the *T. simmonsii* TRS75 strain cultured in media consisting of beet pulp or corn cobs, ensured biomass production at $1.1 \times 10^8 - 1.2 \times 10^8$ CFU/g dm. In the production of biopreparations, attention should be paid to the potential use of substrates with the addition of corn cobs in the SSF cultures of *Trichoderma* fungi, since it was found that the presence of this material, even as a sole carbon source in the medium, in addition to the production of biomass, had a positive effect on the synthesis of enzymes, including FP-ases, CMC-ases and xylanases (Kancelista and Witkowska, 2008).

When obtaining a high yield of the biomass, next to the proper composition of the substrate, it is important to determine the optimal parameters for the growth of filamentous fungi. In this work, the production of biomass of strains tested in SSF cultures lasted for 10 days at the temperature 25°C. Similar duration of the culture of filamentous fungi in SSF media (7 to 10 days) was determined by Kredics *et al.* (2003). The study of Orzua *et al.* (2009) demonstrated that the appropriate range of moisture (30–80%), dependent on the substrate, in the SSF cultures enhanced the efficient production of biomass. Moisture content of the substrates used in the present study ranged from 59.9% to 69.4%, which positively affected the growth of the studied fungi.

At high moisture content, lignocellulosic waste materials swell, thereby facilitating the access of filamentous fungi to polymers, which they are composed of. Furthermore, the high water content improves the solubility of nutrients (Kovacs *et al*., 2009; Xin and Geng, 2010).

Numerous authors, recommended the range of pH 4 to pH 6 as the optimum for the growth of filamentous fungi (Kredics *et al*., 2003; Benitez *et al*., 2004). In the current study, the initial pH value of the substrates was not adjusted, the mean pH of monocomponent media (dried grass, beet pulp or corn cobs) was 5.3, while the pH of bicomponent substrates (dried grass with beet pulp or dried grass with corn cobs) was 5.7. These pH values of substrates also facilitated the biosynthesis of extracellular hydrolytic enzymes, necessary for utilization of substrates, which resulted in a high biomass yield of the tested strains.

Majority of the publications have reported on the use of the following species as biological agents in the biocontrol products: *T. harzianum*, *T. virens*, *T. viride* and *Trichoderma koningi*, while *T. atroviride* was reported less commonly. The present study investigated two strains of the species: *T. atroviride*, TRS7 and TRS14, which similarly as strains of *T. simmonsii* TRS75 and *T. virens* TRS109, demonstrated a high biomass yield, particularly in the bicomponent lignocellulosic substrates ($1.7 \times 10^8 - 3.5 \times 10^8$ CFU/g dm). It has been demonstrated that these strains, by having a high capacity for breakdown of lignnocellulosic materials, also possess a potential as biological agents in biocontrol products for plant protection. Panahian *et al.* (2012) conducted a study on the formulation of biopreparations with *T. atroviride* and reached similar conclusions to ours.

One of the most important issues in studies on the production of biopreparations is their preservation intended for long-term storage. One of the preservation methods is lyophilization involving a variety of protective agents that is mainly applied to stabilize the biomass of pure cultures, including the industrially important strains of filamentous fungi, and allows for the long-term storage while maintaining their high survivability, good growth rate, genetic homogeneity and stability of technological traits. In the present work, the biomass of four strains tested, *i.e*., *T. atroviride* TRS7, *T. atroviride* TRS14, *T. simmonsii* TRS75 and *T. virens* TRS109, obtained on lignocellulosic by-products, was preserved in the process of lyophilization with the addition of maltodextrin as a protective agent by reducing the moisture of the products to the desired range from 7.9% to 9.4%. Guijarro *et al.* (2006) reported that the moisture content of biopreparations produced on the basis of conidia stored at room temperature should be within the range of 5–15%.

While the survival rate of strains tested immediately after lyophilization depended solely on the preservation procedure of the biomass, there were no significant differences in the survivability of fungi with respect to the strains studied, the biomass carrier, or the

presence of protective medium. The study of Panahian *et al.* (2012) evaluated various protective agents including maltodextrin during the lyophilization process of species *T. atroviride* and *T. koningi* in SSF culture on molasses and corn grain, and also did not find a beneficial effect of maltodextrin on the survival of the controlled strains. In the current study, strains demonstrated survival rate in the range of 54.99% to 70.27% ($9.01 \times 10^7 - 1.48 \times 10^8$ CFU/g dm). The most effective biomass carrier in terms of the highest survival rate was corn cobs (75.45%), while beet pulp was the least efficient substrate (56.88%). Based on these results, it can be assumed that the applied lignocellulosic by-products were already relatively good preservation agents for retaining sufficient survivability of the strains tested as well as the high growth rate.

After lyophilization, the tested strains exhibited proper growth rates. The only significant differences were recorded in the length of the lag phase, depending on the biomass carrier. Longer adaptive phase had no effect on the maximum rate of specific growth ($\mu_{max} = 0.1156\ h^{-1} - 0.2212\ h^{-1}$) and the final maximum biomass yield ($\Delta OD_{max} = 1.497–1.675$) of the strains analyzed. The results obtained are a continuation of previous studies. In the previous studies (Kancelista *et al*., 2013) we showed an increased growth rate and higher biomass yield of *T. harzianum* and *T. virens* strains that grew on lignocellulosic substrates ($\mu_{max} = 0.135\ h^{-1} - 0.194\ h^{-1}$ and $\Delta OD_{max} = 1.660–1.786$) when compared to strains cultured on a standard fungi medium (PDB $\mu_{max} = 0.081\ h^{-1} - 0.136\ h^{-1}$ and $\Delta OD_{max} = 1.397 - 1.479$). However, after the preservation of the biomass in the drying process, these strains were characterized by the reduced values of growth kinetics parameters. Simões *et al.* (2009) controlled the influence of different carbon sources on the growth of *T. viride* strain during the 60-hour culture in a Bioscreen C system. The lowest biomass yield was reported in cultures with glucose $\Delta OD_{max} = 1.100$, while the highest in the culture with sorbitol $\Delta OD_{max} = 2.100$. Other authors (Rossi-Rodrigues *et al*., 2009) evaluated the growth of *Trichoderma* fungi of species *Trichoderma hamatum*, *T. harzianum*, *T. viride* and *Trichoderma longibrachiatum*, in relation to the carbon sources (glucose, sucrose) as well as nitrogen sources (yeast extract and tryptophan). They found that the growth rate was a species-specific trait, and the most effective carbon source was glucose in combination with yeast extract, on which the *T. hamatum* strain ensured biomass yield at the approximate level of $\Delta OD_{max} = 1.800$, and the *T. harzianum* strain at $\Delta OD_{max} = 1.500$. In the present study, the strain of *T. simmonsii* species derived from lyophilizates obtained on lignocellulosic substrates showed a slightly higher biomass yield ($\Delta OD_{max} = 1.582$) than in the above-discussed study. The remaining test strains grew to the ΔOD_{max} level of 1.556–1.655.

These results demonstrated the preservation of the high growth rate after the process of lyophilization of the investigated strains. In order to find a commercial application in plant protection for biopreparations based on *Trichoderma* strains, it is necessary to provide a high survival rate and the stability of all the important parameters for at least 18 months of storage (Pedreschi and Aguilera, 1997). Therefore, further studies will focus on the control of viability and retaining the ability for the biosynthesis of enzymes (mainly related to the degradation of cell wall of phytopathogens) during long-term storage of fungal preparations at room temperature and comparatively at refrigerated conditions.

Summary

In conclusion, it was shown that the applied lignocellulosic by-products such as dried grass, beet pulp and to a lesser extent, corn cobs were suitable substrates for the production of *Trichoderma* biomass. However, bicomponent media, composed of dried grass and beet pulp and dried grass with corn cobs, were more effective for fungal growth, compared to monocomponent media. The process of lyophilization of the resultant biomass reduced the survival rate of the strains present in lyophilized biological products by about 50–60%. However, the actual number of viable cells in lyophilized biopreparations was relatively high ($0.58 \times 10^8 - 1.68 \times \times 10^8$ CFU/g dm). Maltodextrin used in the lyophilization process had no particular protective effect on the survival rate of the strains. A very similar number of viable cells was detected either in the presence or absence of maltodextrin in the lyophilizates on lignocellulosic carriers ($0.62 \times 10^8 - 0.84 \times 10^8$ CFU/g dm).

This paper presents the results not yet described in the literature and concerning the use of lignocellulosic by-products as carriers of spores and fungal biomass of *Trichoderma* both in the process of their production and preservation by freeze-drying. Studies on the stability of such biopreparations are continued, as one of the most important issues is to maintain the high survivability and growth rate of the fungi in biological products during their long-term storage.

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ORIGINAL PAPER

Gene Expression during BTEX Biodegradation by a Microbial Consortium Acclimatized to Unleaded Gasoline and a *Pseudomonas putida* Strain (HM346961) Isolated from It

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Abstract

Pseudomonas putida strain (HM346961) was isolated from a consortium of bacteria acclimatized to unleaded gasoline-contaminated water. The consortium can efficiently remove benzene, toluene, ethylbenzene and xylene (BTEX) isomers, and a similar capability was observed with the *P. putida* strain. Proteome of this strain showed certain similarities with that of other strains exposed to the hydrocarbon compounds. Furthermore, the toluene di-oxygenase (*tod*) gene was up-regulated in *P. putida* strain when exposed to toluene, ethylbenzene, xylene, and BTEX. In contrast, the *tod* gene of *P. putida* F1 (ATCC 700007) was up-regulated only in the presence of toluene and BTEX. Several differences in the nucleotide and protein sequences of these two *tod* genes were observed. This suggests that *tod* up-regulation in *P. putida* strain may partially explain their great capacity to remove aromatic compounds, relative to *P. putida* F1. Therefore, new *tod* and *P. putida* strain are promising for various environmental applications.

Key words: *Pseudomonas* spp. BTEX, dioxygenases, LC/MS/MS, bioremediation, biodegradation

Introduction

Benzene, toluene, ethylbenzene and xylene (BTEX) isomers are aromatic hydrocarbons that constitute the major components of gasoline (Potter, 1992). The irresponsible use of these compounds and their release into soil and water causes considerable damage to the environment (Díaz *et al*., 2001; Lawniczak *et al*., 2011). In comparison to other gasoline hydrocarbons, these compounds are soluble in water and have genotoxic properties (Dean, 1985; Tsao *et al*., 1998), although under favorable conditions, BTEX is biodegradable by a wide variety of microorganisms (Gibson and Subramanian, 1984; Lawniczak *et al*., 2011; Lisiecki *et al*., 2014). The scientific community is increasingly interested in these microorganisms and their genomes for developing biotechnological processes that remove aromatic compounds (Pieper and Reineke, 2000; Díaz *et al*., 2001; Owsianiak *et al*., 2009).

Numerous bacterial species have been discovered that can metabolize chemical components found in gasoline (Cyplik *et al*., 2011; Dalvi *et al*., 2014). Recently, we reported that a consortium acclimatized to unleaded gasoline degraded 95% of total BTEX and *Pseudomonas*, *Shewanella*, *Burkholderia*, *Alcanivorax*, *Rhodococcus* and *Bacillus*, were identified by 16S rDNA. While, *Pseudomonas putida* strain, isolated from that consortium, was capable of removing 90% of total BTEX (Morlett-Chávez *et al*., 2010). Moreover, bacteria from the genus *Rhodococcus* have the ability to grow under a wide variety of xenobiotic compounds including aliphatic and aromatic hydrocarbons (Kim *et al*., 2004). Other microorganisms that can remove aromatics are *P. putida*-DOT T1E and *Pseudomonas mendocina*-KR1

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that utilize toluene (Ramos-Gonzales *et al*., 2003); *Pseudomonas fluorescens*-CA-4 utilizes ethylbenzene (Díaz *et al*., 2001); and *P. putida* F1 utilizes benzene, toluene and ethylbenzene (Zylstra and Gibson, 1989). The ability to remove BTEX by microorganisms is attributed to the expression of proteins capable of oxidizing and cleaving aromatic-rings; these enzymes are known as dioxygenases (Tarasev *et al*., 2007).

Several studies based on proteins or mRNAs have been employed to elucidate the pathway followed by microorganisms during aromatic compounds biodegradation. The knowledge of genomic and biochemical basis of proteins related with biodegradation could improve degradation efficiency and use them as substrate hydrocarbons broad range (Sabirova *et al*., 2006). The principal main of this study was use semi-quantitative proteomic analysis to investigate the catabolic potential of the new bacterial *P. putida* strain, isolated from an enriched consortium of microorganisms acclimatized to unleaded gasoline and capable of removing BTEX, in the presence and absence of these aromatic compounds. In order to confirm our results, the *bed* and *tod* genes from the new bacterium were amplified by PCR, their mRNAs were quantified by RT-PCR, and their nucleotide sequences were obtained from the amplified products.

Experimental

Material and Methods

Reagents. BTEX isomers (o-,m-,p-) were purchased from Sigma-Aldrich Química (Monterrey, NL, México). Bacteriological agar (BIOXON, Becton-Dickinson, Monterrey, NL, Mexico), mineral medium reagents and other chemicals used in this study were reagent grade or better and purchased from either Sigma-Aldrich Química (Monterrey, NL, México) or CTR Scientific (Monterrey, NL, México).

Culture enrichment. The consortium used in this study was obtained from an acclimatized biomass and kept according to Morlett-Chávez *et al.* (2010). The *P. putida* strain was isolated from this consortium, was fed weekly with 50 mg l^{-1} of BTEX, and maintained in the conditions mentioned above.

Bioassays with BTEX. Bioassays with BTEX at 50 mg l^{-1} were prepared as suggested Acuna-Askar *et al.* (2006) and Morlett-Chávez *et al.* (2010). As final concentration of cell suspension 3×10^{10} cell/ml. were used All bioassays were incubated at 250 rpm at 36 ± 2°C and substrate concentrations were monitored at 0, 8, 16, 24 and 32 h. Three replicates were run for each set of bioassays to evaluate substrate biodegradation kinetics.

Bioassays with separate BTEX substrates. Bioassays to test the degradability of individual BTEX chemicals were run as mention above (Acuna-Askar *et al*., 2006; Morlett-Chávez *et al*., 2010).

Chemical analysis. BTEX concentrations were analyzed by a Varian 3400 GC/FID chromatograph. A Petrocol™ (Supelco, Bellefonte, PA) 100 m × 0.25 mm ID × 0.5 µm film DH fused silica GC capillary column was used. Column, injector and detector conditions were held as reported in a prior study (Acuna-Askar *et al*., 2006). Results were analyzed and statistically evaluated using the computer software SigmaPlot® 10.0 U (Cincinatti, OH, USA)(Standard Methods 1998).

Differential proteomic analysis

Sample preparation and SDS-PAGE. Consortium and *P. putida* strain biomass were recovered from culture by centrifugation (5000 rpm 10 min^{-1}) at 0, 8, 16, 24, 32 h, during BTEX biodegradation. A quantity of 100 mg (wet weight) of this biomass and 1 ml of buffer were added for protein extraction (B-PER- Bacterial Protein Extraction Reagent) per the manufacturer's instructions (Pierce, Rockford IL, USA). The samples were kept at 4°C and a protease inhibitor cocktail was added. Afterwards, cell lysis was performed by sonication (Sonicor-ultrasonic processor Up 400 a, Copiague N.Y. USA) with six cycles (75 W × 30 seg) and intervals of 30 seconds each. The sonicated residue was centrifuged at 14,000 rpm × 30 min at 4°C to remove cellular debris, the rest of protocol was run as described by Sabirova *et al.* (2010).

Protein identification. Protein identification was run as previously described by Morlett-Chávez *et al.* (2010) and Dalvi *et al.* (2014).

Genetic studies. To confirm our proteomic results and unambiguously identify the enzymes associated with biodegradation, the following genetic studies were performed for the *bed* and *tod* genes analyzed.

Amplification of the catabolic *bed* and *tod* genes. Three vials that contained 1 ml of the consortium, *P. putida* strain, and *P. putida* F1, respectively, were centrifuged at 3,000 rpm × 5 min to obtain a pellet, and the supernatant was discarded. Genomic DNA was extracted from each pellet with the DNAeasy kit (Qiagen, GMBH, Hilden, Germany). Also, *E. coli* DNA was extracted to use it as a negative control for the amplification reaction. Using the extracted DNA, *bed* and *tod* genes were amplified by PCR, employing primers described in Table I, and PCR was carried out with an initial denaturing step for 5 min at 94°C followed by 35 cycles at 94°C for 45 s, *bed* 54°C and *tod* 60°C both for 45 s and 72°C elongation for 45 s, followed by a final elongation at 72°C for 5 min. The amplicons were revolved in 1% agarose gels, stained with ethidium bromide (0.5 g ml^{-1}) and detected in

Table I
Primers for amplifying bed and tod genes.

	Name	Code	Sequence
Primers	Bed	Fwdbzm	5' GAAGGGGACGTAGAATCATG
		Rvbzm	3' GCTAACGATTGCGTCTTGA
	Tod	Fwdtolm	5' TGAAAAGTGAGAAGACAATG
		Rvtolm	3' GATTCAGAGTGTCGCCTTCA

a photo-documenter (UVP Inc, Upland, CA, USA). The amplicons were purified using the Wizard SV Genomic DNA Purification System kit (Promega, Madison WI, USA). Sequences and bioinformatics analysis were ran in accordance with Morlett-Chávez *et al*. (2010).

Reverse Transcriptase/Polymerase Chain Reaction. Total RNA was extracted from the consortium, *P. putida* strain and *P. putida* F1, grown in the presence of BTEX as a mixture and from its individual components. The RNA from microorganisms was isolated during the exponential phase ($DO_{600nm} = 1.5$) using trizol and following the manufacturer's protocol (Invitrogen, Carlsbard California, USA). Finally, this was treated with DNAse for 30 min at 37°C (Invitrogen) to eliminate traces of genomic DNA. In addition, total RNA was extracted from *E. coli* (grown in the presence of glucose) to use it as negative control in gene expression analyses. One microgram of total RNA was used as a template for reverse-transcription into complementary DNA (cDNA) using the Reverse AidH Minus First Strand cDNA synthesis kit (Fermentas, ST. Leon-Rot, Germany), and *bed*-reverse and *tod*-reverse oligonucleotides as primers. The program was of 94°C × 5 min; 94°C × 45 sec, 60°C × 45 sec and 72°C × 45 sec; the final extension was 72°C × 10 min. The cDNAs from the consortium, *P. putida* strain and *P. putida* F1 were used as templates for studying *bed* and *tod* gene expression. The genomic and cDNA from *P. putida* F1 were amplified as a positive control, while total RNA was used as a negative control. Also, 16S RNA was retro-transcribed and used as an internal control for verification of cDNA synthesis and amplification.

Results

BTEX biodegradation kinetics. The enriched consortium showed better kinetics to remove these compounds, followed by *P. putida* strain and the reference strain (*P. putida* F1). Our results showed that *P. putida* strain had removed 50% of the compounds in the first 16 h and 85% at 32 h; however, the consortium had already removed more than 95% in 32 h and ~75% at 16 h, while the reference strain removed ~80% in 32 h and less than 25% at 16 h. An important detail to consider is that *P. putida* strain had a slow lag phase (8 h) in comparison to the consortium (5 h) (Fig. 1).

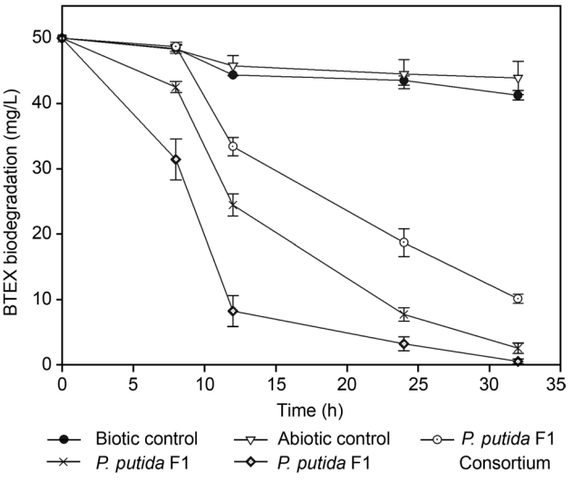

Fig. 1. Removal kinetics of BTEX-mixture (50 mg l^{-1}) by consortium and *P. putida* strain.

Bioassays were set with a BTEX mixture having 50 mg l^{-1} of each chemical. All bioassays were shaken at 45 × g, at a temperature of 36 ± 2°C and monitored for substrate concentrations at 0, 8, 16, 24 and 32 h. Results indicate the *P. putida* strain exposed to ethylbenzene show mayor degradation (97.7%), followed by benzene (94.8%), toluene (94.8%) and xylene (87.8%).

Kinetic results obtained from biodegradation experiments with *P. putida* strain and the consortium exposed to the BTEX or to its individual chemical components are shown in Fig. 2. These results indicated that the *P. putida* strain primarily biodegraded ethylbenzene (97.7%), followed by benzene (94.8%), toluene (90.8%) and xylenes (87.8%).

Differential proteomic analysis

SDS-PAGE. Comparison of SDS-PAGE semi-quantitative results allowed us to identify putative peptide bands that may possibly be related to the aromatic compounds biodegradation. The proteins were extracted from *P. putida* strain and the consortium exposed to BTEX at multiple time points. As expected, the protein banding pattern and the protein abundance within particular bands changed during the time course of BTEX biodegradation. In Fig. 3a, four conspicuous bands with different molecular weights were observed: band 1 (~ 60 kDa), band 2 (~ 48 kDa), band 3 (~ 22 kDa), and band 4 (~ 19 kDa). These bands were activated at the 8th hour. At the 16th and 24th hours, several proteins showed differences in the molecular weight with respect to the proteins mentioned above: band 5 (~ 45 kDa), band 6 (~ 35 kDa), and band 7 (~ 23 kDa). SDS-PAGDE also showed that the consortium and *P. putida* strain expressed different proteins when exposed to glucose.

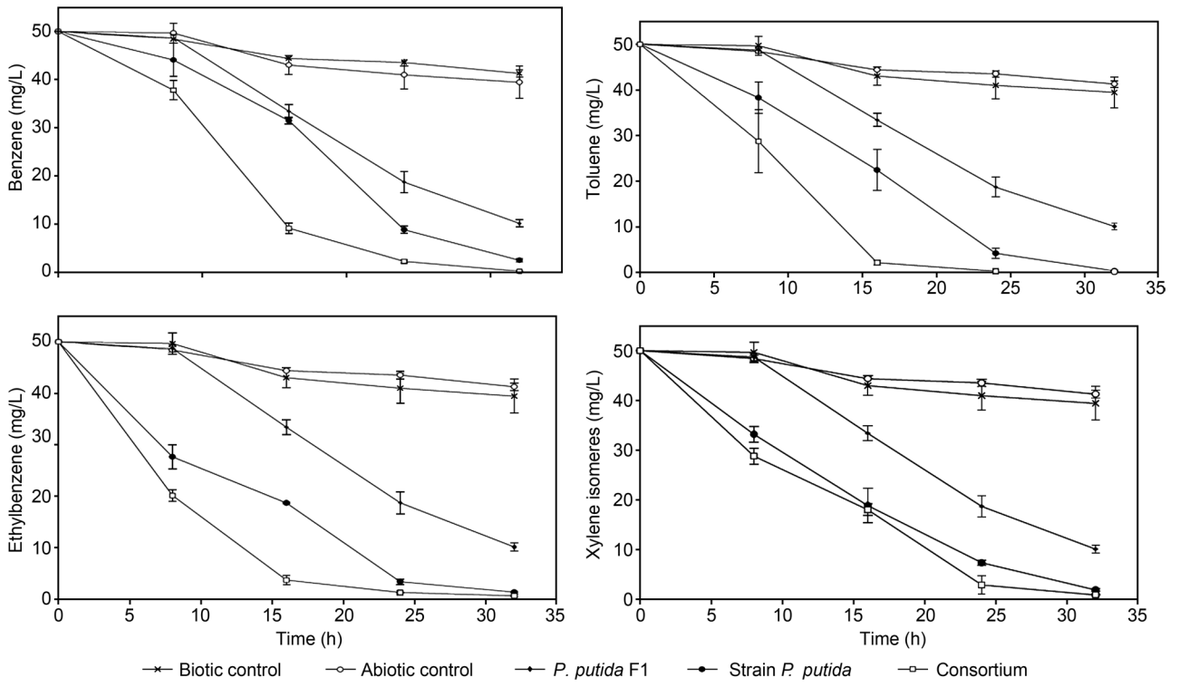

Fig. 2. Kinetics of BTEX-separate biodegradation by consortium and *P. putida* strain.

One set for each individual BTEX chemical at the initial concentration of 50 mg l^{-1} was run with each of the following three cultures: a) consortium, b) *P. putida* strain and c) *P. putida* F1. Similarly, all bioassays were shaken at 45 × g, at a temperature of 36 ± 2°C and monitored for substrate concentrations at 0, 8, 16, 24 and 32 h. Three replicates were also run for each set of samples.

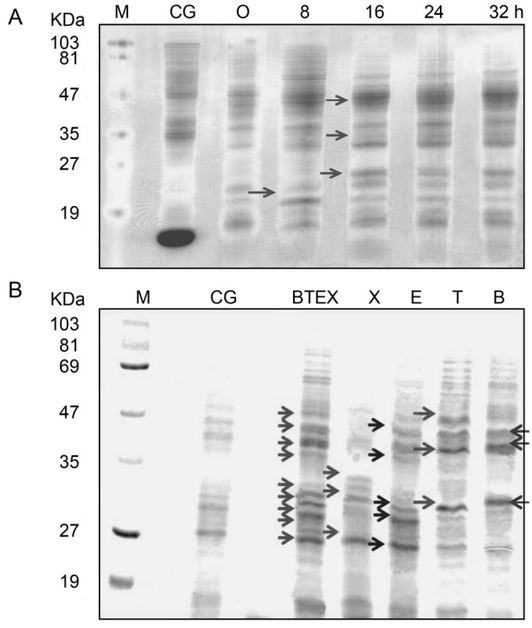

Fig. 3. Protein profile of *P. putida* strain exposed to the BTEX-mixture.

Protein bands for *P. putida* strain bioassays with and without BTEX (using only glucose as source of carbon) were resolved by SDS-PAGE and stained with Coomassie bright blue. A) Cells were collected at indicated different times of cultivation and proteins were recovered and resolved by SDS-PAGE. B) Protein expression profile of *P. putida* strain exposed to BTEX-mixture and -separate. Samples were collected at 24 h. As a control, a *P. putida* strain fed with glucose (CG).

In another experiment, the protein profile of *P. putida* strain and the consortium when exposed to the BTEX components individually were compared. Figure 3b shows proteins differentially expressed in the presence of the individual BTEX chemical components: the more abundant bands were observed in the cells exposed to benzene: 1) ~ 43 kDa, 2) ~ 40 kDa, and 3) ~ 30 kDa; in toluene: 1) ~ 48 kDa, 2) ~ 38 kDa, and 3) ~ 29 kDa; in ethylbenzene: 1) ~ 43 kDa, 2) ~ 29 kDa, 3) ~ 28 kDa, and 4) ~ 27 kDa; and in xylene: 1) ~ 33 kDa, ~ 31 kDa, and ~ 28 kDa. Similar results were observed in the analyses of proteins from the consortium (Fig. 4a and 4b).

Capillary LC/MS/MS with protein database searching. Various peptides revealed by time and carbon source *via* SDS-PAGE (Fig. 3a and 3b), were identified by capillary LC/MS/MS and protein database searching. These include: cytochrome C (35,526 Da); transcriptional regulator Lys R (33,602 Da); hydrogenase Fe-S (19,306 kDa); ferrodoxine, union dominion Fe-S (12,458 Da); NADH dehydrogenase (18,308 Da); 2,3 catecol-dioxygenase (32,189 Da); 4- oxalocrotonate decarboxylase; polihydroxyalconato depolymerase; tioredoxina-disulphure reductase (38,518 Da); formate dehydrogenase (21,699 Da); and *tod*F hydratase (23,902 Da) (Table II). All of these proteins are involved in the metabolism of aromatic/aliphatic compounds.

Genetic studies. The dioxygenases are enzymes involved in oxidative hydroxylation, the initial step of

Table II
PCR conditions to amplify bed and tod genes and PCR cycles.

PCR conditions		PCR Cycles			
DNA (100 ng)	1 µl		Time	Temperature	Cycle
Buffer 10 × PCR	2 µl	Initial denatured	5 min	94°C	1 X
MgCl2	2 µl	Denatured	45 sec	94°C	35 X
dNTPs (10 mM)	0.5 µl	Annealing	45 sec	*Bed* 54°C	
Primers 5' (10 mM)	0.4 µl	Extension	45 sec	72°C	
Primers 3' (10 mM)	0.4 µl	Extension	10 min	72°C	1 X
Taq DNA polymerase	0.2 µl				
Sterile Water	14.5 µl				
Total Volume	20 µl				

aromatic compound biodegradation. Proteomic analysis revealed novel proteins, in addition to dioxygenases, that are also involved in BTEX removal. To confirm that the dioxygenase genes are strongly expressed in response to BTEX, we amplified the α-subunit of the *bed* and *tod* genes involved in the catabolism of benzene and toluene, respectively, and evaluated their expression by RT-PCR.

Amplification of *bed* and *tod* genes. Genomic DNA was extracted from: a) consortium, b) *P. putida* strain, c) *P. putida* F1, and d) *E. coli* DH5α, and used them as templates for PCR, *bed* and *tod* genes were amplified. Results show an amplification of 1400 pb corresponding to the *bed* gene, and 1353 pb for *tod*. In both cases the genes were amplified from the cultures mention above, except for the one corresponding to *E. coli*. The amplicons were directly extracted from the agarose gel, purified and sequenced. Nucleotide sequences obtained were compared with the sequences reported in GenBank (genes *tod* and *bed* from *P. putida* F1). The *bed* gen of *P. putida* strain resulted similar in 98% while the *bed* gen of *P. putida* F1 was similar in 99%, with regard to the *bed* gen from *P. putida* F1-GenBank. Interestingly, the *tod* gene of *P. putida* strain was similar in 90%, while the *tod* gene of *P. putida* F1 showed 99% similarity, with respect to the *tod* gen from *P. putida* F1-GenBank (Fig. 5a). The virtual translation of the *tod* gen of the *P. putida* strain suggests that it encodes a protein with several amino acids changes including amino acids in the enzyme's catalytic site. Also, by comparing the amino acid sequence of Tod, the *P. putida* strain protein is 95% similar to the one of *P. putida* F1 Tod in the GenBank (Fig. 5b).

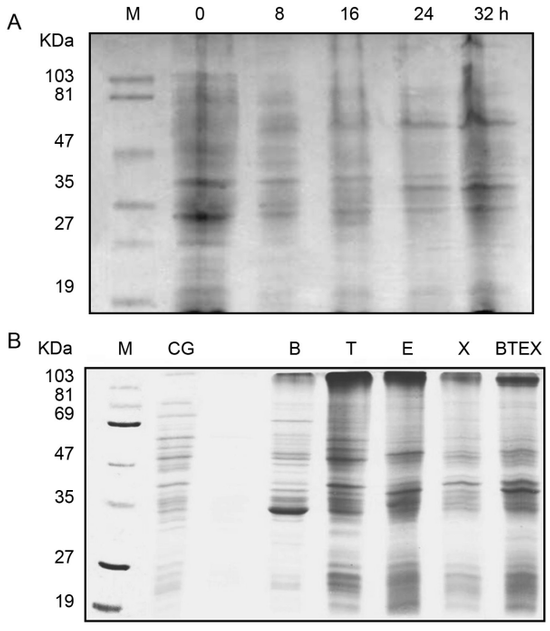

Fig. 4. Consortium proteins expressed in the presence of BTEX. Protein profile for consortium bioassays with and without BTEX (using only glucose as source of carbon) were resolved by SDS-PAGE and stained with Coomassie bright blue. A) Protein expression profile of the consortium exposed to BTEX and the cells were collected at different points of cultivation. B) Protein expression profile of the consortium using BTEX-separate and -mixture as the only source of carbon. Samples were collected at 24 h and 1.5 D.O. ($D.O._{600\ nm}$). The control was a consortium growth in glucose (CG).

RT-PCR of *tod* gene. The dioxygenase enzymes catalyze the first reaction during BTEX degradation; these proteins were strongly observed by proteomic analysis. In this context, the expression of the genes *bed* and *tod* was analyzed in the consortium and the *P. putida* strain grown in the presence of BTEX and with its individual components. To achieve this, cells were collected during the exponential phase and their total RNA was isolated. By RT-PCR *bedα* and *todα* cDNAs were amplified. Fig. 6 shows the results for *todα*, which indicates that its gene was expressed in *P. putida* strain exposed to BTEX, toluene, ethylbenzene and xylene, but not in the presence of benzene. Similar results were observed for the consortium. Table III summarizes these results. Interestingly, in *P. putida* F1, *todα* gene expression was induced only in the presence of BTEX

```
Query  1        TCAGCGTGTCGCCTTCAGCGCGTCCCAGTCGGGGGATGTCATCATCCGCAGCCAATGGGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271737  TCAGCGTGTCGCCTTCAGCGCGTCCCAGTCGGGGGATGTCATCATCCGCAGCCAATGGGC

Query  61       ATAGAGCCCGCGGGCAGCTTCCTCGCTGTAGACGTTGTTGCTGATCCGCCCGGGGTAAAC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271797  ATAGAGCCCGCGGGCAGCTTCCTCGCTGTAGACGTTGTTGCTGATCCGCCCGGGGTAAAC

Query  121      CGGGTCGTTGTCGACGGTCTGGTCCATGCTCATCTCGGCATTGAAAGGGCGGCTCCGCGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271857  CGGGTCGTTGTCGACGGTCTGGTCCATGCTCATCTCGGCATTGAAAGGGCGGCTCCGCGC

Query  181      CTTGTGGCCTCGCAGGATGTGCTGGATCTCGACCCAGTTCTCCCCGTCGTCCTGCTCGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271917  CTTGTGGCCTCGCAGGATGTGCTGGATCTCGACCCAGTTCTCCCCGTCGTCCTGCTCGAA

Query  241      CACGCCACCGGCAGAGAAGGTGCGCAGCGTCTGGCGCCGGAACTCTTCCTTGATATCGTC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271977  CACGCCACCGGCAGAGAAGGTGCGCAGCGTCTGGCGCCGGAACTCTTCCTTGATATCGTC

Query  301      AGGAGCATCAGCATCGACCACCGTAAACGCCCATACCTCGACCTCGTTCGGCCCGCGCGG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272037  AGGAGCATCAGCATCGACCACCGTAAACGCCCATACCTCGACCTCGTTCGGCCCGCGCGG

Query  361      ATGCCATGTCCGGACCGTATTGATACCTGGGAGGAAGGAACACGTGGGGAAGACGGTCAT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272097  ATGCCATGTCCGGACCGTATTGATACCTGGGAGGAAGGAACACGTGGGGAAGACGGTCAT

Query  421      GTGCTCGACCATGAGTTTCGAGCCGCGCTCCACGCTACCCAGACGTTCGGCCGCCTTTTC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272157  GTGCTCGACCATGAGTTTCGAGCCGCGCTCCACGCTACCCAGACGTTCGGCCGCCTTTTC

Query  481      CGACGCGGGGCCTTCGGTCCAGTAGCTGGTGACCTTTGGCCCCATGATGGCAAGCATCAG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272217  CGACGCGGGGCCTTCGGTCCAGTAGCTGGTGACCTTTGGCCCCATGATGGCAAGCATCAG

Query  541      ATTGGGGTCGCCGACATAGAAGCCACTTCCATGTCCGCCCCATGACGCACGGTACTGCTT
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Sbjct  3272277  ATTGGGGTCGCCGACATAGAAGCCACTTCCATGTCCGCCCCATGACGCACGGTACTGCTT

Query  601      GCCAACTGTCGGCGGAGCAAGGTCGGCCATTTCAAGGTCTTCTGGCAGGCCTGCCAGGAT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3272337  GCCAACTGTCGGCGGAGCAAGGTCGGCCATTTCAAGGTCTTCTGGCAGGCCTGCCAGGAT
```

Fig. 5A. Nucleotide sequence and identity search.

The sequence obtained from the *tod* gen of *P. putida* Strain was used to search for matches in the GenBank.

```
tod          MNQTDTSPIRLRRSWNTSEIEALFDEHAGRIDPRIYTDEDLYQLELERVFARSWLLLGHE 60
FMB08        MNQTDTSPIRLRRSWNTSEIEPLFDEHAGRIDPRIYTDEDLYQLELERVFARSWLLLGHE 60
             ********************.**************************************

tod          TQIRKPGDYITTYMGEDPVVVVRQKDASIAVFLNQCRHRGMRICRADAGNAKAFTCSYHG 120
FMB08        TQIRKPGDYITTYMGEDPVVVVRQKDASIAVFLNQCRHRGMRICRADAGNAKAFTCSYHG 120
             ************************************************************

tod          WAYDTAGNLVNVPYEAESFACLNKKEWSPLKARVETYKGLIFANWDENAVDLDTYLGEAK 180
FMB08        WAYDTAGNLVNVPYEAESFACLNKKEWSPLKARVETYKGLIFANWDENAVDLDTYLGEAK 180
             ************************************************************

tod          FYMDHMLDRTEAGTEAIPGVQKWVIPCNWKFAAEQFCSDMYHAGTTSHLSGILAGLPEDL 240
FMB08        IYMDHMLDRTEPRTEAIPGVQKWVIPCNWKFAAEQFCSDIYHAGTTSHLSGILAGLPEDL 240
             :**********. ************************:********************

tod          EMADLAPPTVGKQYRASWGGHGSGFYVGDPNLMLAIMGPKVTSYWTEGPASEKAAERLGS 300
FMB08        EMADLAPPTVGKQYRASWGGHGSGFYVGDPNLRPAIMGPKVTSYWTEGPASEKAAERLGS 300
             ******************************  *************************

tod          VERGSKLMVEHMTVFPTCSFLPGINTVRTWHPRGPNEVEVWAFTVVDADAPDDIKEEFRR 360
FMB08        VERGSKLMVEHMTVFPTCSFLPGINTVRTWHPRGPNEIEVWAFTEVDADAPDDIKEELRS 360
             ************************************:****** ************:**

tod          QTLRTFSAGGVFEQDDGENWVEIQHILRGHKARSRPFNAEMSMDQTVDNDPVYPGRISNN 420
FMB08        QTLSTFSAGGVFEQDDGENWVEIQHILRGHKTRSRPFNAEMSMDQTVDNDPVYPGRISNN 420
             *** ***************************:****************************

tod          VYSEEAARGLYAHWLRMMTSPDWDALKATR 450
FMB08        VYSEEAARGLYAHWLRMMTSPDWDALKATR 450
             ******************************
```

Fig. 5B. Aminoacidic sequences of Tod-*P. putida* strain and Tod-*P. putida* F1.

The aminoacidic sequence deduced for Tod-*P. putida* strain was used to search for matches in the Swiss protein bank. Tod-*P. putida* F1 strain was found to differ only in eleven residues.

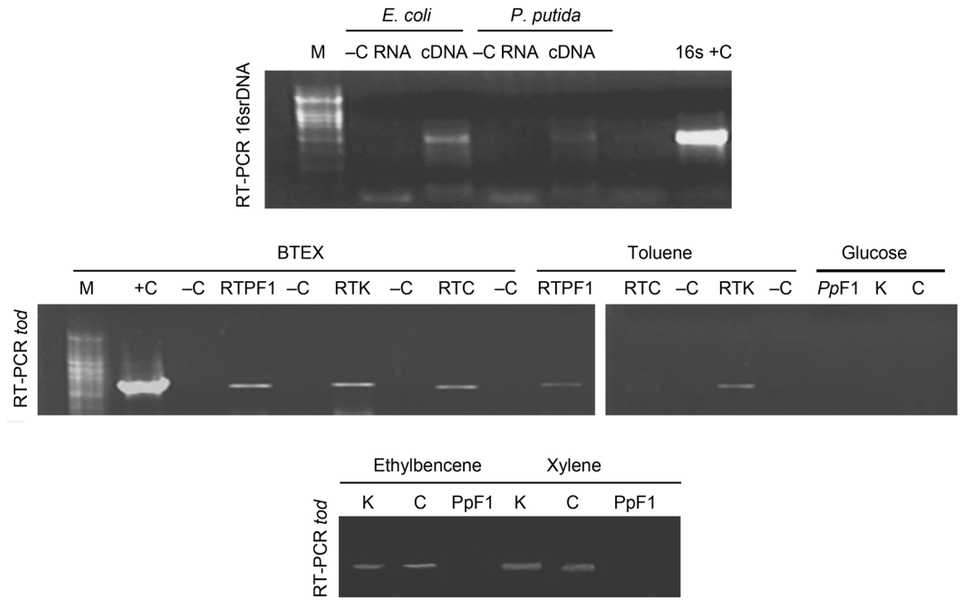

Fig. 6. Gene expression analyses of *P. putida* strain and consortium exposed to BTEX and their individual components. Total RNA was extracted from the consortium, the *P. putida* strain and *P. putida* F1 grown in the presence of BTEX as a mixture and of its individual components. Its retro-transcription yield cDNA was used as template for studying *bed* and *tod* genes expression. +C = positive control, –C = negative control (total RNA, K = consortium, C = *P. putida* strain and PpF1 = *P. putida* F1 (ATCC 700007).

or toluene. In the case of *bedα*, the gene was expressed in *P. putida* strain and the consortium exposed to BTEX and benzene (data not shown).

Table III
Conditions for cDNA formatted from total RNA of the strain FMB08, consortia, reference strain and *E. coli* DH5.

Conditions RT-PCR		
RNA (400 ng)	4 µl	65°C × 5 min
Primer 3' (200 ng µl-1)	1 µl	
dNTPs (10 mM)	1 µl	
Sterile Water	8 µl	
5X first Strand	4 µl	65°C × 2 min
DTT 0.1 M	2 µl	
M.MVLRT (200 U)	1 µl	37°C × 50 min

Inactivate 1 µl RNAase 37°C × 30 min

Discussion

Biodegradation kinetics of BTEX. In this study, we have established that the enriched consortium acclimatized to unleaded gasoline, and *P. putida* strain, are bio-degraders of aromatic compounds (50 mg l^{-1} BTEX). Results show that most of the oxidation occurs in the first 8 and 24 hours (Fig. 1). Our findings are in agreement with previous studies including our prior report (Morlett-Chávez *et al.*, 2010). *Pseudomonas* genus, have been found in BTEX contaminated sites and reported to use a different BTEX metabolic pathway (Lima-Morales *et al.*, 2016). Lee and Lee (2001) reported that *Ralstonia* sp. was capable of completely removing 3 mg l^{-1} of BTEX under aerobic conditions. These results can be explained because a specialized consortium is required to removal different aromatic compounds such as BTEX (Bell *et al.*, 2013). Furthermore, some organisms are capable of growth on the majority of the hydrocarbons, whereas others may be specialized to only a few of the substrates (Ciric *et al.*, 2010). In contrast, bacterium can rarely use multiple aromatic compounds (Gescher *et al.*, 2006). Bell *et al.* (2013); Gescher *et al.* (2006) indicated due to the high toxicity of organic contaminants, these are not completely mineralized by a single bacterium. Moreover, intermediates produced during biotransformation results more toxic than the initial compound (Lawniczak *et al.*, 2011). By last, similar results were reported by Morlett-Chávez *et al.* (2010), where the F distribution and the Tukey's statistical tests provide evidence that the consortium exhibited higher biodegradation efficiency than the FMB08 isolate.

Differential proteomic analysis

SDS-PAGE. Our strategy allowed us to distinguish several proteins up-regulated by BTEX. This strategy had also been used by other authors to identify proteins involved in aromatic compound metabolism (Demanèche *et al.*, 2004; Sabirova *et al.*, 2006; Peters

et al., 2007; Patrauchan *et al.*, 2008). Interestingly, Peters *et al.* (2007) found two protein bands of 57 and 60 kDa when *Geobacter metallireducen* was exposed to to *p*-cresol. In other work, Kim *et al.* (2004) identified α dioxygenase (~ 50 kDa) y β dioxygenase (~ 25 kDa) related to *o*-xylene degradation. Also, Maeda *et al.* (2001) reported a molecular weight of ~ 50 kDa for the enzyme biphenyl-dioxygenase α-subunit and ~ 23 kDa for subunit β and toluene dioxygenase α and β subunits at 52.5 and 21.5 kDa, respectively.

Table IV
Identified proteins of consortium and FMB08 strain exposed to mixture-BTEX.

Protein	Hydrocarbons	Found to be similar to:	Identification No.	MW (Da)	pI	Coverage %
Aromatic ring oxidation						
P1	BTEX	ABC-type oligopeptide transport system	giI84359023	59,033	8.7	40
P2	BTEX	Porine E	giI26986977	48,297	5.4	31
P3	BTEX, B	Outer membrane Protein F	giI85058985	40,061	4.5	55
P4	T	Thioredoxin-disulfide reductase	giI84516681	38,518	5.5	25
P5	T	Outer membrane protein II	giI148368	25,538	4.8	34
P6	BTEX	NADH dehydrogenase	giI113871846	18,308	8.7	97
P7	BTEX, T	OmpF porin	giI15131544	38,442	4.6	100
P8	BTEX	LrgA family protein	giI148653125	18,800	10	93
P9	B	TRAP-T family transporter	giI84385307	21,269		7
Aromatic ring cleavage						
P10	BTEX	Glyoxilase/dioxygenase	giI148548089	32,189	5.8	100
P11	BTEX	Ferredoxin, Fe-S union domain	giI149118333	12,458	4.4	99
P12	BTEX	Cytochrome C family protein	giI117921118	35, 62	8.6	100
P13	BTEX	Catechol dioxygenase	giI78063176	35,000		27
P14	BTEX, T	Formate dehydrogenase subunit B	giI34733215	21,699	6.4	99
P15	BTEX, T	Mot/TolQ/ExbB	giI119774393	19,762	8.9	95
P16	BTEX, T	Fe-S-cluster containing hydrogenase	giI77973841	19,306	8.4	100
P17	BTEX, T	TodF Hydratase	giI135977	23,902	4.7	15
P18	BTEX, X	Transcriptional regulator LysR	giI126990316	33,780	6.9	16
P19	BTEX, E	hypothetical protein ebD82	giI56477892	7,688	4.7	100
P20	BTEX	4Fe-4S cluster binding	giI26250216	17,594	8.1	96
Protein in the fatty acids catabolism						
P21	BTEX	Polyhydroxyalconate depolymerase	giI73538528	46, 97	7.9	100
P22	B	C4-dicarboxylate transport system	giI149187948	21,274	9.0	7
P23	BTEX	4 oxalocronate decarboxylase	giI148548088	28,222	4.9	5
Other proteins identified						
P24	BTEX	BB2842 hypothetical protein	giI33601818	11,724	5.6	100
P25	BTEX	Hypothetic protein OB2597	giI84503169	10,824	6.3	100
P26	BTEX	Hypothetic protein ED21	giI149186419	8,154	4.4	100
P27	BTEX	Hypothetic protein c2B002	giI56476484	9,822		100
P28	BTEX	Conserved hypothetic protein	giI121531209	8,857	6.7	100
P29	BTEX	Hypothetic protein azo 1735	giI119898026	12,147	4.5	100
P30	BTEX	Hypothetical protein Aave1976 protein	giI120610656	12, 662	5.7	100
P31	BTEX, E	Enlongation factor Tu	giI2886756	43,337		8
P32	B	Translation elongation factor of translation	giI96718	43,324	5.3	10
P33	BTEX	Hypothetic protein BammMC	giI118700962	28,692	11.5	3
P34	X	Tryptophan synthetase	giI464911	28,422	5.1	3
P35	BTEX, B, E	50s Ribosomal protein (L1)	giI26987185	24,236	9.5	12
P36	BTEX, B	Hypothetic protein RF_034	giI67458826	21,986	5.0	6
P37	BTEX	Electron transport complex protein Rnfb	giI59711541	20,523	4.5	100
P38	B	putative sulfate transport protein	giI145627930	10,698	6.5	100

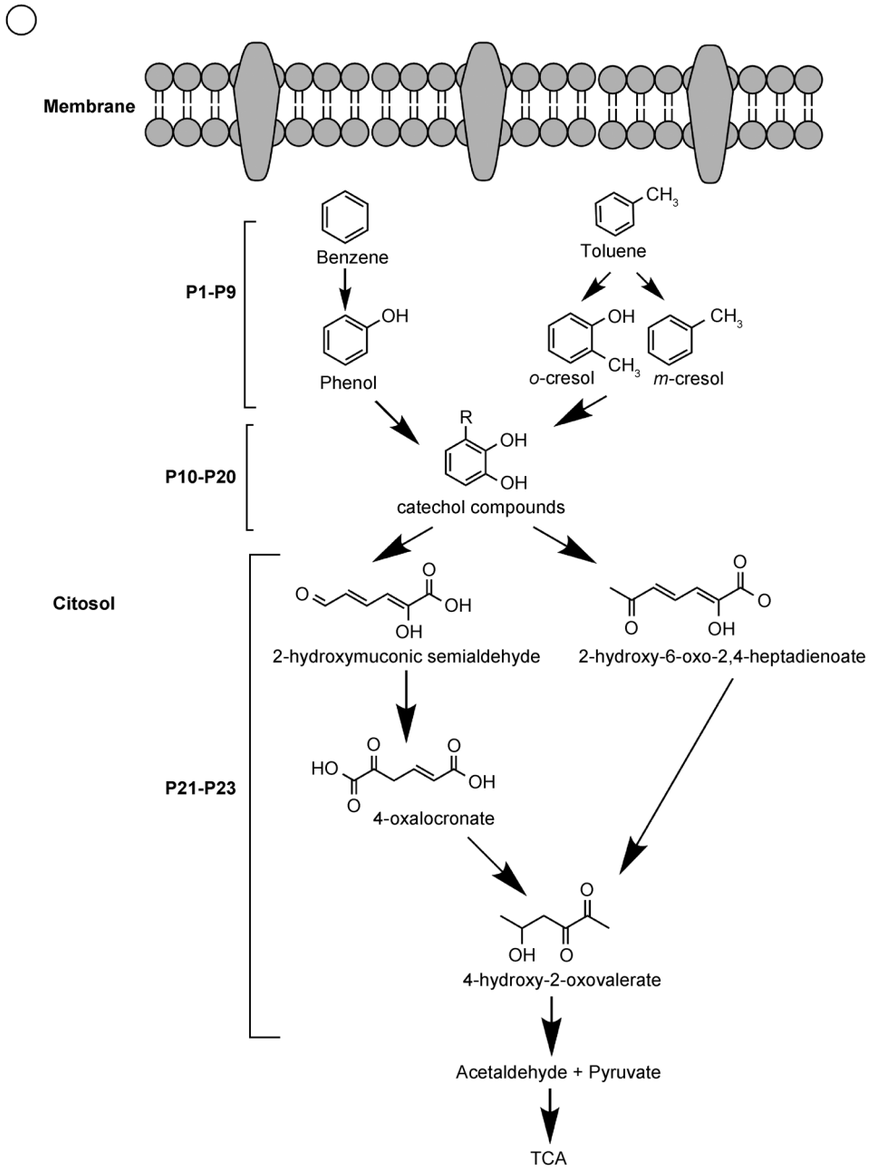

Fig. 7. BTEX catabolism in *P. putida* strain.

Capillary LC/MS/MS and protein database searching. All of the proteins identified by capillary LC/MS/MS and protein database searching are involved in the metabolism of hydrocarbon aromatic/aliphatic compounds. These can be classified by function and subcellular localization as membrane proteins, proteins involved in aromatic ring oxidation and cleavage, and proteins involved in fatty acid catabolism (Fig. 7).

Membrane proteins. The identified membrane proteins are thioredoxin-disulfide reductase (38,518 Da), and formate dehydrogenase (21.6 kDa). In *E. coli* this protein is a membrane protein that uses formate as an electron donor in reduction of nitrate to nitrites (Kane *et al*., 2007). Other membrane proteins identified in the study are: Lrga (18.8 kDa), transport systems ABC (59 kDa), membrane protein F (40 kDa), porin (36 kDa), permease (21 kDa) and membrane proteins II (25 kDa), all of these had been reported previously by Sabirova *et al.* (2006) and Peters *et al.* (2007).

Aromatic ring oxidation. Is well known that BTEX compounds, after penetrating the cell membrane of microorganisms, receive an initial hydroxylation by mono- or dioxygenases. These proteins are formed by a) flavoprotein reductase, b) ferredoxine, and c) the ISP presents α- and β-subunits with the former acting as the catalytic site (Bagnéris *et al*., 2005; Witzig *et al*., 2006, Szczepaniak *et al*., 2016). During initial hydroxylation catechol or protocatechuate is produced and

those products are identified as central intermediate products. On that subject, we identified a) hydrogenase Fe-S (19.3 kDa), b) Ferredoxin-dominion of union Fe-S (12.4 kDa), and c) NADH dehydrogenase (18.3 kDa) and monooxygenases (cytochrome P450). Similar results were reported by Dalvi *et al.* (2014); Patrauchan *et al.* (2008) and Sabirova *et al.* (2006). Prior, Fong *et al.* (1996) reported the presence of a protein (39 kDa) identified as a dehydrogenase NAD^+ dependent, necessary to convert benzene into catechol.

Aromatic ring cleavage. Following with the metabolic pathway of aromatic compounds, central intermediate products are the substrate for ring cleavage mediated by dioxygenases. We identified catechol 2,3-dioxygenase (32.1 kDa); and its transcriptional regulator known as Lys R (33.2 kDa). Lima-Morales *et al.* (2016) identified catechol 2,3 dioxygenases gene in *Pseudomonas* strains. Patrauchan *et al.* (2008) identified ethylbenzene dioxygenases, benzene dioxygenases and catechol dioxygenases enzymes. In addition, from the consortium we identified a protein similar to 4-oxalocrotonate decarboxylase (28.2 kDa) which degrades benzoate, toluene, and xylene. However, intermediates central can follow different routes where cleavage is ran by non oxygenolitic enzymes (Gescher *et al*., 2006).

Protein in the catabolism of fatty acids. The catechol dioxygenase enzyme cleaves the catechol-aromatic ring, allowing formation of ketoadipate enollactone, which is degraded by beta-oxidation pathway (Patrauchan *et al*., 2008; Dalvi *et al*., 2014). Herein, we identified a protein similar to polyhydroxyalkonate depolymerase (46.9 kDa) and 4-oxalocronate decarboxylase. Shöber *et al.* (2000) and Dalvi *et al.* (2014), respectively, indicate that these enzymes are responsible for fatty acid degradation to acetate, butyrate, and succinate. Finally, bacteria must produce precursors of metabolites like acetyl CoA to produce energy and biomass. We also identified an enzyme similar to glyoxylase (32.1 kDa) and linked to tricarboxylic acid cycle.

Genetic analyses. To confirm the key enzymes in BTEX catabolism that were revealed by differential proteomic analysis, we amplified the catabolic genes *bed* and *tod*. The analysis of the *bed* gene sequence indicates that is similar in a 98% to the reference *bed*-gene (GenBank gi|151068). When comparing the nucleotide sequence of *tod*, it showed a 90% similarity to the *tod* gene of *P. putida* F1 (GenBank gi|148512152). The alignment of both amino acid sequences showed 95% similarity. The 5% difference corresponds to amino acid substitutions (A22P, F181I, A192P, M219I, V337I, F357L, and A391T) in the catalytic site. This difference may be linked to the increase of catabolic potential of the *P. putida* strain in comparison with *P. putida* F1. Szczepaniak *et al*. (2016) indicated the alfa subunit is found in dioxygenase enzymes with similar catabolic activity but with different substrate specificity. Bagnéris *et al*. (2005) indicates that amino acid substitution (I301V, T305S, I307L, and L309V) of the *tod* catalytic region increases the preference for ethylbenzene. In addition, genetic studies indicate that the *tod* gene expressed in consortium and *P. putida* strain exposed to BTEX, T, E, and Xylene. In contrast, the *tod* gene is only expressed in the reference strain exposed to BTEX and toluene. Previous studies have reported similar results, Patrauchan *et al*. (2008) amplified the genes *Etb* and *Bph*, being the products of these genes ethylbenzene-benzene and biphenyl enzymes, respectively.

In conclusion, the enriched consortium acclimatized to unleaded gasoline is capable of removing 95% of the BTEX in this experiment, while *P. putida* strain alone removes up to 90%. Differential proteomic analyses allowed us to identify proteins that are up-regulated in the presence of BTEX. These proteins are homologous to other proteins related to BTEX biodegradation that were reported in previous studies. *bed* and *tod* genes were identified, which are up-regulated when exposed to BTEX. The *tod* gene of *P. putida* strain is 90% similar to the counterpart from the reference strain. The protein tod of *P. putida* strain is a 95% to the enzyme Tod in the reference strain. The *bed* gene only gets expressed in consortium and strain exposed to BTEX and benzene. The nucleotide sequence from *bed* gene has a 98% similarity with the one from reference strain. Analyzing the amino acid sequence of Bed, it was possible to prove that it is 100% identical to the Bed enzyme in the reference strain. These results are expected to provide directions for future studies on BTEX removal and other environmental applications.

Acknowledgments

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Literature

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ORIGINAL PAPER

The Diversity, Growth Promoting Abilities and Anti-microbial Activities of Bacteria Isolated from the Fruiting Body of *Agaricus bisporus*

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Abstract

Agaricus bisporus plays an important role in ecological processes and is one of the most widely cultivated mushrooms worldwide. Mushroom growth-promoting bacteria have been isolated from casing soil and compost, but microorganisms in the fruiting body have received only a little attention. To get an overview of phylogenetic diversity of microorganisms in the fruiting body of *A. bisporus*, as well as to screen antimicrobial and mushroom growth-promoting strains, and eventually intensify mushroom production, we isolated and characterized microorganisms from the fruiting body of *A. bisporus*. In total, 55 bacterial strains were isolated, among which nine isolates represented Actinomycetes. All the isolates were analyzed by 16S rRNA gene RFLP and sixteen representative strains by 16S rRNA gene sequencing. According to the phylogenetic analysis, eleven isolates represented the Gram-positive *Bacillus*, *Lysinibacillus*, *Paenibacillus*, *Pandorea* and *Streptomyces* genera, and five isolates belonged to the Gram-negative *Alcaligenes* and *Pseudomonas* genera. The bacteria isolated from the fruiting body of *A. bisporus* had broad-spectrum antimicrobial activities and potential mushroom growth-promoting abilities.

Key words: cellulase, IAA, isolation from fruiting body, mushroom growth promoting bacteria, phosphate-solubilizing

Introduction

Agaricus bisporus, commonly known as the white button mushroom (WBM), is one of the most widely cultivated mushrooms in the world. Although compost grown *A. bisporus* lacks the ability to degrade and consume highly substituted xylan fragments (Jurak *et al.*, 2015), it mainly occupies ecological niches rich in lignocellulose such as forest litter, and grasslands, and thus plays an important role in carbon recycling. *A. bisporus* is the model fungus for the adaptation, persistence and growth in the humic-rich leaf-litter environment (Fermor and Wood, 1981; Burton *et al.*, 1997). Apart from its ecological role, *A. bisporus* has been an important component of the human diet for over 200 years and constitutes the biggest part of the total mushrooms consumed in most western countries. *A. bisporus* was relatively rich in monounsaturated fatty acids and ergosterol (Stojković *et al.*, 2014) and could increase the activity of glutathione peroxidase (Maseko *et al.*, 2014), showing its possible role in nutrient metabolism (Jeong *et al.*, 2010). Because of its importance, the genome of this mushroom has been sequenced recently (Morin *et al.* 2012).

Numerous species of soil bacteria which inhabit soil close to plant roots have been reported to play important roles in nutrient mobilization and plant growth (Kumar *et al.*, 2012). These bacteria are known as plant growth-promoting rhizobacteria (PGPR). Indoleacetic acid production, cellulase production and phosphate-solubilization are three key characteristics of PGPR (Ahmad *et al.*, 2008). The PGPR have been studied in detail, yet relatively little is known about the mushroom growth-promoting bacteria (MGPB). Two *Pseudomonas putida* strains, which were isolated from casing layers of *A. bisporus*, were reported to be potent MGPB and possible inoculants to be applied in mushroom production to increase the yield (Zarenejad *et al.*, 2012). Chao *et al.* screened the mycelial surface of *Pleurotus ostreatus* and found that some fluorescent *Pseudomonas* spp. promoted the formation of primordia and enhanced the development of the basidiome of *P. ostreatus* (Cho *et al.*, 2003). These studies strongly suggest that specific bacteria isolated from mushroom substance may have potential applications in mushroom productions.

The transition from vegetative to reproductive growth stage of *A. bisporus* is stimulated by a number

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of factors, among which bacterial activity was implicated as a major contributor (Eger, 1972; Rainey, 1991). The bacteria inhabit on the casing soil may function *via* secretion of hormone-like compound, phosphate solubilizing and siderophores production (Chen *et al.*, 2013; Ali *et al.*, 2012), and it is possible that the bacteria in casing soil may colonize the hyphae and then enter the fruiting body, making these bacteria as potent MGPB. Most of the reported MGPB were isolated either from mycelium or casing soil, whereas bacteria isolated from the fruiting body were less studied. Although the bacterial community structure contained inside the fruiting body of *Morchella* was examined by using uncultured method like denaturing gradient gel electrophoresis (DGGE) technology (Shen *et al.*, 2008), no pure culture strain had been isolated from mushroom fruiting body. The aim of this study was to evaluate mushroom growth-promoting bacteria resources by isolating microorganisms from the fruiting body of *A. bisporus*, by assessing their potential mushroom growth-promotion and antimicrobial activities, and by analyzing the diversity of the isolates using amplified ribosomal DNA restriction analysis (ARDRA). The diversities and bioactivities of these isolates will be useful to exploit the mushroom growth-promoting bacteria resources.

Experimental

Materials and Methods

Sampling of *A. bisporus*. *A. bisporus* fruiting bodies were collected from five traditional *A. bisporus* production areas in Dayi, Dujiangyan, Chongzhou, Shuangliu and Jintang, SiChuan, China. The fruiting bodies were collected at the first flush. All samples were immediately stored in sterilized valve bags and transported to laboratory.

Isolation of microorganisms. The surface of the fruiting bodies was disinfected in 70% ethanol for 60 sec, followed by 1.0% NaOCl for 5 min, and 70% ethanol for 30 sec, thoroughly washed with sterile distilled water. The surface sterilized fruiting bodies were aseptically cut into 5-mm square and put on the beef extract-peptone medium plates, and incubated at 30°C (Hallmann *et al.*, 2006). Six explants were put on each plate. To confirm the success of sterilization process, aliquots of sterile distilled water from the final rinse were inoculated on beef extract-peptone media. Plates were examined for bacterial growth after 3 days, and then on regularly monitored. Picked colonies were spread on plates and reinoculated until pure cultures were obtained. Pure cultures were grown on freshly prepared beef extract-peptone medium slope medium and kept at 4°C.

Indoleacetic acid production assays. Colorimetric method was used for rapid estimation of Indoleacetic acid (IAA) production (Bric *et al.*, 1991). Fifty microliters of logarithmic-phase culture from beef extract-peptone liquid medium was transferred to 3 ml fresh medium containing 0.5 g l^{-1} tryptophan. The transferred cultures were grown at 140 rpm min^{-1} at 28°C for 36 h to reach logarithmic phase. The assay was done in triplicate for each strain. Fifty microliters culture suspension was absorbed into a white porcelain board and, after adding 100 µl of color reagent (4.5 g l^{-1} $FeCl_3$, 57.6% H_2SO_4), the board was incubated at 25°C for 30 min. Development of pink color indicated positive IAA production. A non-inoculated beef extract-peptone liquid medium with tryptophan was used as a negative control.

To quantify IAA production, 4 ml of color reagent was added to 2 ml of culture supernatant which obtained by centrifugation at 10 000 g min^{-1} for 5 min. The mixture was kept at 25°C for 30 min without light. IAA in each supernatant was measured by reading the optical density at 530 nm. The experiment was done in triplicate. IAA concentration in the supernatant was calculated using an IAA standard curve prepared from pure IAA (Sigma-Aldrich).

Phosphate solubilization assay. Fresh cultures of isolates were inoculated on the standard agar medium (pH 6.8–7.0) containing 0.5% tricalcium phosphate (TCP) as the insoluble P source for testing bacteria capable of releasing inorganic phosphate from TCP (Nautiyal, 1999). Sterile medium served as a control. The plates were incubated at 30°C for 7 days. A clear halo zone formed around colonies indicates phosphate solubilization. Experiments were done in triplicate. The ratio of halo zone to colony diameter (HD/CD value) was used as a measure of phosphate solubilization ability.

Extracellular cellulase assay. Cellulase assay was done as described previously (Teather and Wood, 1982). Five microliters of overnight grown culture was spot inoculated on minimal agar plates (0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.1% KCl, 0.05% yeast extract and 1.5% Bacto agar) with carboxymethylcellulose (CMC) sodium salt as the sole carbon source. The plates were incubated at 37°C for 2 days and flooded with an aqueous solution of Congo red (1 mg ml^{-1}) for 15 min. The Congo red solution was then poured off, and plates containing CMC were flooded with 1 M NaCl for 15 min. Finally, the agar was flooded with 1 M HCl, which changes the dye color to blue and inhibits further enzyme activity. Experiments were done in triplicate. A clear halo zone formed around colonies indicated cellulase production. The ratio of halo zoom to colony diameter (HD/CD value) was used as a measure of cellulase production ability.

Evaluation of antimicrobial activity. Six indicator organisms (*Escherichia coli* [ATCC 35218] and *Sta-*

phylococcus aureus [ATCC 25923], *Curvularia lunata* (Walker) Boedijn [SCAU3697], Rhizoctonia solani Ktihn [SCAU3111], *Fusarium oxysporum* f. sp. vasinfectum [SAUM2312] and *Alternaria solani Sorauer* [SCAU3247], obtained from the Maize research institute of Sichuan Agricultural University), were used to test antimicrobial activity as described previously (Taechowisan *et al*., 2003). Antimicrobial activity was detected by formation of an inhibition zone in 3 days.

ARDRA analysis of 16S rDNA. Genomic DNA was prepared using standard methods (Ausubel *et al*., 1995). The 16S rRNA gene was amplified by using universal forward primer P1 and the universal reverse primer P6. Primer P1 (5'-AGAGTTTGATCCTGGTCAGA ACGCT-3') corresponds to positions 8–37 and primer P6 (5'-TACGGCTACCTT GTTACGACTTCACCCC-3') corresponds to positions 1479–1506 in the *E. coli* 16S rRNA gene (Yanagi and Yamasato, 1993). The thermocycler program was 94°C for 5 min, followed by 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 35 cycles and with a final 10 min extension at 72°C. PCR products were separated by electrophoresis in a 1% agarose gel.

In the ARDRA analysis, the 1.5 kb PCR products (5 µl) were digested at 37°C for 4 h using restriction enzymes *Hha* I, *Hae* III, *Msp* I and *Taq* I. The restriction fragments were separated and visualized by electrophoresis in 2.5% (w/v) agarose gels. Fragments shorter than 80 bp were excluded from the ARDRA analysis because they were very close to the detection threshold. According to the combined ARDRA patterns, isolates were assigned to different rRNA gene types.

Sequencing and phylogenetic analysis. Based on the phenotypic results and 16S rRNA-ARDRA, representative isolates were chosen for 16S rDNA gene sequencing at Shengong Biotechnology Ltd. (Shanghai, China). Sequences from the isolates were compared with GenBank database using the BLASTN. The closest matching sequences were further pairwise aligned by CLUSTAL X (version 2.0) (Larkin *et al*., 2007). A phylogenetic tree was inferred using neighbor-joining method by MEGA6.0 (Tamura *et al*., 2013). The sequences were assigned GeneBank accession number KJ716488-KJ716500 and KM263532-KM263534

Results

Growth promoting potential of the isolates. In total, 55 culturable bacteria were isolated from *A. bisporus* fruiting bodies, among which 9 actinomycetes isolates were found (Table I).

To test whether the isolates have potential as mushroom growth promoting bacteria (MGPB), their abilities to produce IAA and cellulase and to solubilize phosphate were tested. Altogether 41 isolates showed at least one and nine isolates showed all of the three tested abilities (Table I). In total 36 (78.3%) isolates produced IAA at concentrations ranging from 5.34 µg $ml^{-1}$to 19.2 µg ml^{-1}, 19 (41.3%) isolates solubilizes phosphate with HD/CD values from 1.25 to 2.77, and 29 (63.0%) isolates produced cellulose with HD/CD values from 1.43 to 3.14.

Antimicrobial activity. In the antimicrobial activity assay, forty isolates showed activity against one or more pathogens (Table I). Altogether 25 (45.4%) and 23 (41.8%) isolates were active against *S. aureus* and *E. coli*, respectively. The *C. lunata* and *F. oxysporum* f. sp. vasinfectum were inhibited by 27 (49.1%) isolates. However, only 15 (27.3%) and 11 (20%) isolates inhibited the growth of R. solani Ktihn and *A. solani Sorauer*, respectively. The isolates CZ7, DY17, DYA32 and SLA49 showed significant antagonistic activity against the tested pathogen.

ARDRA and phylogenetic analysis. To estimate the diversity of microorganisms in the *A. bisporus* fruiting body, the 16S rRNA genes of the isolates were analyzed by ARDRA. Sixteen different ARDRA patterns were obtained (Table I). From each ARDRA pattern one representative isolate was chosen for16S rRNA gene sequencing. The 16S rRNA genes of the isolates possessed 99–100% similarity with species in the GenBank.

Eleven isolates represented five Gram-positive genera. Five isolates represented five different *Bacillus* species (Fig. 1). In addition to the *Bacillus* spp. isolates, isolates CZ7, DY21 and CZ12 were identified as Gram-positive strains displaying 99% similarity to the type strains of *L. macroides*, *P. barcinonensis* and *P. pnomenusa*, respectively. Three isolates represented *Streptomycetaceae*.

The five Gram-negative isolates were assigned to two genera (Fig. 1). CZ1, CZ8 and CZ13 belonged to the *Alcaligenaceae*. Isolates DJ35 and CZ9 were assigned as *P. fluorescens*.

A. bisporus is one of the most widely cultivated mushrooms in the world, yet it is still necessary to enhance its production to meet the growing demand. Even though PGPR are widely studied, few studies have systematically analyzed mushroom growth promoting bacteria (MGPB). The microorganisms in casing soil or compost have been found to promote the growth of mushrooms (Cho *et al*., 2003; Zarenejad *et al*., 2012). For example, the transition from vegetative to reproductive growth in *A. bisporus* is stimulated by application of a casing layer, in which some bacteria assist the basidiome initiation (Zarenejad *et al*., 2012). One of the mechanisms by which bacteria stimulate fruiting body formation is thought to remove self-inhibitory substances produced by the vegetative mushroom mycelium (Frey-Klett *et al*., 2011). However, whether the microorganisms inside the fruiting body of *A. bisporus* have potential to promote the growth of *A. bisporus* is still unknown.

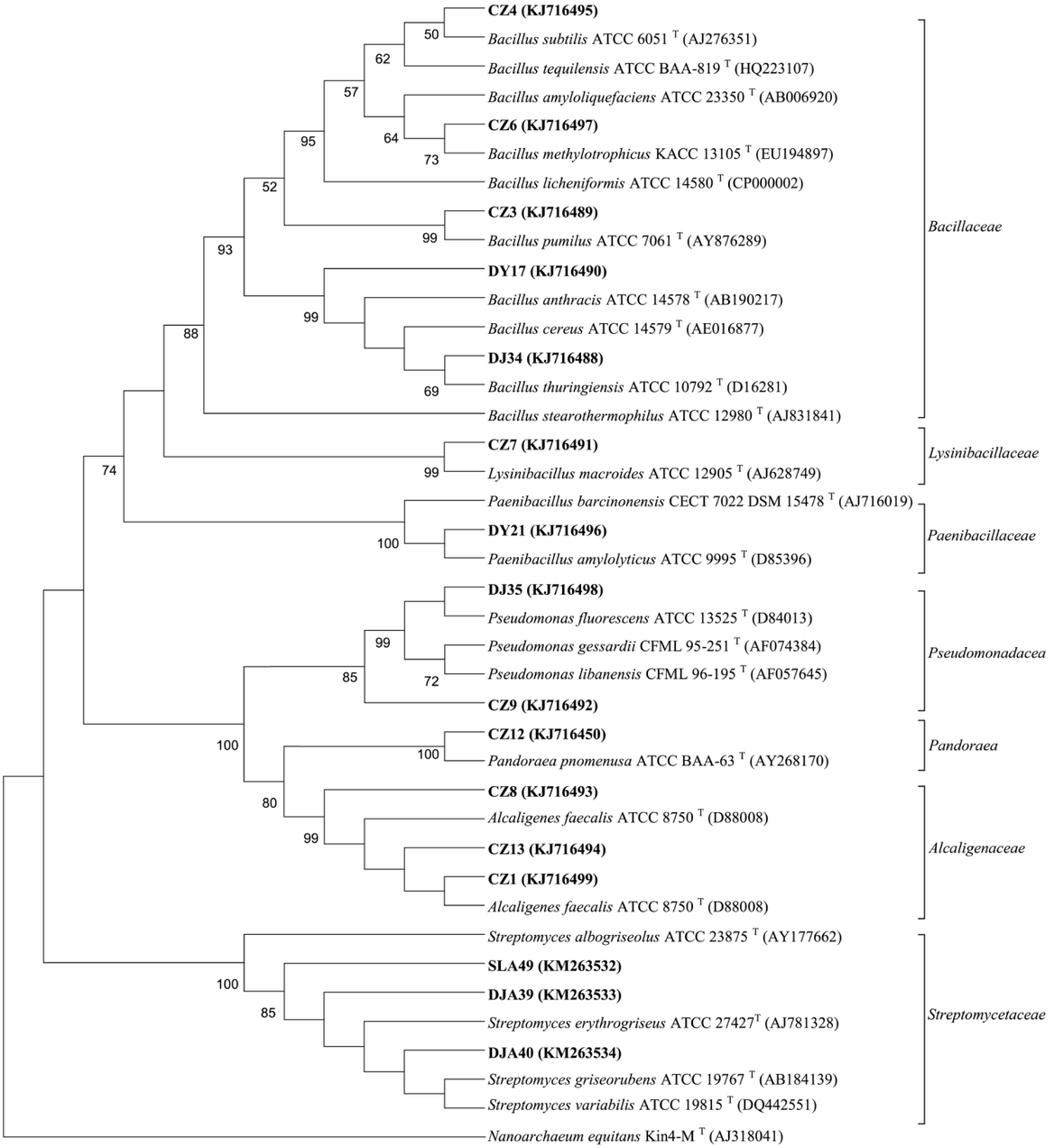

Fig. 1. Neighbor-joining tree based on 16S rRNA sequences of representative isolates (in bold) from the fruiting body of *Agaricus bisporus* and reference strains.

The scale bar corresponds to 0.05 substitutions per nucleotide position. The numbers at nodes indicate the levels of bootstrap support (%) based on 1,000 resampled data sets; GenBank accession numbers in parentheses. Superscript "T" means type stain; *Nanoarchaeum equitans* Kin4-M was used as an outgroup.

The abilities to produce indoleacetic acid (IAA) and cellulase and to solubilize phosphate are key characteristics of plant growth promoting rhizobacteria (PGPR) (Ahmad *et al*., 2008). In our study most of the isolates showed growth promoting abilities. Six, nine and four isolates showed relative strong IAA and cellulase production and phosphate solubilization abilities, respectively. The presence of *P. putida* or *P. fluorescens* in the casing layer is thought to be main contributors to mushroom production of *A. bisporus* (Eger, 1972; Fett *et al*., 1995). The isolate DJ35, assigned as *P. fluorescens*, produced both IAA and cellulase, suggesting it has potential to promote the growth of *A. bisporus*.

A. bisporus can decompose lignocellulosic material (Morin *et al*., 2012), and cellulose is an ideal carbon source for *A. bisporus*. Degradation of the cellulose and

Table I
Geographical origins, rRNA gene type, index of growth promotion factors and antimicrobial activities of endophytes of *A. bisporus*

Geographical origin	Isolate	GenBank accession number	rRNA gene type[a]	Growth promotion factors[b]			Antimicrobial activity[c]					
				PS HD/CD	IAA (μg/ml)	CP HD/CD	1	2	3	4	5	6
Chongzhou	CZ1	KJ716499	14	1.52	8.62 ± 0.22	1.61	–	–	2.11	–	1.55	0.00
	CZ2		2	1.39	5.85 ± 0.37	1.47	2.09	–	1.62	–	–	–
	CZ3	KJ716489	2	1.42	6.62 ± 0.09	–	1.25	1.58	2.36	2.25	1.46	–
	CZ4	KJ716495	9	1.54	8.23 ± 0.59	1.46	1.56	2.11	2.08	–	2.18	–
	CZ5		3	1.63	5.92 ± 0.28	2.00	2.17	1.42	–	–	–	–
	CZ6	KJ716497	4	1.56	7.62 ± 0.20	1.81	2.10	–	1.57	2.09	2.08	1.00
	CZ7	KJ716491	12	1.32	8.38 ± 0.29	3.14	3.22	3.09	1.67	1.75	2.15	0.81
	CZ8	KJ716493	8	–	12.00 ± 0.61	1.43	1.50	2.25	2.00	–	1.70	
	CZ9	KJ716492	13	–	7.85 ± 0.18	1.67	–	–	1.50	–	2.17	0.00
	CZ10		3	–	10.38 ± 0.81	2.67	–	1.27	1.50	–	2.10	–
	CZ11		2	1.49	16.92 ± 1.01	–	–	1.33	1.31	1.57	–	–
	CZ12	KJ716450	6	1.60	6.31 ± 0.37	–	2.18	2.11	–	–	–	–
	CZ13	KJ716494	3	–	7.77 ± 0.66	–	–	2.27	2.09	1.40	1.64	–
	CZ14		3	1.25	–	–	–	1.69	–	–	–	–
	CZ15		4	2.26	–	–	–	–	–	–	–	–
Dayi	DY16		5	1.52	7.46 ± 0.81	–	1.42	–	2.33	1.55	1.67	0.93
	DY17	KJ716490	5	–	8.23 ± 1.12	1.53	2.11	2.30	1.73	2.22	2.33	0.95
	DY18		4	1.63	8.54 ± 0.65	2.71	2.63	1.38	1.42	–	1.45	–
	DY19		4	2.02	9.54 ± 0.34	1.45	–	–	–	–	–	–
	DY20		3	–	6.08 ± 0.31	1.69	1.25	–	1.45	–	–	–
	DY21	KJ716496	10	–	–	2.11	1.18	–	3.18	–	–	–
	DY22		1	–	9.46 ± 0.40	2.00	–	–	–	–	–	–
Dayi	DY23		7	–	6.38 ± 0.52	1.38	–	–	–	–	–	–
	DY24		3	–	–	2.67	–	1.60	–	–	–	–
	DY25		1	–	7.62 ± 0.43	1.50	–	–	–	–	–	–
	DY26		2	–	6.92 ± 0.12	1.64	–	–	–	–	–	–
	DY27		3	–	9.08 ± 0.57	2.00	1.14	–	–	1.50	1.64	–
	DY28		5	1.34	5.54 ± 0.45	–	–	–	–	–	–	–
	DY29		6	–	5.84 ± 0.14	–	–	1.58	–	–	1.31	–
	DYA30		2	nd	nd	nd	2.20	–	1.55	1.45	3.22	–
	DYA31		1	nd	nd	nd	–	–	1.62	1.69	1.64	1.03
	DYA32		2	nd	nd	nd	3.44	3.56	1.83	1.92	2.20	0.87
Dujiangyan	DJ33		1	–	–	1.62	–	2.08	2.45	1.58	1.62	
	DJ34	KJ716488	7	–	5.34 ± 0.22	1.56	–		2.20	1.62	1.78	0.91
	DJ35	KJ716498	11	–	6.38 ± 0.42	2.00	–	2.09	3.44	–	–	–
	DJ36		1	–	–	–	–	1.67	–	–	–	–
	DJ37		3	–	8.23 ±0.32	–	–	–	–	–	–	–
	DJ38		2	–	–	–	–	–	1.54	–	1.38	–
	DJA39	KM263533	16	nd	nd	nd	3.22	3.11	–	–	1.42	–
Dujiangyan Shuangliu	DJA40	KM263534	1	nd	nd	nd	3.00	2.17	–	–	1.92	–
	DJA41		2	nd	nd	nd	3.11	2.30	–	–	1.90	–
	DJA42		5	nd	nd	nd	–	1.62	–	–	–	–
	SL43		1	2.02	12.92 ± 0.65	–	–	–	–	–	–	–
	SL44		2	2.05	7.92 ± 0.52	–	–	1.55	–	–	–	–
	SL45		6	–	19.23 ± 0.43	1.71	–	–	–	–	–	–
	SL46		1	–	–	1.42	1.62	–	–	–	1.62	–

Table I continued

Geographical origin	Isolate	GenBank accession number	rRNA gene type[a]	Growth promotion factors[b]			Antimicrobial activity[c]					
				PS HD/CD	IAA (μg/ml)	CP HD/CD	1	2	3	4	5	6
	SL47		1	–	5.92 ± 0.29	1.67	–	–	–	–	–	–
	SL48		1	–	5.46 ± 0.61	1.33	1.64	–	1.64	–	–	–
	SLA49	KM263532	15				2.25	2.38	1.69	2.33	3.30	0.71
Jintang	JT50		1	–	12.3 ± 0.27	1.50	–	–	–	–	–	–
	JT51		4	–	–	–	–	–	–	–	–	–
	JT52		2	2.77	14.38 ± 0.39	1.80	–	–	–	–	–	–
	JT53		1	1.33	13.62 ± 0.43	–	–	1.69	1.21	–	1.91	–
	JT54		2	–	–	–	–	–	–	–	–	–
	JTA55		1	nd	nd	nd	1.77	–	2.18	2.10	2.17	0.97

The isolated actinomycetes are underlined. The isolates with no accession number were identified by ARDRA only.

[a] rRNA gene types were defined based upon the restriction patterns of ARDRA digested with *Hha* I, *Hae* III, *Msp* I and *Taq* I.

[b] PS: phosphorus solubilization; IAA: Indole-3-acetic acid production; CP: cellulase production; nd: not determined, –,

[c] Indicator strains: 1. *E. coli*, 2. *S. aureus*, 3. *C. lunata* (Walker) Boedijn, 4. *R. solani* Ktihn, 5. *F. oxysporum* f. sp. vasinfectum, 6. *A. solani Sorauer*; (–) no inhibition

hemicellulose in the plant wastes increases the availability of carbon to the cultivated mushroom. Thus, the cellulase producing isolates may promote mushroom growth by degrading cellulose and therefore providing carbon to the host. *Paenibacillus*, which is a predominant endophytic bacterium in colonizing tissue cultures of woody plants (Ulrich *et al*., 2008), are known to produce hormones that stimulate plant growth. In our study, the isolate DY21, assigned as *P. amylolyticus*, showed strong cellulase production ability, which might be one of the mechanisms for *Paenibacillus* to promote growth.

The secondary metabolites produced by microorganisms may confer resistance to pathogenic invasion (Tan and Zou, 2001). The methanolic extract of *A. bisporus* showed relatively high antifungal activity (Stojković *et al*., 2014). In this study, most of isolates exhibited inhibitory activity against the tested pathogens, but the number of strains with antimicrobial activity from different regions were different. Fourteen of the fifteen isolates and half of the isolates from Chongzhou and Jintang, respectively, showed antagonistic activity. The regional difference may be caused by different environmental stress factors. Nine isolates belonging to *Lysinibacillaceae*, *Paenibacillaceae*, *Pseudomonadaceae* and *Streptomycetaceae* genera showed broad antimicrobial activity. Different *B. subtilis* strains produce different antimicrobial compounds (Ongena and Jacques, 2008; Hamdache *et al*., 2011). Our *Bacillus* spp. isolates showed antimicrobial activity against pathogenic bacteria and fungi. Especially, the *B. cereus*-like isolate DY17 inhibited all tested indicator pathogens, making it a good candidate for further studies. Numerous *Streptomyces* strains hosted in medicinal plants produce a wide variety of bioactive metabolites (Li *et al*., 2008). *Streptomyces* spp. are a particularly abundant source of antibiotics and related compounds (Liu *et al*., 2013), and in line with that all our *Streptomyces* isolates showed antimicrobial activity.

In this study, the culturable bacteria isolated from the fruiting body of *A. bisporus* were diverse, representing seven bacterial families. Five of the sixteen sequenced strains were *Bacillus* spp., making *Bacillus* the most dominant genus in *A. bisporus* fruiting body. *Bacillus* spp. and *Pseudomonas* spp. have been isolated from soil samples surrounding *Chroogomphus rutilus* (Wang *et al*., 2011). *Bacillus* spp. that have been isolated from plant rhizospheres and identified as endophytic microorganisms (Forchetti *et al*., 2007) affect the survival of mushroom and decomposition of wheat straw (Bis'ko *et al*., 1995; McDonald *et al*., 1998). *Pseudomonas* spp. are ubiquitous in agricultural soils, and some *Pseudomonas* spp. were found to promote the growth of *P. ostreatus* and *A. bisporus* (Fett *et al*., 1995; Cho *et al*., 2003). Fluorescent *Pseudomonas* spp. accounted for 14–41% of the total bacteria present in casing layer and the populations increased during *A. bisporus* cultivation with positive effects on the mushroom yield (Siyoum *et al*., 2010; Zarenejad *et al*., 2012).

The interaction between bacteria and fungi is complicated. The compost and casing soil layer are the two main possible sources of colonizing bacteria. The timing and means of the fruiting body colonization by bacteria are unknown. However, these microorganisms may coexist with each other. Further studies are needed to test the *in vitro* abilities of these isolates in promoting the growth of *A. bisporus*.

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ORIGINAL PAPER

Molecular Study of Indigenous Bacterial Community Composition on Exposure to Soil Arsenic Concentration Gradient

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Abstract

Community structure of bacteria present in arsenic contaminated agricultural soil was studied with qPCR (quantitative PCR) and DGGE (Denaturing Gradient Gel Electrophoresis) as an indicator of extreme stresses. Copy number of six common bacterial taxa (*Acidobacteria*, *Actinobacteria*, *α-*, *β-* and *γ-Proteobacteria*, *Firmicutes*) was calculated using group specific primers of 16S rDNA. It revealed that soil contaminated with low concentration of arsenic was dominated by both *Actinobacteria* and *Proteobacteria* but a shift towards *Proteobacteria* was observed with increasing arsenic concentration, and number of *Actinobacteria* eventually decreases. PCA (Principle Component Analysis) plot of bacterial community composition indicated a distinct resemblance among high arsenic content samples, while low arsenic content samples remained separated from others. Cluster analysis of soil parameters identifies three clusters, each of them was related to the arsenic content. Further, cluster analysis of 16S rDNA based DGGE fingerprint markedly distributed the soil bacterial populations into low (< 10 ppm) and high (> 10 ppm) arsenic content subgroups. Following analysis of diversity indices shows significant variation in bacterial community structure. MDS (Multi Dimensional Scaling) plot revealed distinction in the distribution of each sample denoting variation in bacterial diversity. Phylogenetic sequence analysis of fragments excised from DGGE gel revealed the presence of *γ-Proteobacteria* group across the study sites. Collectively, our experiments indicated that gradient of arsenic contamination affected the shape of the soil bacterial population by significant structural shift.

Key words: agricultural fields, arsenic (As), bacterial community, DGGE, copy number, qPCR

Introduction

Arsenic is one of the most common toxic metalloid, present in ground water as well as in soil (Xiong *et al.*, 2010; Majumder *et al.*, 2013), and has become a significant problem to environment (Banerjee *et al.*, 2011; Ghodsi *et al.*, 2011). Although arsenic intoxication of humans through drinking water is of the primary concern (Bachate *et al.*, 2009), soil is the secondary source and transfers arsenic to the edible part of the crops (Heikens *et al.*, 2007). Agricultural fields are frequently irrigated with arsenic contaminated ground water and hence a route of arsenic contamination into the food chain was established (Ghosh *et al.*, 2014; Shrivastava *et al.*, 2014). In India and Bangladesh, around 105.000 km^2 fertile deltaic plains are widely reported to have high levels of arsenic (Mukherjee and Bhattacharya, 2001). This region is largely used for the various types of agriculture, seasonal crops and irrigated with arsenic rich ground water creating major environmental threat (Bhattacharya *et al.*, 2002; Guha Mazumder 2003). The impact of environmental contamination on microbial community composition and diversity is being increasingly considered as highly sensitive ecological parameters to provide baseline information about contamination (Dhal *et al.*, 2011). Therefore it is really important to elucidate the diverse population of microorganisms associated with arsenic mobility and transport, as they can be the sensitive indicators for contaminant stress. A follow-up study identified and reported different soil microorganisms to be resistant to lethal concentration of arsenic and linked the progressive arsenic removal to bacterial activities, chiefly their arsenic (III) oxidizing activity (Aksornchu *et al.*, 2008; Mallick *et al.*, 2014). Alternatively, the toxic and bioavailable forms of the metals often affect adversely the diversity and function of indigenous microorganisms inhabiting the environment (Sobolev and Begonia, 2008; Dhal *et al.*, 2011). In order to understand the soil microbial ecology and the biological

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processes occurring *in situ*, multiple studies should be performed to determine the bacterial diversity in the soil and sediment, with high risk of arsenic contamination or already contaminated. The real status of microbial community cannot be reflected by traditional culture methods. Most of the soil microorganisms are uncultivable under the standard laboratory conditions as they are well adapted to their environment (Vartoukian *et al*., 2010). Several studies have revealed that more than 99% of microorganisms present in their innate environment are not readily cultivable and they represent only a minor fraction, usually less than 1% of the whole diversity present in complex environmental samples, such as soil (Sharma *et al*., 2005; Felczykowska *et al*., 2015). Nowadays, prevailing molecular tools endure investigators to frame microbial community function and structure without cultivation at increasingly finer resolution (Mccaig *et al*., 1999; Fakruddin and Mannan, 2013). Development of cultivation-independent techniques can circumvent such pitfalls and permit access to the genomes of entire communities that extend our knowledge about the diversity, ecology, evolution and functioning of the microbial world in considerably more detailed and accurate manner (Ranjard *et al*., 2000; Pogacic *et al*., 2010).

The study of soil microbial communities has strongly advanced with the introduction of sophisticated molecular techniques (Fakruddin and Mannan, 2013). In this study, we have assessed the effect of arsenic contamination on soil bacterial community structure using molecular techniques. qPCR was used to quantify the abundance of the major bacterial groups in the soil environment and to determine any changes that occur in the diversity of these bacterial assemblies at varying amount of arsenic contamination of the selected sampling sites. Furthermore, the effect of arsenic contamination on total bacterial genomic diversity was estimated through 16S rDNA based PCR-DGGE analysis where predominant DGGE bands were further identified by extraction of the band from a gel and sequencing. Although investigation of bacterial communities and its phylogenetic analysis using 16S rDNA signature sequence in arsenic contaminated aquifers were made previously (Bachate *et al*., 2009; Paul *et al*., 2015; Goswami *et al*., 2015), only a few studies using molecular approach have been carried out for arsenic contaminated soil in our study area. According to Hossain *et al*., 2005, arsenic toxicity in the agricultural fields of West Bengal is the major challenge and suitable remediation schemes are still unsuccessful. The study on bacterial community composition from arsenic contaminated soil may provide better understanding about potential biomarkers of this metal contamination as well as may implement suitable and effective potentials for bioremediation.

Experimental

Materials and Methods

Study site and soil sampling. The soil samples were collected from the five different agricultural fields of Gontra village (23° 1' N, 88° 34' E) in Chakdaha, Nadia, West Bengal, India by composite soil sampling and labeled as VS, PS, TS, JS and AS respectively. From each site, three subsamples were collected from 10–15 cm depth in a 2 m^2 area and transported to the laboratory in sterile polyethylene bags at 4°C. All the soil samples were analyzed separately for the physicochemical properties and average data collected from three subsamples were used to establish the physical and chemical characteristics of the particular site. For microbiological work, subsamples were mixed and used as representative sample after clearing root debris from the soil and stored at –20°C. Bacterial community analysis was performed within a week after sampling.

Physical and chemical analysis of soil samples. Soil samples were analysed for a variety of physical and chemical parameters. Total nitrogen and organic carbon content were measured by Macro-Kjeldahl method (Bremner, 1965) and Walkley-Black (Jackson, 1973) method, respectively. Soil pH was determined in suspension of soil in water (1 : 2.5) using pH meter. The same suspension was used to measure electrical conductivity (EC) using a direct reading conductivity meter. Cation exchange capacity was measured by extracting the soil with buffered $BaCl_2$ (Dewis and Freitas, 1984). Particle size distribution was determined by the International Pipette Method (Piper, 1966). Water holding capacity was analysed as described by Black (Black, 1965). Available phosphate (P_2O_5) was determined by the Bray and Kurtz method (Bray and Kurtz, 1945) and potash (K_2O) by using neutral molar ammonium acetate (Hanway and Heidel, 1952). Total arsenic content of the soil samples were determined by atomic absorption spectrophotometer (GBC Scientific Equipment Ltd., Model: GBC 932 B Plus) as mentioned elsewhere (Blas and Mateos, 1996).

Soil DNA extraction. Microbial genomic DNA was extracted from 0.5 g soil sample collected at each agricultural field using commercial kit and following the protocol described by the manufacturer (SoilMaster™ DNA Extraction Kit-Epicentre). DNA were dissolved in 50 µl TE buffer and stored at –20°C until processing. DNA quality was checked by electrophoresis in 0.8% horizontal agarose gel. Band pattern was observed using Gel Doc System (Bangalore Genei, Bangalore, India) after staining with 0.5 µg/ml ethidium bromide solution. Additionally, purity and yield of the extracted DNA were also analysed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). One µl of DNA

solution was taken to determine the quantity, protein contamination ($A_{260/280}$) and co-extraction of other organic acids, mainly humic acid ($A_{260/230}$).

Soil bacterial community analysis by group specific qPCR assay. An abundance of specific phyla/class of bacteria (*Acidobacteria*, *Actinobacteria*, *Proteobacteria- α*, *β*, *γ* and *Firmicutes*) in the selected soil samples were quantified by qPCR assay using taxon-specific primers for 16S rRNA gene as reported by Philippot *et al.* (2011). These PCR primers were also tested previously for the detailed assessments of complex bacterial communities (Fierer *et al.*, 2005; Muhling *et al.*, 2008). Here, we have used SYBR Green dye for quantification as it is described as reliable method for detecting nucleic acid targets (Olioso *et al.*, 2007). The extracted genomic DNA was used as a template in the assay to detect the copy number of taxon-specific 16S rRNA gene present in the soil samples. Individual qPCR reaction was carried out for each bacterial taxa in a real time PCR system (MyiQ2, BioRad). The qPCR reactions were performed in 96 well plate as described previously (Islam and Sar, 2011) with some modification. Each 20 µl reaction mixture contained 4 µl template DNA, 0.5 µl of each forward and reverse primer (concentration: 10 pmol/µl), 10 µl 2 × master mix containing SYBR Green (BioRad) and 5 µl nuclease free water. The reaction conditions were as follows: 5 min at 95°C; 40 cycles of 20 s at 95°C and 30 s at 60°C. The quantification was based on increasing fluorescence intensity of the SYBR Green dye during amplification. The qPCR assays were performed in triplicate with unknown samples, no template control (nuclease free water), and series of plasmid standard in 10 fold dilutions.

Known concentration of plasmid standard containing single copy of 16S rRNA gene (~ 1.5 kb size) of specific bacterial taxa cloned into pGEM-T vector was a base to construct each standard curve. The threshold cycle values (Ct values) were subsequently used to calculate the target copy numbers of 16S rRNA genes in each of plasmid standards using the standard equation (Lee *et al.*, 2008). A linear regression line was obtained by plotting the logarithm of copy numbers of plasmid standards (X-axis) against the corresponding threshold cycle values (Y-axis). The quality of the standard curve was derived from the slope and the correlation coefficient (r). Targeted 16S rRNA gene copy numbers of various bacterial groups of unknown samples were calculated from the respective standard curve. Correlation between arsenic concentration and the copy number of bacterial groups in soil was also performed.

Bio-geochemical data analysis. The relation among the samples with reference to their physicochemical characteristics was determined using between-group linkage (UPGMA) method of hierarchical clustering analysis with SPSS software (21.0 version). A 2D Principal Component Analysis (PCA) was also performed to correlate the samples with respect to their bacterial community compositions. In order to correlate bacterial community composition with the soil chemical properties, correlation coefficient analysis were applied.

Denaturing gradient gel electrophoresis (DGGE) profiling of bacterial communities. DGGE specific primers, 63F (5'-CAGGCCTAACACATGCAAGTC-3') with 40 bases GC clamp at 5'end and 518R (5'-ATTACCGCGGCTGCTGG-3') were selected to produce a 495 bp fragment for the PCR amplification of V2 and V3 regions of 16S rRNA gene (Fantroussi *et al.*, 1999; Breugelmans *et al.*, 2007). Prior to DGGE analysis touchdown PCR for each soil DNA extracts were performed in 20 µl final volume reaction containing 1 µl of extracted DNA, 2 µl of 10 × PCR buffer, 0.5 µl of dNTP mix (10 mM), 1 µl of each primer (10 pmol/µl), 0.1 µl Taq DNA polymerase (5 U/µl, KAPA Biosystems) and 14.4 µl nuclease-free water. Amplification was carried out in a thermal cycler (MyiQ2, BioRad) with reaction conditions as follows: 5 min at 94°C; 10 cycles of 94°C for 30 s, 60–55°C step down for 30 s, 72°C for 30 s; 20 cycles of 94°C for 20 s, 55°C for 30 s and 72°C for 30 s; final extension was at 72°C for 7 min and then 4°C storage (Schabereiter-Gurtner *et al.*, 2003; Yu and Morrison, 2004). The PCR products were checked by 1% agarose gel electrophoresis.

DGGE analysis of PCR amplicons was carried out essentially as described previously with slight modifications (Smalla *et al.*, 2001). The DGGE gel contained 0.5 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8) and 8% (w/v) acrylamide gel with a linear denaturant gradient from 40% to 60% made of urea and formamide (100% denaturant contains 7 M urea and 40% (v/v) formamide) using a DCode System (Bio-Rad, Munich, Germany). Equal amounts of amplified PCR products were loaded onto 1 mm thick vertical gels and electrophoresis was carried at a constant temperature of 60°C, with 150 V for 8 h. After completion of electrophoresis, gel was stained in an ethidium bromide solution (0.5 µg/ml) for 10 min followed by distaining in distilled water for 20 min. The gel band images were visualized using Chemi Doc System (Bio-Rad, Munich, Germany).

Sequencing, phylogenetic analysis and nucleotide accession number. The DNA fragments to be sequenced were excised from DGGE gel, placed in sterilized vials with 20 µl nuclease free water and kept overnight at 4°C. The eluted DNA was used as template for re-amplification using the primers 63F (without GC clamp) and 518R. PCR products were purified and cloned into pTZ57R/T vector (InsTAclone PCR Cloning Kit, Thermo Scientific) and the resulting plasmids were used as templates for sequencing reactions. At least one positive clone from each selected DGGE bands

was sequenced. The sequences were aligned using the CLUSTAL W program (Thompson *et al*., 1994) to the 16S rDNA sequences from the database of the National Center for Biotechnology Information using a BLAST search (Altschul *et al*., 1997) and deposited in NCBI Genbank database under accession numbers KJ136644, KJ136645, KJ403747, KJ403748, KJ403749, KJ403750 and KJ403751. The sequences from DGGE profiling were compared with known sequences listed in the GenBank nucleotide sequence database. The BLAST search of NCBI database was used to find the evolutionary relationship of the sequences. A phylogenetic tree was constructed using MEGA 5.1 by neighbor-joining method (Saitou and Nei, 1987).

Analysis of DGGE banding patterns. DGGE fingerprints were interpreted by cluster analysis, MDS and diversity indices estimation. DGGE image was first digitized and analyzed using Quantity One software (version 4.65, BioRad, USA) in order to compare the fingerprint patterns. Assuming each band in a lane as a single unique phylotype (operational taxonomic unit/OTU), the band number, band intensity and relative position of each band were determined to estimate the richness value (Huang *et al*., 2013). Identification of DGGE bands was based on the magnified image, absorption peak and similarity in each lane. Band analysis was performed using the rolling disk method and setting background subtraction at 15 (Alele *et al*., 2014). Bands with intensity < 0.05 were excluded from the analysis. Unweighted pair-group methods with arithmetic mean (UPGMA) trees were generated using Quantity One software with Sorensen's similarity index. Based on the presence or absence of individual bands in each lane, a binary matrix was constructed. The binary data representing the banding patterns were used to generate a pair wise Dice distance matrix. A dendogram was generated using UPGMA cluster analysis of Quantity One software. For constructing a MDS diagram, the distance matrix was used, where each DGGE fingerprint was placed as one point in respect with a two dimensional map with artificial X and Y axis. The diagram represented similar samples plotted together. The MDS analysis was performed using SPSS software (21.0 version). In order to correlate the DGGE fingerprinting analysis with multiple aspects, the Shannon-Weaver index of diversity (*H*) (Hedrick *et al*., 2000) and equitability index (*E*) (Smit *et al*., 2001) were calculated.

Results and Discussion

Soil characteristics of the study site. The long term and continuous use of arsenic contaminated ground water for irrigation purpose in agricultural fields is a threat to various life forms in the study site. The

Table I
Physical and chemical characteristics of the soil samples (Mean and SE are shown for each soil samples)

Serial Number	Soil type	Moisture content (g)	Particle size distribution			Moisture holding capacity %	Cation exchange capacity [cmol (p^+) kg^{-1}]	Amount of total arsenic in soil (ppm)	Soil pH	Carbon and organic matter content %	Electrical conductivity (d Sm^{-1}); (1:2.5)	Total nitrogen (kg ha^{-1})	Available P_2O_5 (kg ha^{-1})	Available K_2O (kg ha^{-1})
			Sand %	Silt %	Clay %									
VS	Clay loam	0.82±0.01	30.8±0.47	36.0±0.17	33.2±0.2	44.40±0.22	8.45±0.04	6.65±0.04	6.93±0.07	0.36±0.007	0.22±0.004	692±2.73	27.2±0.22	182±2.03
PS	Clay loam	0.63±0.02	26.8±0.5	40.0±0.09	33.2±0.25	46.07±0.57	10.2±0.18	8.25±0.03	6.42±0.12	0.3±0.008	0.14±0.002	494±2.65	30.5±0.15	150±5.52
TS	Clay loam	0.50±0.008	23.1±0.12	41.2±0.22	35.7±0.19	74.51±0.18	13.4±0.15	21.01±0.020	6.96±0.02	0.42±0.003	0.32±0.01	568±2.6	35.7±0.35	193±0.9
JS	Sandy clay loam	0.65±0.02	52.8±0.25	20.0±0.2	27.2±0.22	46.34±0.14	10.2±0.15	23.03±0.02	6.9±0.03	0.47±0.02	0.45±0.01	448±0.9	35.2±0.19	176±0.9
AS	Sandy loam	0.53±0.02	53.5±0.5	34.2±0.15	12.3±0.18	43.60±0.19	5.7±0.09	56.67±0.1	6.55±0.04	0.27±0.006	0.31±0.008	324±0.9	26.2±0.2	124±5.37

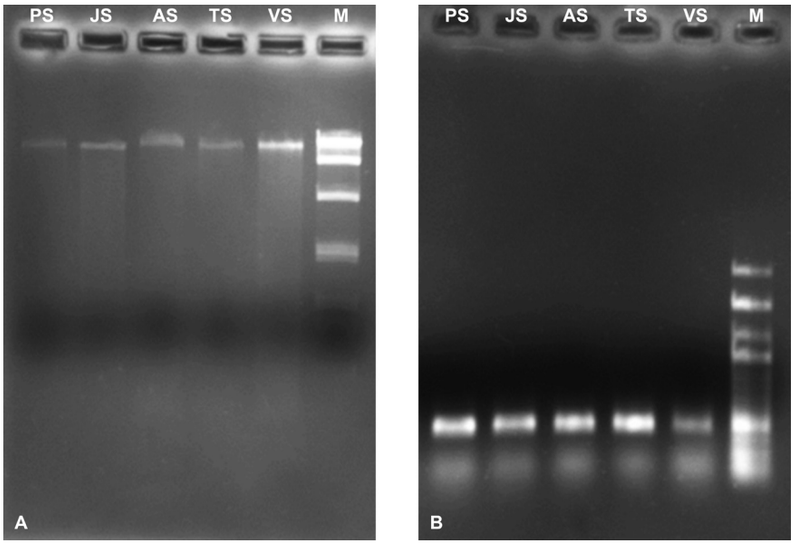

Fig. 1. (A) Agarose gel electrophoresis image of bacterial genomic DNA isolated from arsenic contaminated soil samples. M indicates molecular weight marker (lamda phage DNA/*Hin*dIII digest) and (B) Agarose gel image of PCR amplicons. M indicates 100 bp DNA Ladder.

physical and chemical properties of the soil samples measured in this study are listed in Table I. Soil texture ranged from sandy loam to clay loam with pH close to neutral (6.42–6.96). We found that there are no significant differences in the chosen parameters of the five different agricultural soils including soil type. It confirms that the present irrigation system of the agricultural lands did not affect the general properties of soils. On the contrary, the arsenic contamination varies significantly among these sampling sites. The total arsenic contents ranged from 6.65 to 56.67 ppm creating a gradient of arsenic contamination. Thus, the observed trend of physicochemical characteristics of the selected sites is a key finding of our study. From Table I, it is clear that the selected soil parameters will not influence the microbial community as there is no such variation except soil arsenic content, which may affect the microbial community structure. In our study, it was observed that the maximum arsenic content in agricultural fields of Gontra village in Chakdaha is 56.67 ppm, which is much above the maximum acceptable limit *i.e.* 20 mg per kg as limited by the European Commission (Rahman *et al*., 2007; Bhattacharya *et al*., 2009). Based on our current study it could be concluded that due to continuous and long term irrigation by arsenic contaminated ground water, arsenic content of the studied area is above alarm limit.

Soil DNA extraction. The cultivation independent evaluation of microbial diversity has been mainly based on the extraction of total DNA from environmental samples (Yuan *et al*., 2012). Here, we successfully obtained detectable amount of DNA from soil using commercial kit as shown on agarose gel (Fig. 1A). Agarose gel electrophoresis revealed the yield of high molecular weight DNA approximately 10 kb in the total soil DNA extracts. DNA concentration of the soil samples ranged between 9.0 ng/µl to 11.1 ng/µl as measured by nanodrop (Table II). The $A_{260/280}$ (> 1.7) and $A_{260/230}$ (> 1.9) of extracted DNA indicate minimum protein and other organic contaminations respectively.

Soil bacterial community analysis by group specific qPCR. The qPCR has been used for rapid and reliable quantification of 16S rDNA copy number. Investigation reports suggested that the *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Firmicutes* etc. are the most common phyla of soil bacterial communities (Fierer *et al*., 2005; Muhling *et al*., 2008; Philippot *et al*., 2011). The qPCR data demonstrated that the separate

Table II
Spectrophotometric measurement of extracted DNA from soil obtained by Nanodrop 2000 spcetrophotometer (Thermo scientific)

Sample Name	DNA Concentration (ng/µl)[a]	$A_{260/280}$ [b]	$A_{260/230}$ [c]
VS	9.7	1.74	1.99
PS	9.0	1.70	2.01
TS	11.1	1.77	1.97
JS	9.8	1.73	1.99
AS	9.7	1.77	1.98

[a] Calculated based upon A_{260} against a standard response curve.
[b] $A_{260/280}$, Ratio of A_{260} to A_{280} (a high ratio [> 1.7] is indicative of pure DNA, whereas a low ratio is indicative of protein contamination).
[c] $A_{260/230}$, Ratio of A_{260} to A_{230} (a high ratio [> 2] is indicative of pure DNA, whereas a low ratio is indicative of phenolic and humic acid contamination).

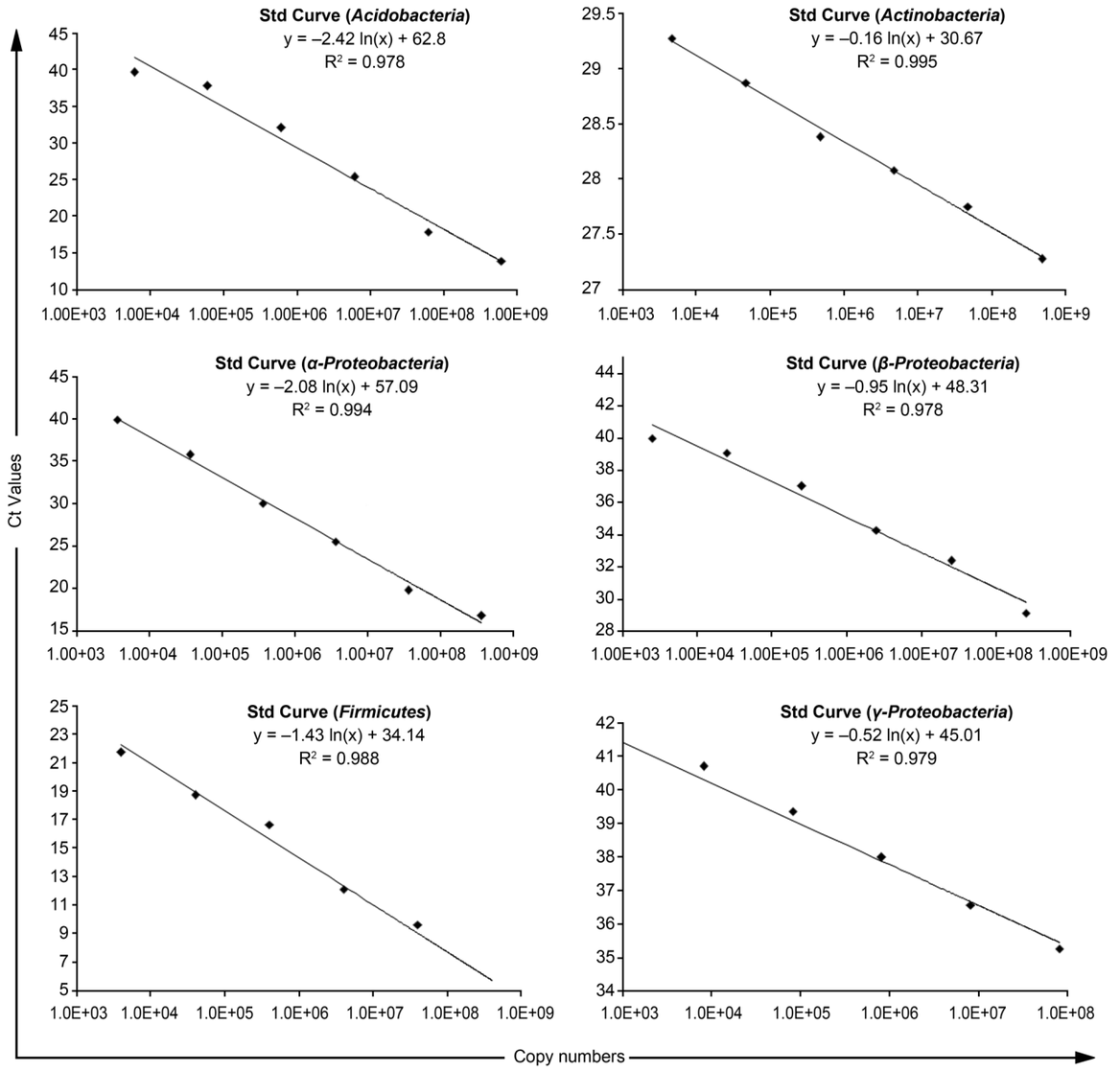

Fig. 2. qPCR standard curves for 16S rRNA gene of different bacterial taxa.

peaks and different threshold values (Ct values) were related to the standard samples. Non-specific amplification was not detected. Copy numbers of the standard samples were calculated and a linear standard curve was generated for each set of qPCR analysis (Fig. 2). Amplification efficiency was calculated from the slops to be ≥ 0.97. Finally, the copy number of the 16S rRNA gene of six targeted bacterial groups in the extracted soil DNA was detected by putting the Ct values in the equation derived from the standard curve. We used the qPCR data to estimate the relative abundance of above mentioned bacterial groups in the five distinct agricultural fields. It was observed that *Actinobacteria* and *Proteobacteria* represented the highest copy number and thus constituted the major bacterial taxa of the selected agricultural fields, whereas *Acidobacteria* and *Firmicutes* with lower copy numbers were estimated as subdominants (Table III). Comparative analysis of the bacterial community composition among the contaminated sites revealed a shift in a relative abundance of dominant group towards increasing arsenic contamination. At lower concentration of arsenic at contaminated sites (< 10 ppm) *Actinobacteria* was the most abundant phylum whereas in the case of higher arsenic contamination (> 10 ppm) the difference in their abundance at phylum level was noted (Fig. 3). Thus this study underlies the fact that the copy number of *Actinobacteria* gradually decreases with increasing concentration of arsenic, on the contrary to the growth of the *Proteobacteria* copy number. Our study also revealed that arsenic contamination influenced the resident microbial communities and dominant group might represent the major arsenic

Table III
The copy number of the bacterial taxa specific 16S rDNA present in the soil samples.

Soil samples	Acidobacteria (μl^{-1})	Actinobacteria (μl^{-1})	Firmicutes (μl^{-1})	α-Proteobacteria (μl^{-1})	β-Proteobacteria (μl^{-1})	γ-Proteobacteria (μl^{-1})
VS	2.41×10^{5}	2.33×10^{9}	9.43×10^{3}	1.48×10^{5}	2.74×10^{8}	8.81×10^{7}
PS	1.16×10^{5}	4.27×10^{8}	4.59×10^{3}	1.79×10^{5}	3.34×10^{8}	8.24×10^{8}
TS	1.75×10^{5}	4.54×10^{8}	4.25×10^{3}	1.41×10^{5}	2.76×10^{9}	3.18×10^{9}
JS	1.74×10^{5}	1.88×10^{7}	2.29×10^{3}	1.89×10^{4}	2.39×10^{9}	1.04×10^{9}
AS	5.95×10^{4}	7.28×10^{5}	2.34×10^{3}	1.11×10^{4}	7.19×10^{9}	5.83×10^{9}

Table IV
Simple linear correlation coefficients relating the abundance of bacterial groups in the different agricultural fields to the varying level of arsenic contamination and other soil parameters (See Table I for soil properties).

Bacterial Groups	Total Arsenic	Total Nitrogen	Carbon & organic matter	Soil pH	Available P_2O_5	Available K_2O
Actinobacteria	–0.94**	0.92**	0.38	0.27	0.27	0.72
Acidobacteria	0.81*	0.88*	0.73	0.63	0.44	0.90**
Firmicutes	–0.72	0.91**	–0.01	0.12	–0.28	0.43
α-Proteobacteria	–0.84*	0.80*	0.12	0.03	0.14	0.53
β-Proteobacteria	0.62	–0.82*	0.02	–0.13	0.41	–0.31
γ-Proteobacteria	0.76	–0.79	–0.14	–0.11	0.41	–0.38

*Significant values ($P < 0.05$) and ** highly significant values ($P < 0.001$) as determined by correlation coefficient analysis.

resistant indicator groups for effective remediation strategies. Thus, the different studies identified *Actinobacteria* as a major heavy metal resistant microorganism in soil (Schmidt *et al*., 2005; Baz *et al*., 2015), however some other reported them as minor group (Sheik *et al*., 2012). In present scenario, it was important to understand the effect of arsenic contamination on the resident soil microbial communities to make effective remediation strategies. Therefore, phylum level shift may give a significant indication suggesting *Proteobacteria* as a major arsenic tolerant organism. Previous studies indicated that *Proteobacteria* are capable for metal transformation (Gillan *et al*., 2005; Cai *et al*., 2009).

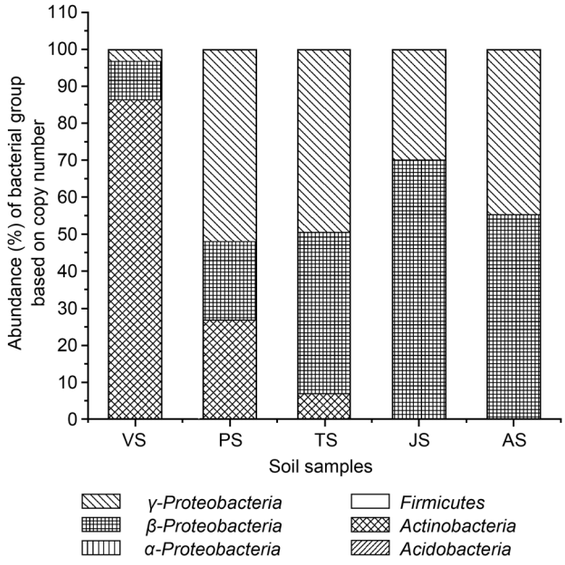

Fig. 3. Relative abundance of major taxonomic groups across the sampling sites representing bacterial community shifting towards *Proteobacteria* with increasing soil arsenic contamination.

Bio-geochemical data analysis. Correlation coefficient analysis revealed a significant relationship between the bacterial population structure and soil arsenic contamination. *Actinobacteria*, *Acidobacteria*, *α-Proteobacteria* and *Firmicutes* displayed significant negative correlation but *β-Proteobacteria* and *γ-Proteobacteria* were positively correlated with soil arsenic contamination. Other soil parameters were also correlated with the bacterial abundance, showing negligible correlation, except total nitrogen content (Table IV). Relations between the samples were assessed separately in respect to their physicochemical properties and bacterial community composition (Fig. 4). The PCA of bacterial community composition among the studied samples were performed (Fig. 4A), where the two principal components (principal component 1 and principal component 2) represented 91.3% of variation among the five soil samples. Principal component plot indicated a distinct resemblance among all the samples collected from high arsenic soil content while PS and VS with low arsenic content remained separated from other clusters. Between-group linkage cluster method

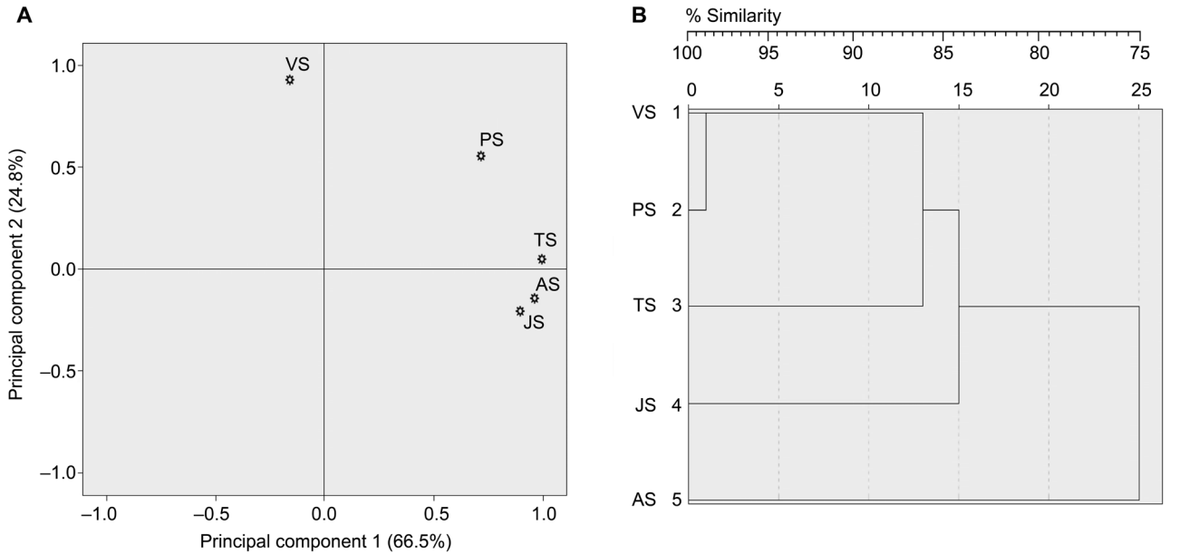

Fig. 4. Relationship among biogeochemical factors of five different soil samples. (A) PCA plot based on bacterial community composition and (B) hierarchical cluster analysis of geochemical data.

(UPGMA) was applied and the measurement was performed on Euclidean distance for the analysis on soil characteristics. The cluster analysis distingushed three clusters, each of which are related to the arsenic concentration of studied samples (Fig. 4B). VS and PS indicated 99% similarity while AS with highest arsenic content remain separated from the other samples showing positive relation with 75% similarities with JS, TS cluster (81.5%). In summary, it was shown that arsenic contamination greatly affects the microbial population.

DGGE profiling of arsenic contaminated soil bacterial communities, diversity estimations and phylogenetic analysis. Bacterial community structure of the defined arsenic contaminated agricultural soils was investigated by PCR-DGGE method based on band intensity and position. The touchdown PCR was performed to avoid nonspecific primer binding and further amplification (Maiwore *et al*., 2012). Agarose gel electrophoresis of PCR amplified products (Fig. 1B) suggested that the extracted DNA was as a good substrate for the amplification of bacterial 16S rDNA fragments. The DGGE image depicted the number and intensity of migrating bands of the DNA profiles from all samples (Fig. 5A) and provided a fingerprint of the microbial community found in soil samples indicating high diversity of 16S rRNA genotypes. The bands from DGGE profile corresponded to the 495 bp 16S rDNA fragments with different nucleotide sequences represented variation in dominant microbial populations in the community. The DGGE profiling showed a large number of bands in case of PS and AS but low number of bands appeared in analysis of VS site. A total of 50 different band positions were detected in the DGGE gel image of the five different soil samples. The number of bands per lane varied from 34 to 40. Thirty seven DGGE bands were very similar in the analyzed samples indicating the presence of a large number of equally abundant ribotypes. The other bands were unique to particular sampling sites suggesting that the samples had different dominant bacterial population.

After the analysis of the digitized image with the Quantity One software it was observed that some bands dominated in samples from particular sites as showed by high band intensity whereas bands in the other samples were always faint. DGGE banding patterns showed that similarity indices varied significantly, suggesting genetic diversity with distinction among the studied samples. The Quantity One software generated dendrogram revealed similarity of PCR-DGGE fingerprints from the gel on the basis of visual comparison. Cluster analysis showed that the samples fell into two groups and correlated with the arsenic concentration of the collection sites (Fig. 5B). The first cluster included samples from VS and PS sites of arsenic contamination below 10 ppm while the second cluster consisted of samples from TS, JS and AS sites of > 10 ppm arsenic contamination. MDS analysis was performed to investigate the changes in the bacterial community structure due to arsenic contamination and illustrated the similarity of all possible pairs of each gel track. The two dimensional MDS plot pointed out that the soil samples containing bacterial community were not grouped together, suggesting significant variations in the community structure (Fig. 6A). Beside these studies, we also compared the bacterial communities of the different samples by calculating the Shannon diversity

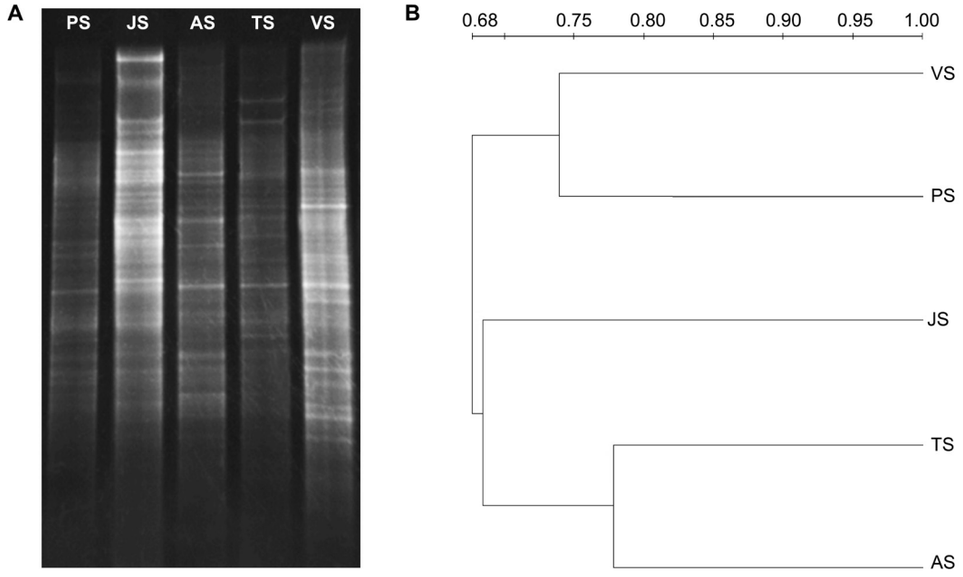

Fig. 5. (A) DGGE fingerprints of PCR-amplified 16S rRNA gene fragments of bacterial communities from five different soil samples and (B) Quantity One generated UPGMA dendogram of DGGE profile showing two clusters: the first cluster (VS and PS) having arsenic contamination < 10 ppm while the second cluster (TS, JS and AS) having arsenic contamination > 10 ppm.

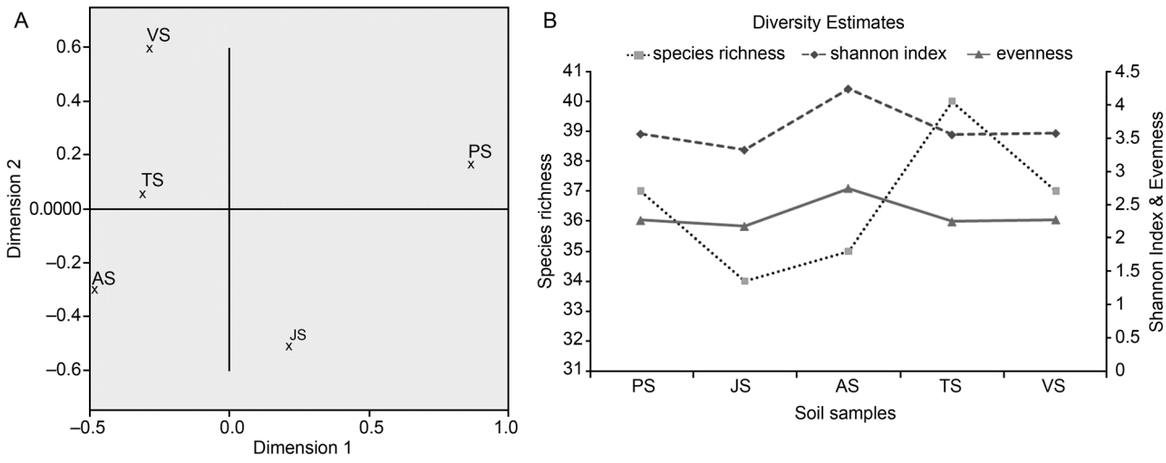

Fig. 6. (A) Two-dimensional MDS plot obtained from DGGE profile representing significant variation in the bacterial community structure of different soil samples and (B) Comparative representation of diversity estimates calculated from DGGE profile.

index (*H*), based on the DGGE banding patterns of the soil samples. The two diversity indices; species richness (*S*) and species evenness (*E*) were determined for each sample. The ability to quantify diversity in this way was then shown to be an important tool for biologists trying to understand community structure (Moura *et al*., 2009). The Shannon diversity index (*H*) has often been discussed in the analysis of DGGE fingerprints (Moura *et al*., 2009; Gafan *et al*., 2005). *H* values differed significantly ranging from 3.32 to 4.24, indicating the difference in bacterial community structures across the samples. When analyzed by Shannon index the highest value ($H = 4.24$) was found in the case of the highest arsenic contaminated soil indicating high diversity in the bacterial community. Simultaneously the bacterial diversity was low in less arsenic contaminated soils according to Shannon index. The observed data of calculated *H* index and its correlation with arsenic content of the soils showed significant positive value (0.81) at $P < 0.05$ level. Moreover, the indices for evenness of the bacterial community were also significantly different among the soils and showed high values ranging from 2.17 to 2.74. Soil bacterial diversity, as estimated by phylotype richness and diversity varied across the arsenic contaminated agricultural fields. Comparison of all diversity estimates is shown in Fig. 6B indicating

Table V
Identification of DGGE bands by 16S rDNA sequencing.

Excised Band No.	Isolation Source	No. of nucleotide compared	Identification	Closest type strain and accession code		Identity	Our GenBank Accession number
G14	Soil	454	Uncultured *Aeromonas* sp.	Aeromonas sp.	KP716703.1	99%	KJ136644
G15	Soil	455	Uncultured *Aeromonas* sp.	*Aeromonas* sp.	KP716703.1	99%	KJ136645
G16	Soil	443	Uncultured *Acinetobacter* sp.	*Acinetobacter* sp.	KT003253.1	97%	KJ403747
G2	Soil	451	Uncultured *Klebsiella* sp.	*Klebsiella oxytoca*	HQ683969.1	99%	KJ403748
G3	Soil	464	Uncultured *Acinetobacter* sp.	*Acinetobacter calcoaceticus*	KM585587.1	99%	KJ403749
G1	Soil	454	Uncultured *Klebsiella* sp.	*Klebsiella oxytoca*	KC462193.1	99%	KJ403750
G4	Soil	456	Uncultured *Aeromonas* sp.	*Aeromonas media*	HF937047.1	99%	KJ403751

distinct bacterial diversity among the samples. By analyzing the DGGE banding patterns using the Shannon index of diversity in combination with the evenness and species richness of the soils, we were able to monitor a whole range of community responses from all the contaminated soil samples.

Among all major bands, seven bands with distinct migration distance in the DGGE gel were successfully sequenced. The bands were selected on the basis of their intensity as well as availability in the study sites. The 16S rDNA sequences of all isolates were subjected to nucleotide BLAST and the bacteria were classified according to their similarity to sequences in the GenBank database. The sequences were derived from unculturable organisms and were representing *Aeromonas* sp. (strain KJ136644, strain KJ403751 and strain KJ136645), *Acinetobacter* sp. (strains KJ403749 and KJ403747), *Klebsiella* sp. (strain KJ403748 and strain KJ403750) (Table V). Phylogenetic analysis revealed that all the isolates belonged to *γ-Proteobacteria*. The organisms belonging to the genera of *Aeromonas*, *Acinetobacter* and *Klebsiella* were previously reported as arsenic resistant and arsenic accumulating organisms and found to grow effectively at more than 100 ppm arsenic (Anyanwu and Ugwu, 2010). The genus *Acinetobacter* is broadly represented as arsenic resistant and isolated from various arsenic contaminated sites exhibiting arsenite oxidase activity (Achour *et al*., 2007). *Klebsiella* was reported as highly resistant to arsenic and able to survive even at high arsenic concentration by converting arsenite into less toxic form arsenate (Singh, 2011). Other reports also showed the presence of the genera *Aeromonas* among arsenic resistant bacteria (Anyanwu and Ugwu, 2010). Our findings re-established these representative species as the valuable indicators of the arsenic contaminated soils and confirm that these arsenic resistant or accumulating bacteria are widespread in the polluted environment. A phylogenetic tree was constructed (Fig. 7) using the nucleotide sequences of major DGGE bands and related sequences obtained from the DNA database. A bootstrap analysis was performed and values greater than 50% were indicated. Phylogenetic analysis of these sequences suggested that they were deeply branching

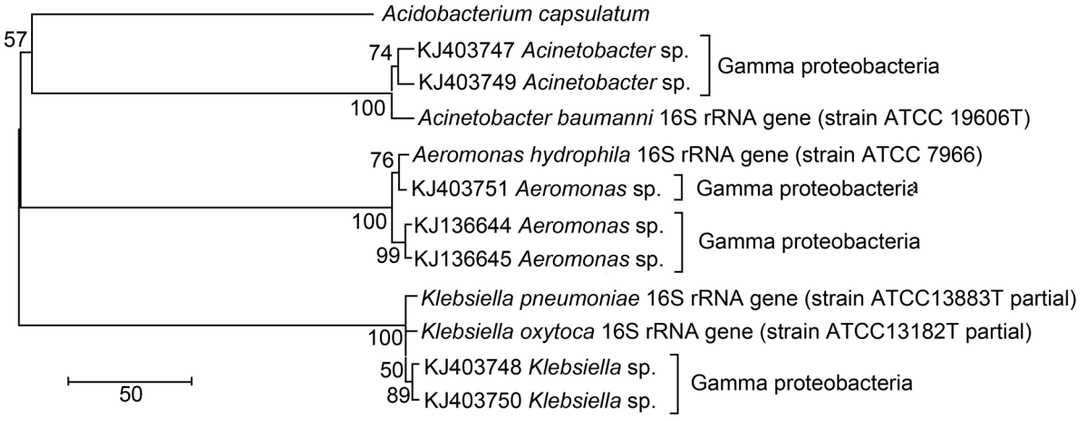

Fig. 7. Phylogentic tree based on 16S rRNA gene sequences of bands excised from DGGE gel and their closet type strains using neighbor joining method. Bootstrap values of > 50% are mentioned at the nodes.

members of the *γ* subclass of the *Proteobacteria* and grouped within a tight phylogenetic cluster. Moreover a significant correlation among bacterial copy number detection and DGGE analysis was observed. Here the combined approach of DGGE and PCR became a promising area of research denoting fluctuations at bacterial phylum level due to arsenic contamination. The consistency between the inferences made by both the molecular approaches again establishes the effect of arsenic contamination on community profiling. All the data in combination provide an indication of the nature of the bacterial communities under arsenic stress and supply important information to microbial ecology. It is therefore reflecting bacterial diversity of soils exposed to arsenic contamination.

Conclusion

In this study we utilized the culture independent techniques to gain a better understanding of the impact of arsenic on soil bacterial community. Overall study indicated significant structural variations between bacterial taxonomic groups throughout the arsenic affected sampling areas. It provides the knowledge of the distribution of bacterial community structure in such contaminated environment. The qPCR data suggested that *Proteobacteria* were the dominant group of the contaminated soils irrespective of the soil type and soil character. Therefore it can be assumed that most of the arsenite oxidizing or arsenate reducing bacteria present in soil may belong to these groups. The bacterial composition of soil contaminated with highly concentrated arsenic significantly differs from that of low arsenic concentration. *Actinobacteria* were mostly present in low arsenic containing regions but bacterial community structure of the selected soils shifted from *Actinobacteria* to *Proteobacteria* with increasing arsenic level. DGGE profiling of soil samples further confirmed the presence of bacteria (*Aeromonas* sp., *Acinetobacter* sp., *Klebsiella* sp.) belonging to *γ-Proteobacteria*. Furthermore, the diversity analysis indicated a diverse bacterial population in the soils as well as high positive correlation with arsenic content and bacterial diversity of the studied area. The observed trend of *Actinobacteria* to be highly sensitive to arsenic concentration indicates the possibility that it may be the stress determinant to monitor arsenic contamination. While dominance of *γ-Proteobacteria* at high arsenic contaminated areas makes it possible to apply this observation to enhance the bioremediation of arsenic contaminated sites. The findings reported here improve the knowledge about the abundance and patterns of soil bacterial community in arsenic contaminated agricultural fields that will enrich the future studies of soil ecology.

Conflict of Interest

The authors declare no conflict of interest.

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ORIGINAL PAPER

Safety Evaluation of Enterocin Producer *Enterococcus* sp. Strains Isolated from Traditional Turkish Cheeses

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Abstract

The purpose of this study was to determine the antimicrobial activity and occurrence of bacteriocin structural genes in *Enterococcus* spp. isolated from different cheeses and also investigate some of their virulence factors. *Enterococcus* strains were isolated from 33 different cheeses. *Enterococcus faecium* (6 strains) and *Enterococcus faecalis* (5 strains) enterocin-producing strains were identified by 16S rDNA analyses. Structural genes *ent*A, *ent*B, *ent*P and *ent*X were detected in some isolates. Multiple enterocin structural genes were found in 7 strains. None of the tested enterococci demonstrated anyβ-haemolytic activity and only one strain had gelatinase activity. Six strains showed multiple antibiotic resistance patterns and in addition, *vanA* and several virulence genes were detected in many strains. Only *E. faecalis* MBE1-9 showed tyrosine decarboxylase activity and *tdc* gene was detected only in this strain.

Key words: *Enterococcus* sp. from cheeses, antibiotic resistance of food borne enterococci bacteriocin production ability, virulence factors

Introduction

Enterococci are an important group of lactic acid bacteria (LAB) that inhabit the digestive tract of humans and animals as well as the surface of waters, soil and plants. They are also found in foods, especially of animal origin, such as traditional cheeses produced in different European and Mediterranean countries (Manolopoulou *et al*., 2003; Yogurtcu and Tuncer, 2013). *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* are the most frequently isolated strains from traditional cheeses, and they play an important role for ripening of these cheeses based on their proteolysis, lipolysis, citrate breakdown abilities and hence they promote product's unique typical taste and flavor formation (Giraffa, 2002; 2003; Moreno *et al*., 2006). They can also inhibit the growth of the food-borne pathogens and spoilage microorganisms by producing bacteriocin known as enterocins. The frequently encountered enterocins produced by enterococci strains are enterocins A, B, P, AS-48, L50A, L50B, 1071A, 1071B, and Q (Moreno *et al*., 2006; Nes *et al*., 2007; Edalatian *et al*., 2012; Özden-Tuncer *et al*., 2013).

Although enterococci have desirable technological and metabolic traits, in recent years they have been found to be associated with clinical infections such as endocarditis, bacteremia and urinary tract. Therefore, enterococci have emerged as an important nosocomial pathogen in recent decades (Franz *et al*., 2003; Moreno *et al*., 2006; Peters *et al*., 2007; Lindenstrauß *et al*., 2011). Several putative virulence factors have been described in enterococci, such as gelatinase (*gel*E), cell wall adhesin ($efaA_{fm}$ and $efaA_{fs}$ from *E. faecium* and *E. faecalis*, respectively), sex pheromones (*cpd*, *cob*, *ccf*, and *cad*), collagen adhesin (*ace*), enterococcal surface protein (esp_{fm} and esp_{fs} from *E. faecium* and *E. faecalis*, respectively), aggregation substance (*agg*), and cytolysin (*cyl*M, *cyl*B, and *cyl*A) (Vankerckhoven *et al*., 2004; Cariolato *et al*., 2008; Chuang *et al*., 2009; Barbosa *et al*., 2010; Ben Belgacem *et al*., 2010; Lindenstrauß *et al*., 2011; Yogurtcu and Tuncer, 2013). In addition, enterococci are resistant to several antibiotics due to intrinsic and/or acquire genes located on the chromosomal DNA, plasmids or transposons. The interesting point about antibiotic resistance of enterococci is the increasing number of vancomycin and multiple antibiotic-resistant strains. The reason for the increase in multiple antibiotic resistance in enterococci is transfer of genetic elements carrying antibiotic resistance genes between pathogenic and non-pathogenic bacteria in the human or animal intestinal tract or even in food (Riboldi *et al*., 2009; Sparo *et al*., 2011).

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Although the technological and metabolic activity of enterococci is essential for some cheeses, the presence of virulence factors and antibiotic resistance in enterococci should be investigated due to their ability of transferring virulence genes and antibiotic resistance determinants through the food chain. In recent years, virulence factors and antibiotic resistance of clinical isolates were mostly examined, but every day interest in food-borne enterococci and their pathogenity is increasing. From these perspectives, the aim of this study was to isolate enterocin producing enterococci strains from various traditional artisanal cheeses in Turkey, and to determine their enterocin genes, antibiotic resistance patterns and virulence factors. Enterocin production, antibiotic resistance and virulence determinants of enterococci isolated from some cheese types in this study were investigated for the first time.

Experimental

Materials and Methods

Cheese samples. Cheese samples were obtained from various provinces (Denizli, Isparta, Balıkesir, Bolu, Kayseri, Van and Manisa) of Turkey and it has been noted that they are especially homemade. A total of 33 cheeses samples, including traditional Turkish white cheese, Tulum cheese, Manyas Kelle and Van Otlu (Herby) cheese made from cow's, sheep's and goat's milk were used in the present study.

Isolation and phenotypically identification of *Enterococcus* sp. strains. Ten grams of each cheese sample were homogenized in 90 ml sterile saline solution (0.85% NaCl, w/v) with waring blender (8011 ES HGB2WTS3, Torrington, Connecticut, 06790, USA). Serial decimal solutions were prepared in saline solution and spread onto Kanamycin Easculine Azide Agar (Lab M, Ltd., Bury, Lancashire, U.K.) and incubated at 37°C for 48 h. Over an incubation period black colonies of presumptive *Enterococcus* sp. strains were randomly picked up and grown in de Man Rogosa Sharpe (MRS) broth medium (Lab M) at 37°C for 24 h. A total of 100 isolates were identified to the genus level by Gram staining, catalase and cultural tests such as growth in MRS broth (10°C, 37°C, 45°C and pH 9.6), tolerance of 6.5% (w/v) NaCl and resistance to heat (60°C for 30 and 60 min).

Detection of bacteriocin production ability. Antimicrobial activities of the isolates were evaluated towards selected indicator bacteria by using the toothpick method as described by van Belkum *et al*. (1989). Briefly, overnight presumptive *Enterococcus* sp. strains were grown on MRS solid medium at 37°C for 18 h. After incubation time, sterile toothpicks were used to ensure create spots on MRS agar. After the incubation period, an indicator lawn of 5 ml of appropriate soft agar (0.5%, w/v), containing 100 µl indicator bacteria was poured onto the surface and incubated at the required temperature for indicators for 18 h. After incubation, all inhibition zones around the colonies were examined and the zones were measured. The growth medium and incubation temperature of the indicator bacteria are listed in Table I. Stock cultures in appropriate medium with 20% glycerol were stored at –20°C.

The protein nature of antimicrobial substances produced by isolates was determined by proteinase K enzyme assay. The 20 µl proteinase K (20 mg/ml) was dropped near 1 cm of the colonies and then the plates were incubated for 1 h at 37°C. Finally, indicator bacteria were poured onto plate with 5 ml soft agar and incubated at an optimum growth temperature for the indicators. After the incubation period, diminishing of inhibition zones shaped like a half-moon was considered as an indication that the antimicrobial agent produced by *Enterococcus* sp. isolates was affected by the enzyme proteinase K and antimicrobial substances were recognized as bacteriocin (enterocin).

Genomic DNA extraction. Genomic DNA's of bacteriocin producing isolates were extracted from 0.5 ml of overnight cultures using the method of Cancilla *et al.* (1992). DNA precipitates were resuspended in 50 µl Tris-EDTA buffer (pH 8.0).

Identification of enterocin producer isolates. 16S rDNA gene sequence of the isolates were amplified with following specific primers: pA (forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pE' (reverse) 5'-CCG TCA ATT CCT TTG AGT TT-3' (Edwards *et al*., 1989) using in a programmable DNA thermocycler (Techne TC3000, Cambridge, U.K.). Fifty microliter PCR reaction mixture consisted of 3 µl of the bacterial DNA solution, 1 µl of each primer, 20 µl nuclease-free water and 25 µl PCR master mix (Fermentas, Vilnius, Lithuania) and following PCR conditions were ensured: initial denaturation cycle at 94°C for 2 min, next 30 cycles of denaturation 94°C for 30 s, annealing at 55°C for 60 s and elongation at 72°C for 90 s, and a final extension cycle at 72°C for 10 min. Amplification products were separated by electrophoresis on 1% agarose (w/v) gels at 85 V for 1.5 h in Tris-acetate-EDTA buffer. Agarose gels were stained with ethidium bromide (20 µg/ml) and photographed under ultraviolet light using a Nikon D5100 digital camera (Nikon Corp., Japan). The size of amplicons was determined by comparison with O'GeneRuler 100-bp DNA ladder (Fermentas). Sequencing of the 16S rDNA gene was performed in RefGen (ODTU Technocity, Ankara, Turkey). The 16S rDNA homology searches were carried out using BLAST software against the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) database.

Table I
Growth medium and incubation temperatures of indicator bacteria, and inhibitory spectrum of *Enterococcus* sp.strains

Indicator strains	Growth medium* and incubation temperature	Inhibition zone (Ø mm)										
		MBE 1–9	MBE 12–3	MBE 15–2	MBE 16–5	MBE 20–5	MBE 22–2	MBE 27–2	MBE 27–3	MBE 29–1	MBE 29–3	MBE 31–4
Lactobacillus plantarum LMG2003	MRS, 37°C	–	–	–	–	–	–	–	–	–	–	–
Lactococcus lactis subsp. *lactis* 1	GM17, 30°C	–	–	–	–	–	–	–	–	–	18	–
Lactococcus lactis subsp. *lactis* 731	GM17, 30°C	7	9	12	16	–	–	13	10	–	10	–
Lactococcus lactis subsp. *lactis* T1	GM17, 30°C	16	13	14	–	–	–	14	13	–	15	–
Lactococcus lactis subsp. *lactis* 105	GM17, 30°C	–	–	–	–	–	–	–	–	–	–	–
Listeria innocua LMG2813	LB, 30°C	7	9	8	6	4	5	9	9	4	6	4
Listeria monocytogenes ATCC15813	LB, 30°C	4	13	17	7	3	5	13	13	3	7	4
Listeria monocytogenes ATCC19115	LB, 30°C	13	5	10	9	6	7	10	6	4	14	6
Listeria monocytogenes ATCC7644	LB, 30°C	12	23	24	10	7	9	26	23	6	11	6
Staphylococcus aureus ATCC25923	TSB, 37°C	7	7	10	9	6	7	9	7	6	8	8
Staphylococcus aureus FRI1003022	TSB, 37°C	7	6	6	5	4	6	7	6	5	6	4
Staphylococcus carnosus LMG2709	TSB, 37°C	8	5	5	6	5	6	7	6	5	15	6
Staphylococcus aureus ATCC29213	TSB, 37°C	11	13	10	15	12	11	10	12	10	12	14
Enterococcus faecalis ATCC29212	GM17, 37°C	8	14	10	6	–	5	8	14	5	8	6
Enterococcus faecalis LMG2708	GM17, 37°C	–	10	–	–	–	–	–	8	–	–	–
Enterococcus faecalis LMG2602	GM17, 37°C	–	15	–	–	–	–	–	–	–	13	–
Bacillus cereus ATCC10876	TSB, 37°C	6	7	5	7	6	6	6	7	5	6	6
Bacillus cereus LMG2732	TSB, 37°C	17	5	16	–	–	–	16	–	–	18	–
Salmonella Typhimurium SL1344	LB, 37°C	10	15	16	14	14	9	12	13	14	17	8
Salmonella Enteritidis ATCC13076	LB, 37°C	7	7	8	7	8	5	16	6	5	7	5
Salmonella Typhimurium ATCC14028	LB, 37°C	–	8	4	4	4	6	–	8	–	–	5
Escherichia coli LMG3083 CFAI (ETEC)	LB, 37°C	5	6	6	4	–	–	6	7	–	7	–
Pediococcus pentosaceus LMG2001	TSB, 37°C	19	15	16	–	–	–	17	14	5	18	–

* MRS: de Man Rogosa Sharpe, GM17: M17 with glucose (5%), LB: Luria Bertani, TSB: Triptic Soy Broth

Table II
PCR primers, annealing temperatures and product sizes for detection of bacteriocin*, virulence** and amino acid decarboxylase*** genes.

Gene	Primer sequence (5' to 3')	Annealing temperature (°C)	Product size (bp)	Reference
**ent*A	AAATATTATGGAAATGGAGTGTAT GCACTTCCCTGGAATTGCTC	56	126	Yousif *et al.* 2005
**ent*B	GAAAATGATCACAGAATGCCT A GTTGCATTTAGAGTATACATTTG	50	162	Yousif *et al.* 2005
**ent*P	TATGGTAATGGTGTTTATTGTAAT ATGTCCCATACCTGCCAAAC	50	120	Yousif *et al.* 2005
**entL50A/B*	TGGGAGCAATCGCAAAATTAG ATTGCCCATCCTTCTCCAAT	52	98	Ben Belgacem *et al.* 2010
**bac31*	TATTACGGAAATGGTTTATATTGT TCTAGGAGCCCAAGGGCC	50	123	Yousif *et al.* 2005
**entAS48*	GAGGAGTTTCATGATTTAAAGA CATATTGTTAAATTACCAAGCAA	50	340	Yousif *et al.* 2005
**entQ*	ATGAATTTTCTTCTTAAAAATGGTATCGCA TTAACAAGAAATTTTTTCCCATGGCAA	56	105	Ben Belgacem *et al.* 2010
**ent1071A/B*	CCTATTGGGGGAGAGTCGGT ATACATTCTTCCACTTATTTTT	51	343	Ben Belgacem *et al.* 2010

Table II continued

Gene	Primer sequence (5' to 3')	Annealing temperature (°C)	Product size (bp)	Reference
**cylL$_{L/S}$*	GTGTTGAGGAAATGGAAGCG TCTCAGCCTGAACATCTCCAC	60	324	Brandao *et al.* 2010
**ent*X	GTTTCTGTAAAAGAGATGAAAC CCTCTTAATCATTAACCATAC	50	500	Edalatian *et al.* 2012
***efa*A$_{fm}$	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	54	735	Reviriego *et al.* 2005
***efa*A$_{fs}$	GACAGACCCTCACGAATA AGTTCATCATGCTGTAGTA	54	705	Reviriego *et al.* 2005
***ccf*	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	54	543	Reviriego *et al.* 2005
***cpd*	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	54	782	Reviriego *et al.* 2005
***cob*	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCA	54	1405	Reviriego *et al.* 2005
***esp$_{fm}$*	TTGCTAATGCAAGTCACGTCC GCATCAACACTTGCATTACCGAA	54	955	Reviriego *et al.* 2005
***esp$_{fs}$*	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	54	933	Reviriego *et al.* 2005
***ace*	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	54	350	Ben Belgacem *et al.* 2010
***gel*E	ACCCCGTATCATTGGTT ACGCATTGCTTTTCCATC	54	419	Reviriego *et al.* 2005
***cad*	TGCTTTGTCATTGACAATCCG ACTTTTTCCCAACCCCTCAA	54	1299	Reviriego *et al.* 2005
***agg*	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	56	1553	Eaton and Gasson 2001
***cyl*A	TGGATGATAGTGATAGGAAGT TCTACAGTAAATCTTTCGTCA	54	517	Reviriego *et al.* 2005
***cyl*B	ATTCCTACCTATGTTCTGTTA AATAAACTCTTCTTTTCCAAC	54	843	Reviriego *et al.* 2005
***cyl*M	CTGATGGAAAGAAGATAGTAT TGAGTTGGTCTGATTACATTT	54	742	Reviriego *et al.* 2005
****hdc*	GGNATNGTNWSNTAYGAYMGNGCNGA ATNGCDATNGCNSWCCANACNCCRTA	53	372	de las Rivas *et al.* 2006
****tdc*	TGGYTNGTNCCNCARACNAARCAYTA ACRTARTCNACCATRTTRAARTCNGG	53	825	de las Rivas *et al.* 2006
****odc*	TWYMAYGCNGAYAARACNTAYTTYGT ACRCANAGNACNCCNGGNGGRTANGG	53	1440	de las Rivas *et al.* 2006
****ldc*	CAYRTNCCNGGNCAYAA GGDATNCCNGGNGGRTA	53	1185	de las Rivas *et al.* 2006

PCR primers, annealing temperatures and product sizes for detection of bacteriocin*, virulence** and amino acid decarboxylase*** genes
Y: C or T; R: A or G; W: A or T; S: C or G; M: A or C; D: A, G or T.

Detection of enterocin genes. The presence of enterocin-encoding genes was detected according to Edalatian *et al.* (2012), by using the most common enterocin primers. Interaction primers, annealing temperatures and the size of amplicons are given in Table II. PCR for enterocin genes were performed using the following parameters: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at an appropriate temperature for 1 min, and elongation at 72°C for 40 s and final extension at 72°C for 10 min. PCR products were analyzed by agarose gel (1.5%, w/v) electrophoresis with reference to O'GeneRuler 100-bp DNA ladder (Fermentas), the bands stained with ethidium bromide and photographed with a Nikon D5100 digital camera under ultraviolet light. *E. faecium* EYT31 strain (*ent*A$^+$, *ent*B$^+$ and *ent*P$^+$) obtained from Özden-Tuncer *et al.* (2013), was used as positive control.

Cross-protection activity test. The purpose of this assay was to detect the susceptibility of each strain to

antimicrobial substances produced by all others. Consequently, enterococci strains were used as producers and indicators. Producer bacteria were spotted on appropriate solid media and then enterocin producer strains were poured onto plates with soft MRS agar as indicators. After incubation at 37°C for 18 h inhibition zones were examined.

Haemolytic and gelatinase activity. *Enterococcus* sp. strains were grown in MRS broth medium at 37°C for 18 h and then streaked onto Columbia Agar (Laboratorios Conda S.A., Madrid, Spain) containing 5% defibrinated sheep blood for determining haemolytic activity. After an incubation period (37°C for 48 h) presence of zones of clearing around the colonies were interpreted as β-haemolysis. The absence of zones around the colonies or green zones around the colonies was interpreted as γ-haemolysis and α-haemolysis, respectively (Cariolato *et al*., 2008). Gelatinase production abilities of the enterocin producer strains were investigated on Todd-Hewitt agar presence of gelatinase (30 g/l). Briefly, strains were grown in MRS broth medium (at 37°C for 24 h) then streak onto Todd-Hewitt agar (Liofilchem, Roseto degli Abruzzi, Italy). After incubation at 37°C for 24 h, petri dishes were placed 4°C for 5 h before examination for the zone of turbidity around the colonies, indicating hydrolysis (Eaton and Gasson, 2001).

Antibiotic susceptibility. Antibiotic susceptibility of enterocin producer *E. faecium* and *E. faecalis* strains were determined by disc diffusion method on Mueller Hinton agar (Lab M) plates according to the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2012). The antibiotics used in this study were as follows: doxycycline (30 μg), norfloxacin (10 μg), vancomycin (30 μg), chloramphenicol (30 μg), rifampicin (5 μg), minocycline (30 μg), penicillin (10 μg), streptomycin (300 μg), levofloxacin (5 μg), ciprofloxacin (5 μg), ampicillin (10 μg), quinupristin-dalfopristin (15 μg), nitrofurantoin (300 μg), tetracycline (30 μg), gentamicin (120 μg), erythromycin (15 μg), linezolid (30 μg) and teicoplanin (30 μg).

Presence of *van*A and *van*B genes. *E. faecium* and *E. faecalis* strains were searched for the presence of *van*A and *van*B genes with the following primers: VanA1 [5'-GGG AAA ACG ACA ATT GC-3'] and VanA2 [5'-GTACAA TGC GGC CGT TA-3'] for *van*A gene (Dutka-Malen *et al*., 1995) and VanB [5'-GTG CTG CGA GAT ACC ACA GA-3'] and VanBrev [5'-CGA ACA CCA TGC AAC ATT TC-3'] for *van*B gene (Reviriego *et al*., 2005). Product size of 732 bp and 1,145 bp were screened for *van*A and *van*B genes, respectively. PCR conditions were performed using the following cycling parameters: initial denaturation cycle at 94°C for 2 min, next 30 cycles of denaturation 94°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 1 min, and a final extension cycle at 72°C for 10 min. Electrophoresis of the amplification products was performed on 1.5% (w/v) agarose gel.

Detection of virulence factors. Presence of the *gel*E (gelatinase), *efa*A_{fm} and *efa*A_{fs} (cell wall adhesin for *E. faecium* and *E. faecalis*, respectively), *cpd*, *cob*, *ccf* and *cad* (sex pheromones), *ace* (collagen adhesin), esp_{fm} and esp_{fs} (cell wall-associated protein in *E. faecium* and *E. faecalis*, respectively), *agg* (aggregation substance), *cyl*M, *cyl*B and *cyl*A (cytolysin) genes were investigated by PCR. PCR primers, annealing temperatures and product sizes for detection of virulence factors are listed in Table II. PCR conditions were performed using the following cycling parameters: initial denaturation cycle at 95°C for 5 min, next 35 cycles of denaturation 95°C for 30 s, annealing at an appropriate temperature for 30 s and elongation at 72°C for 1 min, and a final extension cycle at 72°C for 10 min. Electrophoresis of the amplicons was performed on 1.5% (w/v) agarose gel.

Detection of decarboxylase activity and amino acid decarboxylase genes. The amino acid decarboxylase activity of *Enterococcus* sp. strains was estimated according to the method proposed by Bover-Cid *et al*. (1999). Briefly 5 g of amino acid precursors (histidine, lysine, ornithine and tyrosine (Merck) was added to 1 l decarboxylase screening medium. Enterococcal cultures were spotted onto these plates with and without amino acids and incubated at 37°C for 2–5 days. After incubation time, the decarboxylation activity of the *Enterococcus* sp. strains was determined phenotypicaly by yellow to purple color changes on the petri dishes. Tyraminogenic *E. faecium* NYE54 strain obtained from Inoglu and Tuncer (2013) was used as the positive control.

PCR amplification of histidine, lysine, ornithin and tyrosine decarboxylase genes (*hdc*, *ldc*, *odc* and *tdc*, respectively) were carried out based on the method of de las Rivas *et al.* (2012). PCR primers, annealing temperatures and product sizes for detection of amino acid decarboxylase genes are listed in Table II. PCR conditions were performed using the following cycling parameters: initial denaturation cycle at 95°C for 10 min, next 30 cycles of denaturation 95°C for 30 s, annealing at an appropriate temperature for 30 seconds and elongation at 72°C for 2 min, and a final extension cycle at 72°C for 20 min. Electrophoresis of the amplicons was performed on 1.5% (w/v) agarose gel. *E. faecium* NYE54 strain (tdc^+) (Inoglu and Tuncer, 2013) was used as the positive control.

Results

Isolation and phenotypic identification of *Enterococcus* sp. strains. A total of 100 presumptive *Enterococcus* strains isolated from 33 different cheese samples

on KAA medium and grown in MRS broth. All isolates were Gram-positive, catalase-negative and grown at 10°C, 37°C and 45°C, in the presence of NaCl (6.5%) and at pH 9.6 in MRS broth medium. In addition, all of them were found to be resistant to heat at 60°C for 30 and 60 min.

Detection of bacteriocin production ability. Antimicrobial activities of the isolates were evaluated towards selected indicator bacteria by using toothpick method. The 11 presumptive *Enterococcus* sp. isolates showed inhibition zones against different indicator microorganisms (Table I). The Proteinase K treatment showed that antimicrobial substances of 11 isolates have proteinaceous nature. The isolates were given inhibition zones with different diameter against all *Listeria* and *Staphylococcus* sp. strains tested in this study. On the other hand, inhibition of some Gram negative bacteria (*Salmonella* Typhimurium and *E. coli*) by *Enterococcus* sp. strains was evaluated as a striking result. Based on the obtained results these isolates were considered to be enterocin producers.

Identification of enterocin producer isolates. Presumptive *Enterococcus* sp. strains were identified genotypically by 16S rDNA homology. Sequence similarity of an approximately 900-bp PCR amplification products were determined by BLAST software. The 11 enterocin producer isolates were identified as *E. faecalis* (5) and *E. faecium* (6) with the similarity percentage of 93–99% when 16S rDNA PCR amplicons were compared to GenBank.

Detection of enterocin genes. Enterocin genes were detected by using most common enterocin primers. *E. faecalis* MBE1-9 and *E. faecalis* MBE20-5 strains were not give application with the enterocin primers. The *ent*A gene was detected in 7 strains while 8 strains carried *ent*X gene. The e*nt*B and *ent*P genes were detected in 4 and 2 strains, respectively. The genes coding for enterocin L50A/B, bacteriocin 31, enterocin AS-48, enterocin Q, enterocin 1071 and enterocin $cylL_{L/S}$ were not detected in any *Enterococcus* sp. strains (Table III).

Cross-protection activity test. Based on the data obtained from cross-protection test, all of the *Enterococcus* sp. strains were not inhibited by their enterocins when used as an indicator. *Enterococcus* sp. strains used in the present study showed resistance to own bacteriocins as expected. Observations of cross reaction are given in Table IV.

Haemolytic and gelatinase activity. Three of the strains (*E. faecium* MBE27-2, MBE27-3 and *E. faecalis* MBE29-3) showed α-haemolytic activity while another 4 strains (*E. faecalis* MBE1-9, MBE29-1, MBE31-4 and *E. faecium* MBE12-3) had weak α-haemolytic activity. Non-haemolytic activity was detected in *E. faecium* MBE15-2, MBE16-5, MBE22-2 and *E. faecalis* MBE20-5. Only *E. faecalis* MBE29-3 strain showed gelatinase activity on Todd-Hewitt agar.

Antibiotic susceptibility profiles of *Enterococcus* sp. strains. Antibiotic susceptibility profiles of enterocin producer strains against 18 antibiotics are shown as R (Resistant), I (Intermediate) and S (Sensitive) in Table V. All of the strains were found to be sensitive against gentamicin, teicoplanin, doxycycline, ampicillin, nitrofurantoin, linezolid and levofloxacin while 6 of the 11 *Enterococcus* strains showed multiple antibiotic resistance patterns. All strains found to be susceptible to vancomycin except *E. faecalis* MBE29-1 which has intermediary resistance. Resistance to streptomycin, norfloxacin, tetracycline and chloramphenicol was observed in different strains. The highest resistance rate of 72.72% was found against rifampicin.

Table III
Presence of enterocin genes in the *Enterococcus* sp. strains.

Strains	Source	Structural genes of enterocins									
		xxxxxx	*entB*	*entP*	*ent1071*	*ent50A/B*	*bac31*	*entAS48*	*entQ*	*entX*	$cylL_{L/S}$
E. faecalis MBE1-9	Tulum cheese (sheep milk-Denizli)	–	–	–	–	–	–	–	–	–	–
E. faecium MBE12-3	White cheese (cow milk-Denizli)	+	+	–	–	–	–	–	–	–	–
E. faecium MBE15-2	White cheese (goat milk-Denizli)	+	+	–	–	–	–	–	–	+	–
E. faecium MBE16-5	White cheese (cow milk-Denizli)	+	–	–	–	–	–	–	–	+	–
E. faecalis MBE20-5	Tulum cheese (sheep milk-Denizli)	–	–	–	–	–	–	–	–	–	–
E. faecium MBE22-2	Tulum cheese (sheep milk-Kayseri)	+	–	+	–	–	–	–	–	+	–
E. faecium MBE27-2	Tulum cheese (sheep milk-Bolu)	+	+	+	–	–	–	–	–	+	–
E. faecium MBE27-3	Tulum cheese (sheep milk-Bolu)	+	+	–	–	–	–	–	–	+	–
E. faecalis MBE29-1	Kelle cheese (sheep-cow milk-Manyas/Balıkesir)	–	–	–	–	–	–	–	–	+	–
E. faecalis MBE29-3	Kelle cheese (sheep-cow milk-Manyas/Balıkesir)	–	–	–	–	–	–	–	–	+	–
E. faecalis MBE31-4	White cheese (cow milk-Denizli)	+	–	–	–	–	–	–	–	+	–

Table IV
Cross protection activity of the enterocin producer *Enterococcus* sp. strains.

Producing strains	Indicator strains										
	MBE 1-9	MBE 12-3	MBE 15-2	MBE 16-5	MBE 20-5	MBE 22-2	MBE 27-2	MBE 27-3	MBE 29-1	MBE 29-3	MBE 31-4
E. faecalis MBE1-9	–	+	+	+	–	+	+	+	+	–	+
E. faecium MBE12-3	–	–	–	+	+	+	–	–	+	+	+
E. faecium MBE15-2	–	–	–	+	–	+	–	–	+	–	+
E. faecium MBE16-5	–	–	–	–	–	–	–	–	+	–	+
E. faecalis MBE20-5	–	–	–	–	–	–	–	–	–	–	–
E. faecium MBE22-2	–	–	–	–	+	–	–	–	+	–	–
E. faecium MBE27-2	–	–	–	+	+	+	–	–	+	–	+
E. faecium MBE27-3	–	–	–	+	+	+	–	–	+	–	+
E. faecalis MBE29-1	–	–	–	–	–	–	–	–	–	–	–
E. faecalis MBE29-3	–	+	+	+	+	+	–	–	+	–	+
E. faecalis MBE31-4	–	–	–	–	–	–	–	–	–	–	–

Table V
Antibiotic susceptibility of enterocin producer *Enterococcus* sp. strains.

Strains	Antibiotics*																	
	S	CN	MH	TEC	DO	CIP	NOR	QD	RD	VA	TE	P	E	AMP	F	LZD	C	LEV
E. faecalis MBE1-9	R[2]	S	S	S	S	S	I	R	S	S	I	S	I	S	S	S	R	S
E. faecium MBE12-3	S	S	S	S	S	I	S	S	R	S	S	S	I	S	S	S	S	S
E. faecium MBE15-2	S	S	S	S	S	R	R	S	R	S	S	R	I	S	S	S	S	S
E. faecium MBE16-5	S	S	S	S	S	I	S	I	R	S	S	R	R	S	S	S	S	S
E. faecalis MBE20-5	S	S	I	S	S	S	S	S	R	S	R	S	I	S	S	S	S	S
E. faecium MBE22-2	S	S	S	S	S	I	S	I	R	S	S	S	R	S	S	S	S	S
E. faecium MBE27-2	S	S	S	S	S	S	S	S	R	S	S	S	I	S	S	S	S	S
E. faecium MBE27-3	S	S	S	S	S	R	I	S	R	S	S	S	I	S	S	S	S	S
E. faecalis MBE29-1	S	S	S	S	S	S	S	R	I	I	S	S	S	S	S	S	S	S
E. faecalis MBE29-3	S	S	S	S	S	S	S	R	I	S	S	S	I	S	S	S	S	S
E. faecalis MBE31-4	S	S	S	S	S	I	S	R	R	S	S	S	S	S	S	S	S	S

* DO: doxycycline (30 µg), NOR: norfloxacin (10 µg), VA: vancomycin (30 µg), C: chloramphenicol (30 µg), RD: rifampicin (5 µg), MH: minocycline (30 µg), P: penicillin (10 µg), S: streptomycin (300 µg), LEV: levofloxacin (5 µg), CIP: ciprofloxacin (5 µg), AMP: ampicillin (10 µg), QD: quinupristin-dalfopristin (15 µg), F: Nitrofurantoin (300 µg), TE: tetracycline (30 µg), CN: gentamicin (120 µg), E: erythromycin (15 µg), LZD: linezolid (30 µg), TEC: teicoplanin (30 µg). Susceptibilities of *Enterococcus* sp. strains were determined according to CLSI 2012. [2]R: Resistant, I: Intermediary, S: sensitive.

Presence of *van*A and *van*B. *E. faecalis* MBE1-9, *E. faecalis* MBE20-5, *E. faecalis* MBE29-1 and *E. faecalis* MBE29-3 strains carry the *van*A gene among the 11 enterocin producer. Remaining enterocin producers do not contain both *van*A and *van*B genes (Table VI).

Detection of virulence factors. The $efaA_{fs}$, *cad* and cyl_M genes were not detected in any strains while *ccf* and *cyl*B genes were amplified by all the strains. The esp_{fm} gene was found only in *E. faecalis* MBE20-5. esp_{fs} was found in *E. faecalis* MBE20-5 and MBE31-4 and *agg* was found in *E. faecalis* MBE1-9 and MBE29-3, respectively. *gel*E gene was detected in all *Enterococcus* stains except *E. faecium* MBE22-2. *cpd*, *cob*, *ace* and *cyl*A were also found in several isolates. Virulence traits of all *Enterococcus* sp. strains are given in Table VI.

Detection of decarboxylase activity and amino acid decarboxylase genes. The isolated DNA of the *Enterococcus* sp. strains was subjected to PCR amplification to detect the presence of the histidine, lysine, ornithin and tyrosine decarboxylase genes (*hdc*, *ldc*, *odc* and *tdc*, respectively). All of the enterocin producer strains except *E. faecalis* MBE1-9, have phenotypically tyrosine decarboxylase activity and *tdc* gene was also detected in the same strains. The *hdc*, *ldc* and *odc* genes were not detected in any strains (Table VI).

Table VI
Presence of virulens factors, vancomycin and biogenic amine genes in *Enterococcus* sp. strains.

Strains	Virulens factors*													Vanco-mycin**			Biogenic amine***			
	efaAfm	*efaAfs*	*ccf*	*cpd*	*cob*	*cad*	*espfm*	*espfs*	*ace*	*gelE*	*agg*	*cylA*	*cylB*	*cylM*	*vanA*	*vanB*	*hdc*	*tdc*	*odc*	*ldc*
E. faecalis MBE1-9	–	–	+	+	+	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–
E. faecium MBE12-3	+	–	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	–
E. faecium MBE15-2	+	–	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	–
E. faecium MBE16-5	–	–	+	+	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	–
E. faecalis MBE20-5	+	–	+	+	+	–	+	+	+	+	–	+	+	–	+	–	–	+	–	–
E. faecium MBE22-2	+	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–	–
E. faecium MBE27-2	+	–	+	+	–	–	–	–	–	+	–	+	+	–	–	–	–	+	–	–
E. faecium MBE27-3	+	–	+	+	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	–
E. faecalis MBE29-1	+	–	+	+	+	–	–	–	+	+	–	+	+	–	+	–	–	+	–	–
E. faecalis MBE29-3	–	–	+	+	+	–	–	–	+	+	+	+	+	–	+	–	–	+	–	–
E. faecalis MBE31-4	–	–	+	+	–	–	–	+	+	+	–	–	+	–	–	–	–	+	–	–

*$efaA_{fm}$ and $efaA_{fs}$: cell wall adhesin (from *E. faecium* and *E. faecalis*, respectively); *ccf*, *cpd*, *cob* and *cad*: sex pheromone; esp_{fm} and esp_{fs}: enterococcal surface protein (from *E. faecium* and *E. faecalis*, respectively); *ace*: collagen adhesion; *gel*E: gelatinase; *agg*: aggregation substance; *cyl*M, *cyl*B, and *cyl*A: cytolysin.
**vanA: vancomycin A structural gene; *van*B: vancomycin B structural gene.
***hdc: histidine decarboxylase gene; *tdc*: tyrosine carboxylase gene; *odc*: ornitin decarboxylase gene; *ldc*: lisin decarboxylase gene.

Discussion

Enterococci easily adapt to several food systems due to their high salt and acid tolerance. It is believed that these bacteria contribute to the flavor and the aroma formation of some different European and Mediterranean cheeses. *E. faecium*, *E. faecalis* and *E. durans* species were frequently isolated from traditional dairy products (Giraffa, 2002; 2003; Martin-Platero *et al*., 2009; Yogurtcu and Tuncer, 2013; Furlaneto-Maia *et al*., 2014). In the present study a total of 100 *Enterococcus* strains were isolated from various artisanal cheese samples and 11 strains were determined as enterocin producers and also characterized on species level as *E. faecium* (6 strains) and *E. faecalis* (5 strains). The diversity of *Enterococcus* species from cheese depends on the ecological niche that they were isolated from (Rivas *et al*., 2012). Similar to other studies we were found that *E. faecium* and *E. faecalis* were mostly isolated enterococcal strains from traditional Turkish cheeses (Tuncer, 2009; Yogurtcu and Tuncer, 2013).

Some *Enterococcus* sp. strains show inhibition activity with their enterocins against food spoilage and food pathogen microorganisms (Khan *et al*., 2010; Javed *et al*., 2011; Özden-Tuncer *et al*., 2013). It was observed that all *E. faecium* and *E. faecalis* strains showed inhibition zones to all *Listeria* sp.and *Staphylococcus* sp. strains used in this study besides closely related species. On the other hand, inhibition zones against 2 *Salmonella* and 1 *E. coli* strains were also determined. Garcia-Cano *et al*. (2014), indicated that 1 *E. faecium* and 1 *E. faecalis* strains isolated from Cotija cheese displayed inhibitor activity against *S. enterica* Typhimurium, *E. coli* besides *L. monocytogenes* and *S. aureus* similar to our findings.

E. faecalis MBE29-1 and *E. faecalis* MBE29-3 strains were found to harbor only *ent*X structural gene while *E. faecium* MBE16-5 and *E. faecalis* MBE31-4 strains carried *ent*A and *ent*X; *E. faecium* MBE15-2 and *E. faecium* MBE 27-3 strains carry *ent*A, *ent*B and *ent*X; *E. faecium* MBE12-3 strain carried *ent*A and *ent*B; *E. faecium* MBE22-2 strain carried *ent*A, *ent*P and *ent*X structural genes. *E. faecium* MBE27-2 was identified as the only producer strain that harbored 4 structural genes (*ent*A, *ent*B, *ent*P and *ent*X) at the same time. It is not unusual that multiple structural enterocin genes may be present in one strain and it is indicated that many *Enterococcus* sp. strains produce more than one bacteriocin similar to our results (Edalatian *et al*., 2012; Özden-Tuncer *et al*., 2013; Rehaiem *et al*., 2014). On the other hand, *E. faecalis* MBE1-9 and MBE20-5 strains have not given the PCR product with the most common enterocin primers tested in this study suggesting that bacteriocins produced by these strains could be new enterocins. Further analyses should be needed for characterization of these enterocins.

However, many *Enterococcus* sp. strains adapt to food fermentation processes as a starter or as a protective culture, they may harbor antibiotic resistance and some virulence determinants that increase the

risk of pathogenicity of these strains (Martin-Platero *et al*., 2009; Edalatian *et al*., 2012). In the present study, 11 enterocin producer strains were found to be resistant to one or more antibiotics. All strains showed sensitivity against vancomycin but four of them were carrying *vanA* genes. Furlaneto-Maia *et al*. (2014), reported that 5 *Enterococcus* sp. isolates harbored *van*A gene, while they were susceptible to vancomycin phenotypically. In addition the same results for ampicillin, tetracycline and linezolid susceptibilities were attained in the study of Pesavento *et al.* 2014. We determined that all enterocin producer strains showed susceptibility against gentamicin, teicoplanin, doxycycline, ampicillin, nitrofurantoin, linezolid and levofloxacin. On the other hand, most of the resistance in the strains obtained against rifampicin and quinupristin-dalfopristin similar to other results obtained in *Enterococcus* sp.strains isolated from Turkish Tulum cheese (Özden-Tuncer *et al*., 2013).

Only *E. faecalis* MBE29-3 showed gelatinase activity on Todd-Hewitt agar but all the enterocin producers strains except *E. faecium* MBE 22-2 had gene *gel*E. These results suggest that in strains which have no gelatinase activity phenotypically associated with *gel*E the gene was silent or expressed at a low level (Lopes *et al*., 2006). Similar observations were indicated for clinical and food originated *Enterococcus* isolates (Cariolato *et al*., 2008; Ben Belgacem *et al*., 2010; Özden-Tuncer *et al*., 2013; Inoğlu and Tuncer, 2013; Tuncer *et al*., 2014; Medeiros *et al*., 2014). In addition, many of the virulence genes were detected in enterocin producer strains. PCR analysis revealed that all strains contained at least 3 virulence genes. However, sex pheromone genes (*cpd*, *cob*, *ccf* and *cad*) were not accepted as virulence factors their production in *Enterococcus* sp. strains are assume that dissamination of virulence factors, and these genes were detected frequently in *Enterococcus* sp. isolates from cheese (Eaton and Gasson, 2001; Valenzuela *et al*., 2008; Ben Belgacem *et al*., 2010; Inoğlu and Tuncer, 2013). Martin *et al*. (2006), confirmed that $efaA_{fs}$ and $efaA_{fm}$ were only found in *E. faecalis* and *E. faecium*, respectively. However, Barbosa *et al*. (2010) and Inoğlu and Tuncer (2013) were indicated that $efaA_{fs}$ gene found in *E. faecium* and $efaA_{fm}$ was found in *E. faecalis* strain, as confirmed in this study. The *agg* gene product mediates the establishment of contact between bacteria and host cells and facilitating colonization, therefore *agg* gene harboring strains were unwanted in food. The *agg* gene was detected only in *E. faecalis* strains by other researchers (Eaton and Gasson, 2001; Franz *et al*., 2001; Martin *et al*., 2006; Inoğlu and Tuncer, 2013), as confirmed in this study. However, Barbosa *et al*. (2010), showed that *agg* gene may be found in *E. faecium* strains too. The *ace* gene was also having an important role in colonization like *agg* gene and it is reported that *Enterococcus* sp. isolates containing *ace* gene were found to be positively correlated with biofilm formation (Singh *et al*., 2010, Medeiros *et al*., 2014). In our study, the strains containing the *agg* and *ace* genes individually and the strains possesing also both genes should be investigated for biofilm formation in a further study.

Cytolysin is a bacterial toxin involved in the formation of haemolytic activity. Several studies confirmed that *Enterococcus* sp. strains harboring cytolysin genes may have β-haemolytic activity (Eaton and Gasson, 2001; Theppangna *et al*., 2007). However, different researchers found that, non β-haemolytic strains may carry the cytolysin genes similar to the present study (Cosentino *et al*., 2010; Medeiros *et al*., 2014). Medeiros *et al.* (2014), showed that *Enterococcus* sp. isolate originated from food had non β-hemolytic activity, but carried *cyl*A gene, as confirmed in this study. It is also reported that in non haemolytic strains cytolysin determinant may behave as silent. On the other hand, the lack of a correlation between phenotypic and genotypic cytolysin production may suggest some genetic rearrangements in *cyl* operon like missing genes (Semedo *et al*., 2003, Gaspar *et al*., 2009). In our study *cyl*B gene was detected in all strains while *cyl*A gene was detected only in 5 strains. However, *cyl*M was not detected in any strains and in all strains the lack of β-haemolytic activity was determined.

Another undesirable case for *Enterococcus* sp. strains is biogenic amine production ability (Valenzuela *et al*., 2008). In the present study, tyrosine decarboxylating ability was determined in 10 *Enterococcus* sp. strains phenotypically and gene *tdc* was also detected in the same strains. Only *E. faecalis* MBE1-9 has no decarboxylating tyrosine does not harbor *tcd* gene as well. Similar to our results, several researchers indicated that tyramine was frequently produced by *Enterococcus* sp. strains (Tuncer, 2009; Komprda *et al*., 2010; Kalhotka *et al*., 2012; Inoğlu and Tuncer, 2013). PCR study showed compatibility with the result of the phenotypically tyrosine decarboxylating ability, as confirmed by Inoğlu and Tuncer (2013) and Yüceer and Özden-Tuncer (2015).

Conclusion

Many of the enterococci originating from food may produce enterocins are active against pathogenic or spoilage microorganisms, particularly *Listeria* spp. and *Staphylococcus aureus*. In the present study enterocins were identified using known enterocin primers and multiple enterocin genes were detected in 7 strains. Two enterocins could not be identified and need to be investigated whether they are new bacteriocins.

However, all enterocin producers were found to have several virulence factors such *i.e. gel*E, *agg*, *cpd*, *cob*, *ace*, *cyl*A and *tdc* genes. In addition 6 strains had multiple antibiotic resistance patterns. Lack of hemolytic and gelatinase activity (except *E. faecalis* MBE29-3) in bacteriocin producing strains are advantageous, although their virulence characteristics make them risky in terms of consumer health.

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ORIGINAL PAPER

Intracellular Siderophore Detection in an Egyptian, Cobalt-Treated *F. solani* Isolate Using SEM-EDX with Reference to its Tolerance

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Abstract

An Egyptian, plant pathogenic *Fusarium solani* isolate was grown on cobalt concentrations of 0, 50, 200, 500, 800, and 1000 ppm. The isolate survived concentrations up to 800 ppm, however failed to grow at 1000 ppm. Morphology and elemental analysis of the isolate under the investigated Co concentrations were studied using Scanning electron microscopy (SEM) and energy dispersive X-ray microanalysis (EDX). The isolate reserved its morphology up to a concentration of 200 ppm. Morphological distortions were dramatic at 500 and 800 ppm. EDX detected Co uptake through the hyphae, microconidia, macroconidia, and chlamydospores. Iron, calcium, and potassium were also detected. EDX results showed a linear relationship between Co% and Fe% up to a concentration of 500 ppm reflecting the possible ability of the isolate to synthesize intracellular siderophores storing iron and their release from the vacuoles. The participation of such siderophores in conferring tolerance against cobalt is discussed. At 800 ppm, the % of Fe was greatly reduced with an accompanying increase in morphological distortions and absence of microconidia. Increasing the implicated cobalt concentrations resulted in increasing the percentages of the chelated cobalt reflecting the possible implication of the cell wall as well as extracellular siderophores in the uptake of cobalt. The current results recommend the absence of cobalt in any control regime taken to combat the investigated *F. solani* isolate and highlights the accomplishment of biochemical, ultrastructural, and molecular studies on such isolate to approve the production of siderophores and the role of cell wall in cobalt uptake.

Key words: *Fusarium solani*, cobalt uptake, cobalt tolerance, siderophores

Introduction

Ions of the metals sodium, magnesium and potassium participate in the basic metabolism of cells and are therefore absolutely essential for life. They are present in high quantities in microbial cells with concentrations in the mM range (Heldal *et al*., 1985). The metals iron, cobalt, nickel, copper, manganese, zinc and molybdenum are also involved in cellular processes and are present in mM concentrations (Heldal *et al*., 1985). Most of the other metals, including lead, mercury, aluminum and silver, have absolutely no biological function and are therefore termed abiotic elements. Metals involved in cellular processes are often cofactors of enzymes. A systematic analysis of 1371 enzymes showed that 47% contain a metal ion (Andreini *et al*., 2008).

Hence, cobalt is an essential metal and is needed in trace amount by the organisms; it is used as a cofactor of vitamin B12 and other enzymes in yeast, animals, bacteria, archaea and plants. However, at higher concentrations, cobalt becomes toxic for living systems but the exact mechanism of this toxicity is still poorly understood. Metal resistant microorganisms are important as they help in understanding metal-microbe interactions (Saad, 2014).

In general, two mechanisms have been proposed for heavy metal tolerance in fungi: 1. Extracellular (chelation and cell-wall binding) sequestration. 2. Intracellular physical sequestration of metal by binding to proteins or other ligands to prevent it from damaging the metal sensitive cellular targets (Anahida *et al*., 2011). Binding to the cell wall is called biosorption. The cell surface of fungi is negatively charged owing to the presence of various anionic structures, such as glucan and chitin (Bellion *et al*., 2006; Maghsoodi *et al*., 2007). This gives fungi the ability to bind metal cations. In the intracellular mechanism, metal transport proteins may be involved in metal tolerance, either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into vacuolar compartment (Bellion

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et al., 2006; Abdul-Tawab and Maqsood, 2007). Besides, the mechanisms of heavy metals toxicity and resistance are different and they depend on the fungal genera and species (Al-Yemeni and Hashem, 2006).

Iron is an essential nutrient for virtually all forms of life (Winkelmann, 1991). All microorganisms examined so far, with the exception of certain lactobacilli, require iron (Haas *et al*., 1999). Iron participates in a large number of cellular processes, the most important of which are oxygen transport, ATP generation, cell growth and proliferation, and detoxification. It is a coenzyme or enzyme activator of ribonucleotide reductase, a key enzyme for DNA synthesis, which catalyzes the conversion of ribonucleotides to deoxyribonucleotidides and particularly of deoxyuridine to thymidine (Thelander *et al*., 1983).

In the aerobic environment, iron exists mainly as Fe(III) and tends to form insoluble hydroxides and oxyhydroxides (Lesuisse and Labbe, 1994), making it largely unavailable to microorganisms. Iron uptake by fungi is accomplished by specific transport systems, in which an initially Fe^{3+} form is reduced to Fe^{2+} iron, through the action of specific cell surface reductases (ferroxidases). Ferrous iron is then internalized by three different mechanisms, one of such mechanisms or iron acquisition involves the production of siderophores, which are excreted through the fungal wall in the deferric form, bind iron, and then are taken up by the fungi (de Locht *et al*., 1994; Nyilasi *et al*., 2005).

Siderophores are low molecular weight (500–1000 Da), iron-chelating ligands synthesized by microorganisms (Winkelmann, 1991). Most bacteria and fungi produce siderophores (Neubauer *et al*., 2000). Most of the characterized fungal siderophores belong to hydroxamate-type whose basic structural unit is N^5-acyl-N^5-hydroxyornithine (Winkelmann, 1991; 1992). With one exception (neurosporin), all are derived from L-ornithine. Fungal hydroxamate siderophores can be divided into three structural families: fusarinines, coprogens and ferrichromes (van der Helm and Winkelmann, 1994).

Since the Fe level in the cell is a critical factor, microorganisms possess a tight regulation in enzymes and transport systems for siderophore biosynthesis, secretion, siderophore-delivered iron uptake, and iron release. Nonribosomal peptide synthetases (NRPS) pathway and independent of NRPS are the major pathways that are reported for siderophore biosynthesis (Miethke and Marahiel, 2007). It has been reported that many siderophores are peptides biosynthesised by the well-studied nonribosomal peptide synthetase (NRPS) multienzyme family (Seneviratne and Vithanage, 2015).

Fusarium solani, belongs to ascomycetes, is widely found in soil and constitutes one of the most important phytopathogens in agriculture. It infects cultivars like soybean, bean, cassava, and potato, causing root and fruit rot, as well as wilting of the plant upper parts (Olivieri *et al*., 2002; Poltronieri *et al*., 2002).

Expert *et al*. (2012) reported that the plant iron status can influence host-pathogen relationships in different ways by affecting the pathogen's virulence as well as the host's defense. Also, Haas (2012) reported that the control over access to iron is one of the central battlefields during infection as pathogens have to "steal" the iron from the host.

Like other members of the microbial world interacting with animals and humans, plant pathogenic microorganisms have evolved a diversity of systems allowing them to capture iron from various environments in response to their metabolic needs. Indeed, experimental investigations have permitted to underscore the importance of siderophores and corresponding transport machineries in iron nutrition of phytopathogenic species (Expert *et al*., 2012).

Regarding the ascomycete *Fusarium graminearum*, two NRPSs were identified, one encoded by the *NPS2* gene and the other by the *NPS6* gene; *NPS2* gene responsible for the production of an intracellular siderophore while *NPS6* gene for an extracellular one (Oide *et al*., 2006; 2007). Another study on *Fusarium oxysporum* (strain FGSC 9935) reported that this fungus produces three different ferrichrome-type siderophores, ferricrocin, ferrichrome C, and malonichrome (Lopez-Berges *et al*., 2012)

The current work aims at investigating the effect of different cobalt concentrations on an Egyptian, plant-pathogenic *F. solani* islolate using the rapid, cost-effective, successful SEM-EDX technique in an attempt to study the possible tolerance approaches exhibited by the fungal isolate to cope with cobalt.

Experimental

Materials and Methods

Fungal isolate. *F. solani* was isolated from an Egyptian broad bean cultivar suffering root rot and identified at the culture collection unit of the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University.

Media and growth conditions. Czapek's Dox medium was supplemented with different cobalt concentrations of 0, 50, 200, 500, 800 and 1000 ppm (mg Co/l). Except for the control, cobalt replaced ferrous sulfate. Cobalt chloride was the salt used to prepare the different cobalt concentrations. Media were autoclaved and poured into sterile Petri dishes (two Petri dishes for each cobalt concentration). The plates were inoculated with a 7-day old *F. solani* and incubated at 25°C for seven days.

F. solani was grown on media supplemented with different cobalt concentrations (50, 200, 500, 800 and

1000 ppm). Morphological investigations were carried out using scanning electron microscopy. Energy-dispersive X-ray spectroscopy (EDX) was used to identify the elements associated with *F. solani* together with their percentage with respect to one another at the different investigated cobalt concentrations. The distribution of the elements in macroconidia, microconidia, chlamydospores and hyphae was also investigated.

Scanning electron microscopy (SEM). Blocks of the investigated fungal isolate were prepared for SEM at The Regional Center for Mycology and Biotechnology, Al-Azhar Univ. according to Zain (1998). Fixation and dehydration procedures were performed using the programmable LEICA EM TP tissue processor model (A-1170), where six to eight millimeter squares of agar with fungal growth were cut from the 14-day old cultures. The squares were then fixed by immersion in 2% (w/v) aqueous osmium tetroxide (OsO_4) at 4°C for 12 h. Fixed material was allowed to attain room temperature and then washed in distilled water (3 times, 10 min each) to remove excess of OsO_4. Fixed and washed materials were submerged and dehydrated through a graded, 10% steps, ethanol series from 10% to 90% and finally absolute ethanol. Dehydrated specimens were critical point-dried using the Critical Point Dryer EMS (Electron Microscopy Sciences) model EMS 850. The critical point-dried specimens were then attached to 0.9 mm diameter copper stubs using a carbon adhesive. Specimens were gold-coated (nearly 50 nm thickness) using an SPI ModuleTM Sputter Coater and then examined using the high-vacuum mode of a JEOL JSM-5500LV Scanning Electron Microscope. Energy-dispersive X-ray spectroscopy (EDX). Elemental analysis (the percentage of the detected elements with respect to one another) of the samples was carried out using the X-ray detector (INCAxsight, Oxford Instruments) of the scanning electron microscope (Jeol JSM-5500LV). Window Integral was the mode of analysis. The given individual percentages represent the average of ten measurements for each of the macroconidia, microconidia, hyphae and chlamydospores, and then the total average of each element for each cobalt concentration was calculated. It should be noted that SEM-EDX measures the percentage of the detected elements with respect to one another so that the sum of percentages of all the elements detected is 100.

Results

The investigated *F. solani* isolate exhibited growth on 50 and 200 ppm. Poor growth was observed at 500 and 800 ppm, while no growth was found at a cobalt concentration of 1000 ppm.

Scanning electron micrographs (Figs. 1 and 2) illustrate the ability of the investigated *F. solani* isolate to cope with high Co concentrations up to a Co conc. of 800 ppm.

Clumps or aggregates of hyphae appeared at a concentration of 50 ppm (Fig. 1d and e) and 200 ppm (Fig. 1f). Additionally, at 200 ppm, fusion among the hyphae was observed (Fig. 1h). Hyphae were greatly distorted at higher cobalt concentrations of 500 ppm and 800 ppm (Fig. 2).

At a concentration of 200 ppm, conidia were found to decrease in amount and were not easy to find. At such concentration, most of the found conidia possessed rough surfaces which, on a high magnification of 25000X (Fig. 1g), appeared like having tiny protrusions. At 500 and 800 ppm (Fig. 2), more reduction in the amount of conidia was observed. Furthermore, macroconidial shape was greatly distorted (Figs. 2b, e). Microconidia were completely absent at a conc. of 800 ppm. Additionally, chlamydospore production was greatly enhanced at Co concentrations of 500 and 800 ppm, where most of the hyphae possessed chlamydospores (Figs 2 a, c, and d).

Results of EDX on *F. solani* grown are shown in Table I. The elemental analysis was conducted on

Table I
EDX of different morphological structures of *F. solani* isolate grown at cobalt concentrations of 0, 50, 200, 500, and 800 ppm.

Cobalt concs.	Hyphae Individual average % of detected elements				Macroconidia Individual average % of detected elements				Microconidia Individual average % of detected elements				Chlamydospores Individual average % of detected elements				Total average %			
	Co	Ca	Fe	K	Co	Ca	Fe	K	Co	Ca	Fe	K	Co	Ca	Fe	K	Co	Ca	Fe	K
Control	–	8 C: 92	–	–	–	6.7 C: 93.3	–	–	–	3.6 C: 96.4	–	–	No chlamydospores were detected				–	7.35 C: 93.9	–	–
50 ppm	35.5	60.2	2.4	1.9	33.55	49	7.35	10.1	28.3	54.5	3.8	13.4	32.15	46.5	3.3	18	33.73	51.9	4.35	10
200 ppm	43.8	35.5	4.9	15.9	40.65	51.35	2.15	5.9	39	42.8	2	16.4	34.1	46.2	7.4	12.3	39.52	44.35	4.82	11.37
500 ppm	58.1	25.3	6.53	10	58.82	27	4.23	9.95	53	30.1	14.6	2.3	52.4	27.42	4.23	15.95	56.44	26.57	5	12
800 ppm	92.5	3.3	0.7	3.5	96.45	1.6	0.45	1.5	No microconidia were detected				95.5	3.2	0.3	1.1	94.81	2.73	0.45	2.03

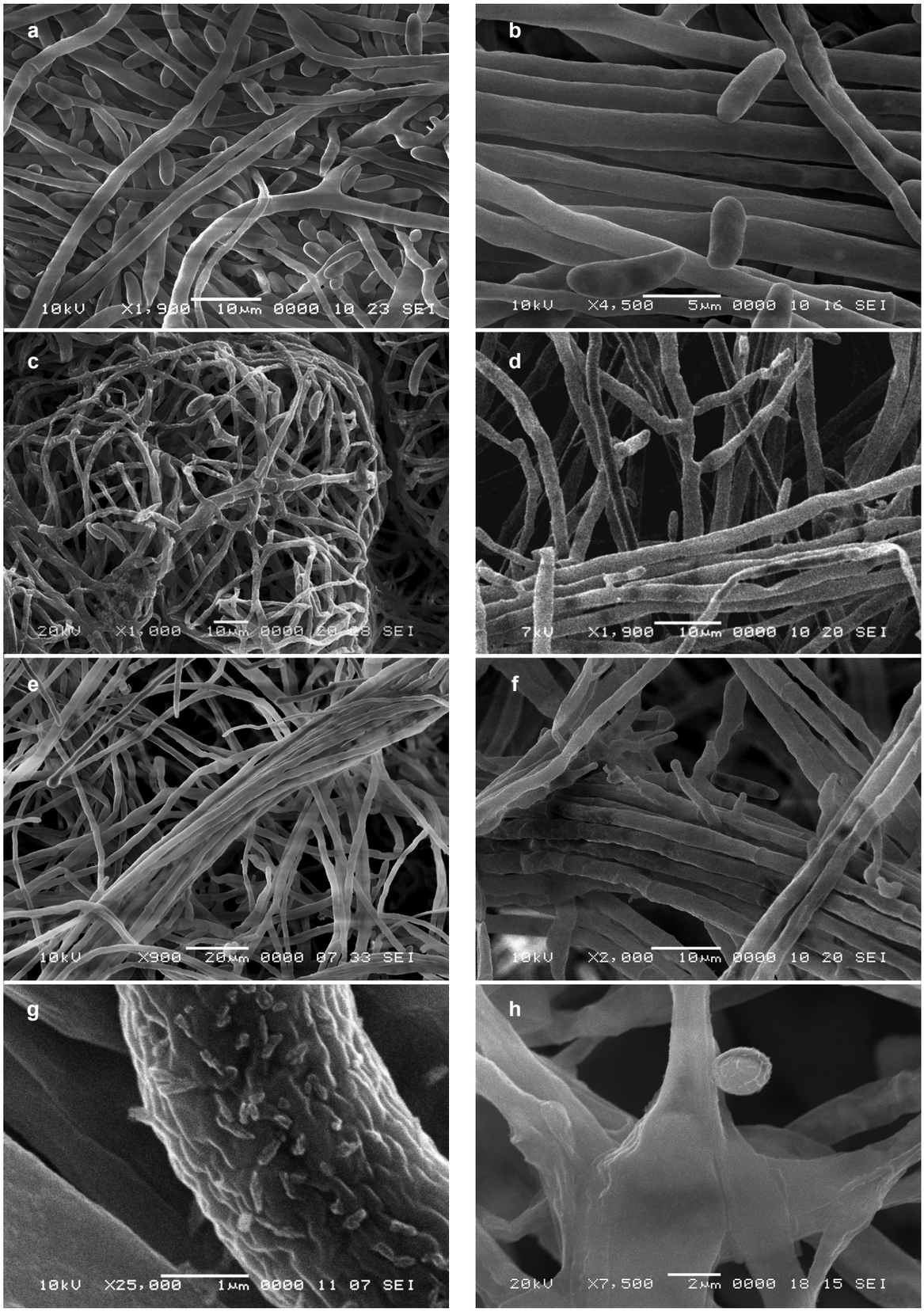

Fig. 1. Scanning electron micrographs of *F. solani*; control, a and b; 50 ppm Co, c, d, and e; 200 ppm Co, f, g, and h.

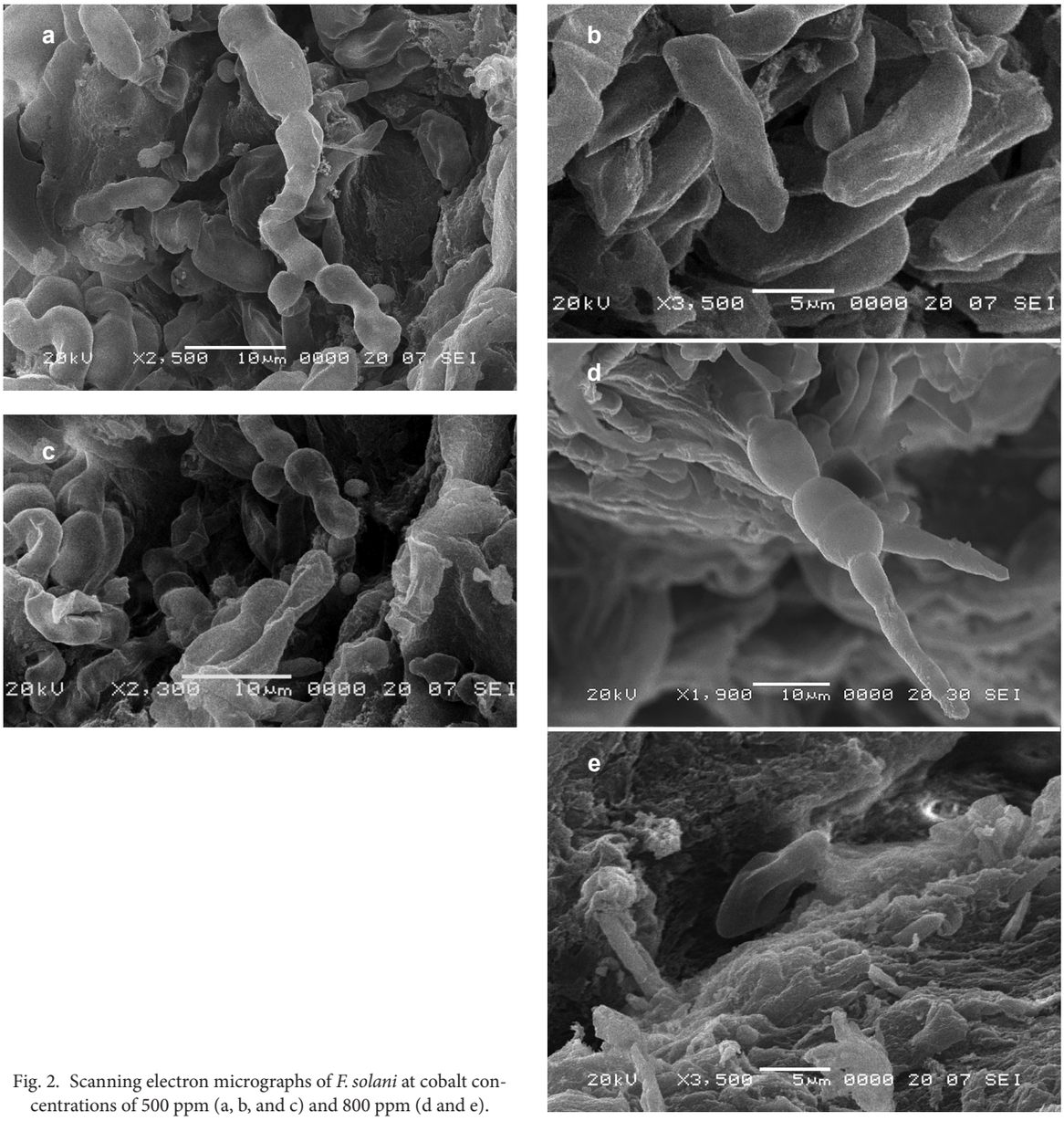

Fig. 2. Scanning electron micrographs of *F. solani* at cobalt concentrations of 500 ppm (a, b, and c) and 800 ppm (d and e).

the hyphae, macroconidia, microconidia, and chlamydospores of *F. solani* at all the investigated cobalt concentrations.

Regarding the control, in the absence of cobalt, only calcium was the detected element. The increase in the concentration of cobalt resulted in increasing cobalt uptake by the investigated *F. solani* isolate from a total average % of 33.73 at 50 ppm to 94.81 at 800 ppm. Increasing cobalt concentration also resulted in the detection of other elements which were not detected in the control, iron and potassium.

For calcium, its total average percentage jumped from 7.35, in case of the control, to 51.9 at a cobalt conc. of 50 ppm. Increasing Co conc. resulted in a gradual decrease in the total average percentage of Ca; 44.35, 26.57, and 2.73 at 200, 500, and 800 ppm respectively, however still high with respect to Fe and K. Regarding the *F. solani* morphological structure having the maximum Ca %, it was found to be different with the difference in Co Conc.; being the hyphae at 50 ppm (60.2%), the macroconidia at 200 ppm (51.35), the microconidia at 500 ppm (30.1), and the hyphae again at 800 ppm (3.3%). Chlamydospores never ranked the first as the structure possessing maximum Ca % at any of the investigated concentrations.

However for iron, increasing cobalt concentration up to a conc. of 500 ppm resulted in an increase in its total average percentage starting at a percentage of 4.35

at 50 ppm to 4.82% and then 5% at 200 and 500 ppm respectively. At a Co Conc. of 800 ppm, the pattern was reversed and the total average percentage of Fe dropped to a value of 0.45. Additionally, it is worth noting that different parts of *F. solani* responded differently to the increase in Co conc in the view of Fe detection; where at a conc. of 50 ppm, the maximum iron average % was detected in macroconidia, at 200 ppm in chlamydospores, at 500 ppm in microconidia, and at 800 ppm in hyphae.

Potassium followed the same pattern as iron showing a gradual increase in its values with increasing cobalt concentration up to 500 ppm, after which, at 800 ppm, the total average % of K dropped from 12% (at 500 ppm) to 2.03%. Chlamydospores represent the site where potassium percentage is usually elevated, at a Co conc of 50 and 500 ppm. However at 200 and 800 ppm, K % was the highest in microconidia and hyphae respectively.

Discussion

Fusarium, as a plant pathogen, have higher requirements for Fe or higher utilization efficiency compared with higher plants. Therefore, Fe differs from the other micronutrients such as Mn, Cu and B, for which microbes have lower requirements (Dordas, 2008). Furthermore, Sow *et al*. (2009) reported that particularly for fungi, iron is a major virulence factor.

F. solani is a widely distributed soil-borne fungus causing wilt and rot diseases on a wide variety of crops (Bogale *et al*., 2009). According to the available research, the effect of cobalt on *Fusarium* spp. has been investigated with little reference to the species *solani*. Hashem and Bahkali (1994) showed that a strain of *F. solani* could grow in the presence of cobalt up to a concentration of 300 ppm, however the scavenging mechanisms of *F. solani* towards cobalt was not studied. Hong *et al*. (2009) studied the effect of *F. solani* isolates on Cu, Zn, and Pb, *i.e*., cobalt wasn't involved in the study.

Hence according to the available research, the current study could be regarded the first dealing with the effect of cobalt on plant-pathogenic *F. solani* with reference to the possible tolerance mechanisms implicated using SEM-EDX.

Scanning electron micrographs revealed that different structures of the investigated isolate responded morphologically to the different cobalt concentrations implicated through forming hyphal clumps or aggregates (at 50 and 200 ppm), hyphal fusion (at 200 ppm), hyphal distortion (at 500 and 800 ppm), reduced macroconidial amounts (at 200, 500 and 800 ppm), absence of microconidia (at 800 ppm), and excessive formation of hyphal chlamydospores (at 500 and 800 ppm). Bautista-Baños *et al*. (2012) studied morphological and cellular alterations in *A. alternata*, *F. oxysporum* and *R. stolonifer* due to chitosan application using SEM and reported the value of EM technology as a tool that enables visualizing internal and external changes occurring in fungi treated with the investigated natural compounds.

Energy dispersive X-ray microanalysis (EDX) of the current study revealed that iron was not detected in the control, however the introduction of cobalt concentrations resulted in considerable iron levels detected by EDX in the form of considerable percentages. Such detectable levels could never be only due to iron sequestration from the distilled water used in preparing the growth medium from which ferrous sulphate was removed to eliminate any source for heavy metal other than cobalt. Hence, the only possible explanation for Fe detection is the ability of the investigated plant pathogenic *F. solani* isolate to synthesize intracellular siderophores capable of storing iron inside vacuoles. Being subjected to high toxic cobalt concentrations, the *F. solani* isolate responded by releasing the vacuolar Fe-siderophore complexes in an attempt to survive the stress exerted by such cobalt concentrations.

This conclusion could be supported by not detecting any iron percentages in the control sample for being stored inside the vacuoles away from the sensitivity of the electron beam which detects elements only in the cell wall, cell membrane and cytoplasm but cannot penetrate deep into the cellular organelles (Farrag *et al*., 2008; Farrag, 2009).

As revealed by SEM micrographs, morphological distortions in hyphae and macroconidia regarding shape and number were observed at 500 and 800 ppm with the last concentration resulting in more adverse morphological effects and accompanied with the least individual and total iron percentages as well as the highest cobalt percentages.

Hence, without refraining the role of cobalt, a relationship between such distortions and the release of vacuolar intracellular siderophores in response to cobalt might be assumed, supported by Eisendle *et al*. (2006) who reported that deficiency in intracellular siderophores causes a reduction of asexual conidiospore production in *Aspergillus nidulans*, which can be partly cured by supplementation with high concentrations of iron. They also concluded that the intracellular siderophore is a central component of the fungal physiology, as it is involved in iron storage, oxidative-stress resistance, and germination. Additionally, it has been demonstrated that intracellular siderophores in *F. graminearum* have roles in certain types of fungal development such as asexual and sexual sporulation (Eisendle *et al*., 2003; Oide *et al*., 2007; 2015).

Schrettl *et al*. (2007) and Wallner *et al*. (2009) reported that intracellular siderophores are believed to function in iron storage.

Recently, Oide *et al*. (2015) reported the presence of the intracellular siderophore ferricrocin in the culture filtrate of *F. graminearum* (meaning it could also be regarded as an extracellular siderophore) and stated that designation of ferricrocin as strictly intracellular appears to be an oversimplification. They also reported its doubtful role in iron uptake as an extracellular siderophore. They additionally added that due to its property as an iron storage molecule, ferricrocin iron release may be strictly regulated, and therefore iron bound to ferricrocin may not be a good source of nutritional iron.

However, several studies indicated that cobalt might compete with iron for specific binding sites in certain proteins, thereby impairing their functions (Goldberg *et al*., 1988; Bunn *et al*., 1998). Also, Stadler and Schweyen (2002) reported that cobalt might displace iron in yeast iron-sensing factors.

In the current study and in the light of the above mentioned references, it could be assumed that the presence of cobalt facilitated the release of iron from the vacuolar-free, intracellular siderophore so that the released iron can participate in overcoming cobalt stress and at the same time cobalt is chelated by forming a Co-siderophore complex.

Another possible assumption, is the ability of the *F. solani* isolate to synthesize other types of extracellular siderophores, forming Co-siderophore complexes, in response to cobalt presence. This could be supported by the fact that intracellular siderophores are only found in siderophore-producing fungi (Haas, 2012).

EDX results could support the first assumption regarding iron release where a pattern of increase in the total average % of Fe with increasing cobalt concentrations was observed up to a concentration of 500 ppm. However, at 800 ppm, the total average % of Fe was decreased to a very low % of 0.45. At 1000 ppm, the fungus failed to grow reflecting the possible inactivation of the enzymes controlling the release process of iron whose activity was greatly reduced with the high cobalt conc. of 800 ppm and completely inhibited at 1000 ppm.

The above results could be supported by Philpott (2006) who reported the presence of enzymes responsible for catalyzing the reduction or degradation of intracellular siderophores. Additionally, the strong inhibition of enzyme activity exerted by high heavy metal concentrations has been well documented by many researchers (Oliviera and Pampulha, 2006; Wang *et al*., 2008).

Furthermore, cobalt competition might have impaired iron-dependent enzymes such as aconitase and catalase A, thereby decreasing conidial size and conidial resistance to oxidative stress (Schrettl *et al*., 2007; Wallner *et al*., 2009).

In support for cobalt uptake by siderophores, it has been reported that not only with Fe, siderophores are able to form complexes with other metals as well (Yeterian *et al*., 2010). Additionally, it has also been reported that toxic metals induce the production of some siderophores suggesting that these chelators may play a role in microbial heavy metal tolerance (Schalk *et al*., 2011). Braud *et al*. (2010) reported that growth assays showed that *Pseudomonas aeruginosa* strains capable of producing pyoverdine and pyochelin, siderophores, appeared to be more resistant to metal toxicity than a siderophore non-producing strain.

Additionally, *F. solani* production of siderophores for sequestering heavy metals was reported by Hong *et al*. (2009) who observed that all the *F. solani* isolates tested showed positive CAS reactions (chrome azurol S agar plate siderophore assay) and hence reported that it is possible that the *F. solani* siderophores may have solubilized and sequestered Cu and Zn from wastes contaminated singly or in combination with these heavy metals.

Cell wall can be assumed to participate in elevating the observed percentage of the chelated cobalt due to the observed elevations in the % of Ca and K, where studies have shown that Mg, Ca, K and hydrogen ions were released from fungal biomass as a result of biosorption (Akthar *et al*., 1996; Kapoor and Viraraghavan, 1997). Muraleedharan *et al*. (1994) reported that the majority of the metal taken up through biosorption was exchanged with calcium present on the cell wall. Additionally, Tsekova *et al*. (2006) studied heavy metal biosorption in *Penicillium cyclopium* and reported that the biosorption of copper and cobalt on raw biomass was accompanied with the release of Mg, Ca and K ions into the reaction mixture.

It is worth noting that SEM-EDX has proved successful in explaining the tolerance of *Penicillium brevicompactum* against cobalt where Farrag *et al*. (2008) reported that SEM-EDX might reflect the possible presence of two mechanisms conferring tolerance to *P. brevicompactum* against cobalt; cell wall and thiol peptides. Then, Farrag (2009) confirmed such conclusions through biochemical and ultrastructural studies proving the efficiency of SEM-EDX as a rapid, cost-effective, and successful technique to investigate and explain tolerance of fungi to heavy metals. SEM-EDX (used in Farrag *et al*., 2008) reached the same results that were reached using the simultaneous work of high performance liquid chromatography (HPLC), gel electrophoresis, capillary electrophoresis, and transmission electron microscope (TEM) (used in Farrag, 2009).

It should also be noted that SEM-EDX guides and paves the way to reach the correct chosen tolerance mechanism(s) of the fungus. For instance, the detection of sulphur by SEM-EDX in *P. brevicompactum* in response to cobalt highlights the possible ability of the fungus to imply metallothioneins in its tolerance

(Farrag *et al*., 2008; Farrag, 2009). However, the detection of Fe in the current study underscores the possible implication of siderophores in the tolerance of the investigated *F. solani* isolate against cobalt.

For the investigated *F. solani* isolate, due to the presence of a relationship between cobalt and increased iron demands, even at the least investigated cobalt concentration, as a mean to overcome the stress and toxicity of cobalt, it is not recommended to use cobalt or cobalt-amended fungicides for its combating as cobalt might induces the *in vivo* uptake of iron in such isolate and hence its survival and virulence. Also, this increased iron uptake boosts the competition on iron and affects the iron levels available for the plant.

Further biochemical, ultrastructural and molecular studies will be conducted on the investigated *F. solani* isolate to ensure the possible role of siderophores and cell wall in tolerance to cobalt.

Conclusively, the current study reflects the possible ability of the investigated plant pathogenic *F. solani* isolate to synthesize intracellular siderophores for iron storage and highlights the importance of such iron in conferring tolerance against cobalt. The role of extracellular siderophores as well as cell wall in cobalt tolerance is also possible.

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ORIGINAL PAPER

In vitro Interaction between Fumonisin B_1 and the Intestinal Microflora of Pigs

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Abstract

The caecal chyme of pigs was incubated anaerobically in McDougall buffer with and without fumonisin B_1 (5 μg/ml) for 0, 24 and 48 h. The plate count agar technique was applied for enumerating the amount of bacteria including aerobic, anaerobic bacteria, coliform, *Escherichia coli* and *Lactobacillus* sp. The quantitative polymerase chain reaction was also performed to estimate the number of copies of the total bacteria, *Lactobacillus*, *Bacteroides* and *Prevotella*. No significant differences in the amount of bacterial groups between the experimental (buffer, chyme, and fumonisin B_1) and control 1 groups (buffer + chyme) were observed in both methods. Fumonisin B_1 and hydrolysed fumonisin B_1 concentration were analysed by liquid chromatograghy – mass spectrometry. There was no significant difference in FB_1 concentration between the experimental and the control 2 group (buffer and fumonisin B_1) at 0 h incubation, 5.185 ± 0.174 μg/ml compared with 6.433 ± 0.076 μg/ml. Fumonisin B_1 concentration in the experimental group was reduced to 4.080 ± 0.065 μg/ml at 24 h and to 2.747 ± 0.548 μg/ml at 48 h incubation and was significantly less than that of in the control group. Hydrolysed fumonisin B_1 was detected after 24 h incubation (0.012 ± 0 μg/ml). At 48 h incubation time, hydrolysed fumonisin B_1 concentration was doubled to 0.024 ± 0.004 μg/ml. These results indicate that fumonisin B_1 can be metabolised by caecal microbiota in pigs though the number of studied bacteria did not change.

Key words: caecal microbiota, fumonisin B_1, intestinal microflora pig

Introduction

The fumonisins are known as important mycotoxins produced mostly by several *Fusarium* species which are found mainly in maize and its products all over the world. Because of the similarity between fumonisins and the sphingoid bases sphinganine (Sa) and sphingosine (So), fumonisin disrupts the sphingolipid metabolism and impacts on animal health. Pigs are high sensitive species to fumonisin B_1 (FB_1), the lowest observed adverse effect level (LOAEL) of fumonisin is 200 μg/kg b.w. per day (EFSA, 2005). It is very important to know the activities of fumonisin from the digestion on the cell systems. However, only a few studies have been conducted to determine fumonisin activities in the gastrointestinal tract of pigs, especially interaction between fumonisin and gut microbiota. Only one report showed the fumonisin was metabolised by the caecal microorganism (Fodor *et al.*, 2007) while there was no complete research about the effect of fumonisins on the intestinal microbiota in pigs. Some *in vivo*, not *in vitro* studies were carried out to estimate the impact of fumonisin on individual microbial species such as *Escherichia coli* and *Salmonella* sp. According to the results, intestinal colonization of pathogenic *E. coli* strain was significantly increased when pigs were treated with 0.5 mg of FB_1/kg of body weight for 7 days (Oswald *et al.*, 2003) while no change of *Salmonella enterica* growth was presented in pig intestine with the moderate dose of fumonisin (8.6 mg FB_1 and 3.2 mg FB_2 per kilogram feeding intake), it transiently affected the digestive microbiota balance (Burel *et al.*, 2013). It is essential to have more information about the impact of fumonisin mycotoxin on gastrointestinal microbiota in pigs. The gut microbiota balance should be concerned as a crucial factor keeping body health. From the gut microbiota ecosystem point of view, it is good to understand the activities of all kinds of bacteria rather than one or few particular types of them when treated with fumonisins. In the presented *in vitro* study, we investigated the interaction between FB_1 and caecal microorganism in pigs i.) to seek signs

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of biological effect on FB_1 induced by gut microbiota and ii.) to determine the impact of FB_1 on the growth of pig caecal bacteria.

Experimental

Materials and Methods

Sampling and processing. Samples of caecal content were collected from adult pigs (n = 2; Hungarian large white race) right after slaughtering from a slaughter house and transferred into sterile bottles. The bottles were put in anaerobic plastic bags with Anaerocult gas generator (Merck, Darmstadt, Germany). The pre-incubated (24 h/37°C/anaerobic) McDougall buffer solution (9.8 g $NaHCO_3$, 3.7 g anhydrous Na_2HPO_4, 0.57 g KCl, 0.47 g NaCl, 0.12 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2$ and 1000 ml aquadest; pH 8.3) were prepared to homogenise samples and make the solution for control groups.

The experiment was designed to 3 groups as shown in Table I. Two control groups were set up. The first controls contained buffer and chyme, while the second controls were prepared including buffer and FB_1.

Caecal chyme was homogenised and divided into the first control and experimental groups. An aliquot of 3.33 g of caecal chyme was suspended in pre-incubated McDougal buffer tubes (experimental and the first control group). After a pre-incubation for four hours at 37°C, FB_1 (50 µg/g; Sigma-Aldrich, Darmstadt, Germany) was added to each tube (experimental and the second control groups) to get a final concentration of 5 µg/ml. Samples were taken at 0, 24 and 48 h of anaerobic incubation for determination of bacterial numbers and FB_1 concentration.

Media and enumeration methods. The plate count technique on selected media was applied for determining the amount of bacteria. Approximately 1 g of post-incubated sample was collected and subsequently homogenised with 9 ml of peptone salt solution. Then the 10-folds series dilution was conducted from 10^{-1} to 10^{-8}. An aliquot (100 µl) was pipetted and add on the surface of each respective selected agar to culture bacteria. Five groups of bacteria were enumerated including aerobic bacteria, anaerobic bacteria, coliform, *E. coli* and *Lactobacillus* sp. The aerobic and anaerobic bacteria were cultured in commercial blood agar (BA; Bak-Teszt Ltd., Budapest, Hungary). Coliform and *E. coli* population were estimated on ChromoBio Coliform Agar (BioLab). The amount of *Lactobacillus* sp. was determined by using MRS agar (BioLab).

The colony forming units/g (CFU/g) were calculated using the formula:

$$N = \Sigma C / Vx1, 1xd$$

- ΣC is the sum of the colonies counted on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies
- V is the volume of inoculums placed in each dish, in millilitres
- d is the dilution corresponding to the first dilution retained

DNA extraction and QPCR. The DNA extraction was carried out with approximately 200 mg of the frozen caecal sample using the QIAamp®DNA Stool Mini Kit according to the manufacturer's instructions. DNA concentrations were measured using Smart Spec Plus Spectrophotometer (Maestrogen Inc.) based on the ratio of absorbance at 260 nm and 280 nm.

The standard curve was created by dilution series of purified PCR products for *Lactobacillus* sp. while the dilution series of plasmid concentration were used to prepare the standard curve for total bacteria, *Bacteroides* and *Prevotella*.

The quantity of total bacteria, *Lactobacillus* sp., *Bacteroides* and *Prevotella*, were determined by Quantitative PCR (QPCR) using SYBR Green. The primers for investigated bacterial groups were selected based on previous literature (Table II). QPCR was conducted in a 25 µl/tube reaction mixture containing 12.5 µl Brillant II SYBR QPCR Low Rox Master Mix (Agilent Technologies, CA, USA), 0.2 µM of each primer, 10.5 µl sterile DEPC treated distilled water and 1 µl of DNA extract. The PCR program consisted of 10 min at 95°C, 40 cycles of 30 sec at 95°C, 1 min at 60°C. All samples were measured in triplicates. The bacterial content of the sam-

Table I
Experimental design.

Incubation (h)/treatment	Experimental group (Buffer+Chyme+FB_1)	Control 1 group (Buffer+Chyme)	Control 2 group (Buffer+FB_1)
0 h	4	4	4
24 h	4	4	4
48 h	4	4	4
Description	12 × 3,33 g chyme 12 × 5,67 ml buffer 12 × 1 ml 50 µg/g FB_1	12 × 3,33 g chyme 12 × 5,67 ml buffer 1 ml H_2O	12 × 9 ml buffer 12 × 1 ml 50 µg/g FB_1

Table II
Oligonucleotide sequences used for QPCR in caecal samples from pigs.

Investigated group	Oligonucleotide sequence (5'-3')	Amplicon length (bp)	Refs.
Total bacteria	Fwd: GCAGGCCTAACACATGCAAGTC Rev: CTGCTGCCTCCCGTAGGAGT	292	Castillo *et al.*, 2006; Marchesi *et al.*, 1998 Amann *et al.*, 1995
Bacteroides and *Prevotella*	Fwd: GAAGGTCCCCCACATTG Rev: CAATCGGAGTTCTTCGTG	418	Kim, 2011 Bartosch *et al.*, 2004
Lactobacillus sp.	Fwd: AGCAGTAGGGAATCTTCCA Rev: CACCGCTACACATGGAG	340	Su *et al.*, 2008; Walter *et al.*, 2000 Heilig *et al.*, 2002

ples was calculated by comparison with the standard curve derived from dilution series. The obtained copy numbers of the samples were adjusted to one gram of caecum contents.

Mycotoxin extraction and analysis. For FB_1 extraction, the post-incubated samples from the experimental group and the control 2 group (Table I) were diluted 2-fold (7 ml sample and 7 ml distilled water) and centrifuged for 5 minutes (3000 rpm). The supernatant was used for FB_1 extraction followed by the modified protocol of Sep-Pak C18 cartridges (Waters Co., Milford, MA, USA) (Szabó-Fodor *et al.*, 2014). The preconditioning column was conducted with 2 ml of methanol then 2 ml of distilled water. The diluted chyme (2 ml) was subsequently loaded onto the columns then washed again with 2 ml of distilled water. The elution of FB_1 was completed by 2 ml of water/acetonitrile mixture, 1:1 v/v.

Liquid chromatography and mass spectrometry (LC-MS) analysis were performed by a Shimadzu Prominence UFLC separation system equipped with an LC-MS-2020 single quadrupole (ultra-fast) liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan) with the electrospray source. Optimised mass spectra were obtained with an interface voltage of 4.5 kV, a detector voltage of 1.05 kV in negative mode, 1.25 kV in positive mode. Samples were analysed on a Phenomenex Kinetex 2.6 µ X-C18 column (100 mm × 2.1 mm). The column temperature was set to 50°C; the flow rate was 0.3 ml/minute. The gradient elution was performed using LC-MS grade water (VWR Hungary, Debrecen) (eluent A) and methanol (eluent B), both acidified with 0.1% acetic acid. One µl of each samples were analysed with a gradient: (0 min) 5% B, (3 min) 60% B, (8 min) 100% B, followed by a holding time of 3 min at 100% eluent B and 3 min column re-equilibration at eluent A. Romer Mix 3 (containing $FB_1 + FB_2$ at 50 mg/l) and HFB_1 primary stock solution used as reference. MS parameters: source block temperature 90°C; desolvation temperature 250°C; heat block temperature 200°C; drying gas flow 15.0 l/min. Detection was performed using selected ion monitoring (SIM) mode.

The efficiency of FB_1 conversion to fully hydrolysed FB_1 (HFB_1) was calculated on the basis of the molecular weight of the compounds (FB_1: 721 g/mol; HFB_1: 405 g/mol) and described as below:

$$\frac{\text{Hydrolysed fumonisin B1 (mol/g)} \times 721\ \text{g/mol}}{405\ \text{g/mol x Fumonisin B1 (mol/g)}}$$

Statistical analysis. The R i386 3.1.2 program was applied for statistical analyses. The comparative means were performed by Independent Samples t-Test, one-way ANOVA with Tukey post-hoc test and non-parametric Kruskal-Wallis test if the normal distribution was not presented.

Results and Discussion

Effect of microflora on fumonisin B_1. At 0 h incubation time, no significant FB_1 concentration difference between the experimental group (buffer, caecal content, FB_1) and control 2 group (buffer, FB_1) was observed; 5.185 ± 0.175 µg/ml compared with 6.433 ± 0.076 µg/ml, respectively. FB_1 concentration in experimental groups was significant lower than control-2 group after 24 h and 48 h incubation period, 4.080 ± 0.065 µg/ml and 2.747 ± 0.548 µg/ml compared to 6,338 ± 0,108 µg/ml and 4.587 ± 0.085 µg/ml, respectively. FB_1 concentration also decreased during incubation time in the experimental group (Fig. 1). HFB_1 concentration has

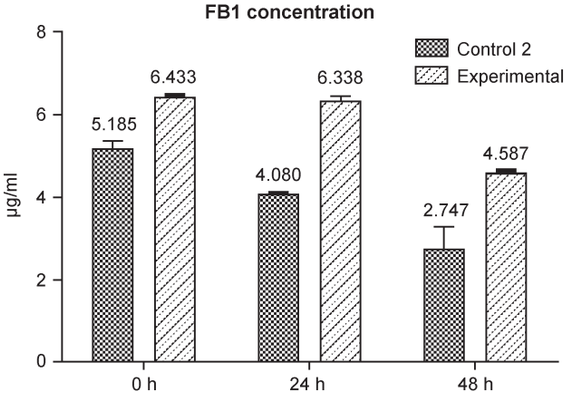

Fig. 1. Fumonisin B_1 concentration in experimental groups and control 2 groups during the incubation time.

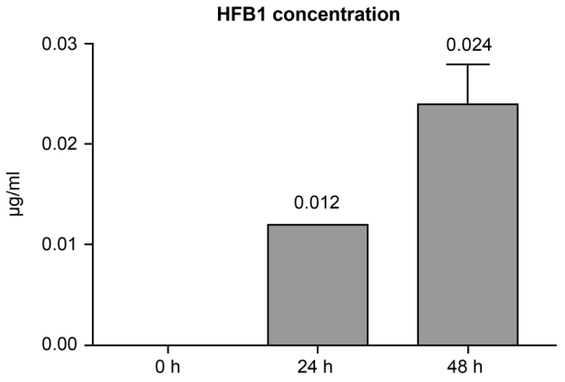

Fig. 2. Hydrolysed fumonisin B_1 concentration in experimental groups during the incubation time.

also been determined at different incubation times. Due to the appearance of main products of the metabolism (HFB_1) only in the experimental group (Fig. 2), we can conclude that FB_1 may be metabolised by microbiota in the caecum of the pig.

The capability of bacteria to influence fumonisins was by Niderkorn *et al.* (2009) and Zoghi *et al.*, (2014). Peptidoglycan, the component of bacterial cell wall, plays a crucial role in binding many mycotoxins including fumonisins. *Lactobacillus* sp. is the class of bacteria having a significant impact on fumonisins. The FB_1 level in maize was decreased by lactic acid bacterial activity after 3-day fermentation (Mokoena *et al.*, 2005). To determine the effect of the microorganism to fumonisins, most of the studies were conducted to estimate the impact of bacteria to fumonisin produced by *Fusarium* sp. such as binding or inhibition of fumonisin production while few of them were concerned with fumonisin metabolism. The concentration of FB_1 was reduced by *Lactobacillus paracasie* subsp. Paracasie after 20-day incubation (70.5 µl/ml compared with 300 µl/ml FB_1 in the control group) and *L. paracasie* subsp. Paracasie could inhibit FB_1 production in a 10-day incubation period (Gomah and Zohri, 2014). Becker *et al.* (1997) reported that FB_1 was not degraded by *Enterococcus faecium* while the binding of FB_1 and FB_2, up to 24 and 62%, respectively by *Enterococcus* sp. was determined (Niderkorn *et al.*, 2007).

In agreement with former results reported by Fodor *et al.* (2007), the conversion of FB_1 to HFB_1 was less than 1% where there was no change in the degree of the conversion of FB_1 to aminopentol (fully hydrolysed FB_1). In this study, conversion of FB_1 to HFB_1 increased significantly from 0.33% to 0.66% after 24 h and 48 h incubation time, respectively. Differences in the HFB_1 related results can be explained on the basis of the different bacterial ecosystem in the gut of experimental pigs. The various structures of gut microbiota may be derived from different diets, time of the sampling or individual enterotypes of the porcine gut microbiota (Pajarillo *et al.*, 2014; Frese *et al.*, 2015).

Effect of fumonisin B_1 on caecal microbiota in pigs. Five groups of bacteria were quantitatively determined by microbial culturing including aerobic bacteria, anaerobic bacteria, coliform, *E. coli* and *Lactobacillus* sp. There was no significant difference in the groups without FB_1 during the period of the incubation time except the anaerobic bacteria group. The $\log_{10}$ number of anaerobic bacteria decreased from 9.046 ± 0.036 (0 h incubation) to 8.389 ± 0.143 (48 h incubation) (Table III). In the caecal bacteria with FB_1 groups, reduction of the $\log_{10}$ number of anaerobic bacteria were identified, from 9.017 ± 0.054 to 8.340 ± 0.082, while there was an increase in *Lactobacillus* sp. group from 7.764 ± 0.040 to 8.006 ± 0.106 after 48 h incubation (Table III). Nonetheless, there was no detectable change in microbial culturing method between the groups of caecal bacteria with and without FB_1 during the incubation time.

The quantitative PCR was also performed to determine the effect of FB_1 on total bacteria, *Bacteroides* and *Prevotella* and *Lactobacillus* sp. The $\log_{10}$ copy-numbers were applied for data analysis (Table IV). The $\log_{10}$ of

Table III
Number of bacteria in the pigs' caecal chyme incubated with (experimental group) and without (control 1 group) fumonisin B_1 measured by culturing ($\log_{10}$ CFU[1]/g, means ±SD).

Bacteria	Period of the incubation time					
	0 hour		24 hour		48 hour	
	Experimental group	Control 1	Experimental group	Control 1	Experimental group	Control 1
Aerobic	7.49 ± 0.09	7.58 ± 0.07	7.55 ± 0.15	7.49 ± 0.258	7.26 ± 0.22	7.31 ± 0.19
Anaerobic	9.02 ± 0.05[c]	9.05 ± 0.04[c]	8.74 ± 0.19[b]	8.76 ± 0.05[b]	8.34 ± 0.08[a]	8.39 ± 0.14[a]
E. coli	5.89 ± 0.07	5.87 ± 0.07	5.58 ± 0.11	5.99 ± 0.33	6.16 ± 0.83	5.87 ± 0.66
Coliforms	5.33 ± 0.06	5.39 ± 0.12	5.44 ± 0.11	5.69 ± 0.29	5.99 ± 0.86	5.84 ± 0.55
Lactobacillus	7.76 ± 0.04[a]	7.87 ± 0.09	7.99 ± 0.06[b]	8.04 ± 0.09	8.01 ± 0.11[b]	7.93 ± 0.12

[1]CFU: colony forming unit
[a, b, c] significant ($P < 0.01$) difference between incubation times within groups.

Table IV
Number of bacteria in the pigs' caecal chyme incubated with (experimental group) and without (control 1 group) fumonisin B_1 measured by QPCR (log_{10} copies number/g, means ±SD).

Bacteria	Period of the incubation time					
	0 hour		24 hour		48 hour	
	Experimental group	Control 1	Experimental group	Control 1	Experimental group	Control 1
Total bacteria	11.52 ± 0.16^a	11.33 ± 0.38	11.91 ± 0.03^b	11.68 ± 0.21	11.79 ± 0.05^b	11.66 ± 0.13
Bacteroides and *Prevotella*	7.41 ± 0.14^a	7.32 ± 0.28^a	7.83 ± 0.13^b	7.95 ± 0.16^b	7.97 ± 0.11^b	7.83 ± 0.12^b
Lactobacillus	9.80 ± 0.25^a	9.61 ± 0.40^a	11.23 ± 0.17^b	11.35 ± 0.11^b	11.33 ± 0.14^b	11.13 ± 0.15^b

[a, b] significant ($P < 0.05$) difference between incubation times within groups.

Lactobacillus, *Bacteroides* and *Prevotella* in control 1 and experimental groups augmented after 24 h incubation ($P < 0.05$). A number of total bacteria was stable during the incubation time in the control groups while there was an increase in the experimental group from 11.520 at 0 h to 11.912 at the 24 h incubation. However, no significant difference between the control groups and the experimental groups in all kinds of investigated bacteria was observed. FB_1 did not affect the number of caecal bacteria in pigs.

As we have detected both in the microbial culture and in QPCR experiment during the incubation time, the anaerobic bacteria decreased while the amount of *Lactobacillus* sp. increased. According to QPCR results the amount of *Bacteroides* and *Prevotella* also increased. The primary difference between the results of two methods is that anaerobic bacteria enumerating by culture is alive organisms whereas *Lactobacillus* sp., *Bacteroides* and *Prevotella* estimating by QPCR based on DNA copy-number. The decline of other, not investigated anaerobic bacterial species (*i.e. Clostridium* sp.), might be another reason in this situation. Further experiments should be focused on other kinds of anaerobic bacteria or all bacterial species using a next generation sequencing approach.

To the best of our knowledge, there was is no complete report about the effect of fumonisin on caecal bacteria in pigs. Becker *et al.* (1997) isolated some strains of *Lactobacillus* sp. from pig intestine and determined the effect of FB_1 (50 and 500 μM) on the growth of these strain by turbidometric Bioscreen system. As shown in the report, no difference in the growth kinetics between the experimental and control groups was observed. The DNA of *E. coli* was not affected by FB_1 (Knasmüller *et al.*, 1997) and the number of *E. coli* showed no change in the presence of FB_1 in this study. However, the intestinal colonisation by pathogenic *E. coli* in pigs treated FB_1 was strengthened in an *in vivo* experiment (Oswald *et al.*, 2003). The indirect impact of fumonisin on bacteria was also demonstrated in some documents; because of immune suppressive effects and decrease the specific antibody response of pathogenic microorganisms (Taranu *et al.*, 2005; Iheshiulor *et al.*, 2011), fumonisin can influence activities of colonised bacteria in the body such as *E. coli* and *Salmonella* sp. (Deshmukh *et al.*, 2005; Burel *et al.*, 2013).

Fumonisin B_1 was metabolised by pig caecal microorganisms. The reduction of FB_1 concentration in chyme containing groups was sharper than it was in control 2 group. FB_1 concentration decreased while the HFB_1 increased. The amount of screened bacterial species has not changed between the groups with and without FB_1. During the period of incubation, the total number of cultured anaerobic bacteria decline while *Lactobacillus* sp. increased. Anaerobic bacteria such as *Lactobacillus* sp., *Bacteroides* and *Prevotella* had tended to increase revealed by QPCR. FB_1 did not impact on the growth of investigated bacteria. Other kinds of microorganisms should be concerned in the similar experiments and the interaction between fumonisins and gut microbiota in the *in vivo* experiments is to be conducted.

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SHORT COMMUNICATION

Early KPC-Producing *Klebsiella pneumoniae* Bacteremia among Intensive Care Unit Patients Non-Colonized upon Admission

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Abstract

Among 140 patients colonized by KPC-producing *Klebsiella pneumoniae* (KPC-Kp) between fourth and seventh day of Intensive Care Unit stay, 24 developed bacteraemia immediately after colonization. Colistin-resistance of the colonizing isolate was the factor significantly associated with early KPC-Kp bacteraemia ($P < 0.001$; OR 6.6, 95% CI 2.4–18.4), a worrisome finding since infections by colistin-resistant isolates is associated with increased mortality due to limited remaining therapeutic options.

Key words: bacteremia, carbapenemase, colistin-resistance, critically ill patients

Carbapenemase-producing *Klebsiella pneumoniae*, and especially KPC-producing (KPC-Kp) constitutes an important worldwide issue since it is endemic in many countries and provokes serious infections associated with increased mortality especially among patients hospitalized in Intensive Care Units (ICUs) (Falcone *et al*., 2016). Many patients develop primary bacteremia probably due to bacterial translocation, suggesting that enteric colonization is an important first step for the induction of infection (Schechner *et al*., 2013; Giannella *et al*., 2014; Giacobbe *et al*., 2015). It is reported that 16.5% (7.6–44.4%) of colonized patients develop a bloodstream infection (BSI), which renders the surveillance with rectal swabs imperative for every infection control program (Borer *et al*., 2012; Tischendorf *et al*., 2016).

The aim of the present study was to determine the risk factors for early KPC-Kp BSI (during the first nine days of ICU stay) among non-colonized patients upon admission in two Greek ICUs.

This retrospective study was performed in the ICUs of the University General Hospital of Patras, Greece (13 beds) during a 28-month period (November 2009-February 2012) and of the General Hospital of Patras "Agios Andreas" (6 beds) during a 16-month period (November 2009-February 2011). Epidemiologic data were collected from patients' chart reviews. The study was carried out under the Hospital Surveillance Programme for multi-drug resistant infections of hospitalized patients, and was approved by the University Hospital Ethics Committee (HEC No: 571).

According to CDC definition, BSI was defined as presence of at least one positive blood culture for *K. pneumoniae* and clinical symptoms consistent with bacteremia. Rectal samples were obtained upon ICU admission and afterwards at days 4, 7 and once weekly until discharge. Only patients that had an ICU stay of at least seven days were included. Swabs were inoculated on a selective chromogenic agar (CHROMagar™ KPC, Paris, France) and incubated at 37°C for 24 hours. Representative colonies were identified as *K. pneumoniae* by standard methods and Vitek 2 Advanced Expert System (bioMerieux, Marci l'Etoile, France).

Susceptibility to carbapenems (imipenem, meropenem, ertapenem), colistin and tigecycline was determined by Etest (bioMerieux) whereas, susceptibility to

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Table I
Univariate and multivariate analyses of risk factors for KPC-producing *K. pneumoniae* (KPC-Kp) bloodstream infection (BSI) immediately after early enteric colonization during Intensive Care Unit (ICU) hospitalization.

Characteristics	Univariate analysis			Multivariate analysis	
	KPC-Kp colonization (n = 116)	KPC-Kp infection (n = 24)	*P*	*P*	OR (95% CI)
Demographics					
Age (years)	57.6 ± 18.4	54.8 ± 17.8	0.500		
Male gender	83 (71.6%)	16 (66.7%)	0.629		
Chronic diseases (number)	0.9 ± 1.0	0.8 ± 1.0	0.929		
Diabetes Mellitus	15 (12.9%)	1 (4.2%)	0.307		
Chronic Obstructive Pulmonary Disease	16 (13.8%)	1 (4.2%)	0.306		
Chronic Heart Failure	13 (11.2%)	1 (4.2%)	0.464		
Chronic Renal Failure	4 (3.4%)	2 (8.3%)	0.273		
Malignancy	11 (9.5%)	3 (12.5%)	0.708		
Cortisone use	8 (6.9%)	1 (4.2%)	1.000		
Obesity	30 (25.9%)	8 (33.3%)	0.458		
Admission data					
APACHE II Score upon admission	17.1 ± 7.0	16.0 ± 8.1	0.499		
SAPS II Score upon admission	39.2 ± 13.4	34.8 ± 9.5	0.156		
SOFA Score upon admission	8.1 ± 3.4	8.9 ± 3.4	0.296		
Respiratory insufficiency	25 (21.6%)	6 (25.0%)	1.000		
Prior emergency surgery	46 (39.7%)	10 (41.7%)	1.000		
Prior abdominal surgery	32 (27.6%)	6 (25.0%)	1.000		
Antibiotics administered					
Carbapenems	101 (87.1%)	23 (95.8%)	0.307		
Quinolones	19 (16.4%)	4 (16.7%)	1.000		
3rd- and 4th-generation cephalosporins	16 (13.8%)	2 (8.3%)	0.738		
Piperacillin/tazobactam	30 (25.9%)	10 (41.7%)	0.139		
Colistin	29 (25.0%)	10 (41.7%)	0.132		
Aminoglycosides	30 (25.9%)	10 (41.7%)	0.139		
Glycopeptides	95 (81.9%)	21 (87.5%)	0.766		
Metronidazole	17 (14.7%)	4 (16.7%)	0.759		
Tigecycline	3 (2.6%)	0 (0.0%)	1.000		
Linezolid	19 (16.4%)	3 (12.5%)	0.766		
Mean antibiotic use per day	2.6 ± 1.0	2.6 ± 0.6	0.324		
Hospitalization data					
Mechanical ventilation	115 (99.1%)	24 (100%)	1.000		
Tracheostomy	49 (42.2%)	16 (66.7%)	0.042		
Number of catheters[a]	1.1 ± 1.3	1.4 ± 1.4	0.208		
Abdominal catheter and/or colostomy	27 (23.3%)	6 (25.0%)	0.798		
Dialysis	12 (10.3%)	5 (20.8%)	0.172		
Cortisone administration	58 (50.0%)	16 (66.7%)	0.179		
Parenteral nutrition	49 (42.2%)	14 (54.2%)	0.367		
Enteral nutrition	74 (63.8%)	16 (66.7%)	1.000		
Resistance of colonizing isolate					
Imipenem resistance	56 (48.3%)	18 (75.0%)	0.024		
Gentamicin resistance	36 (31.0%)	14 (58.3%)	0.018		
Colistin resistance	22 (19.0%)	14 (58.3%)	< 0.001	< 0.001	6.6 (2.4–18.4)
Tigecycline resistance	18 (15.5%)	9 (37.5%)	0.021		

Data are number (%) of patients or mean ±standard deviation
Abbreviations: APACHE II, Acute Physiology and Chronic Health Evaluation I; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment; [a] All patients after ICU admission were intubated, mechanically ventilated and were continuously monitored with a central venous catheter, an arterial catheter and a urinary catheter. Number of catheters does not include the aforementioned catheters.

other antimicrobials (amoxicillin/clavulanic acid, piperacillin, cefoxitin, ceftriaxone, aztreonam, ciprofloxacin, co-trimoxazole, amikacin, gentamicin) was determined by the disk diffusion method. Results were interpreted according to EUCAST guidelines (EUCAST, 2016). Presence of bla_{KPC} gene in all *K. pneumoniae* strains was confirmed by PCR (Queenan and Bush, 2007)

Statistical analysis was performed with SPSS version 22.0 (SPSS, Chicago, IL) software. Categorical variables were analyzed by using the Fisher exact test or chi^2 and continuous variables with Mann-Whitney U test, as appropriate. Backward stepwise multiple logistic regression analysis used all those variables from the univariate analysis with a $P<0.1$. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to evaluate the strength of any association. All statistic tests were 2-tailed and $P<0.05$ was considered statistically significant.

From 349 patients that had an ICU stay of at least seven days in both ICUs, 304 were not colonized by KPC-Kp upon admission. Among them, 140 patients (46.1%) were colonized in day seven and they were included in the analysis. No patient was colonized in fourth day. In total 58 patients (16.6%) developed KPC-Kp BSI within an average of 19.3 days, from which 24 (41.4%) within nine days of ICU stay (two days after colonization). Table I shows the univariate and multivariate analyses of factors that differed among patients that developed a KPC-Kp BSI until day nine of ICU stay and those that did not. Multivariate analysis revealed that colistin-resistance of the colonizing isolate was significantly associated with early KPC-Kp BSI.

A high percentage of patients admitted in the ICU (46.1%) were colonized during their first seven days of their stay, while 17.1% among them developed a BSI within two days from enteric colonization. In contrast, in previous studies (Schechner *et al*., 2013; Giannella *et al*., 2014), colonization preceded infection by 11 (range 3–27) and 19 days (range 6–28), far later than in our study (8 days; range 1–25).

The only independent factor associated with the occurrence of bacteremia was the resistance of colonizing isolate to colistin, since among 36 colistin-resistant KPC-Kp colonizing isolates, 14 (38.9%) resulted in immediate bacteremia, while no difference in colistin administration was observed among infected and colonized patients (25.9% vs 41.7%; *P* 0.132). This finding suggests that not all KPC-Kp isolates share the same virulence capacity, consistent with results of previous studies (Diago-Navarro *et al*., 2014; Chiang *et al*., 2016). The capacity of colistin-resistant isolates to induce infection is a worrisome finding since BSI from such isolates is associated with increased mortality due to limited remaining therapeutic options (Giacobbe *et al*., 2015, Falcone *et al*., 2016). As previously shown, colonization by colistin-resistant isolate was a prerequisite to an infection by such isolate (Giacobbe *et al*., 2015).

Chronic diseases, invasive procedures, severity of disease and antibiotic administration were commonly identified as risk factors for infections by carbapenemase-producing *K. pneumoniae* (Borer *et al*., 2012; Schechner *et al*., 2013; Giannella *et al*., 2014; Giacobbe *et al*., 2015, Falcone *et al*., 2016). In contrast to previous studies, none of aforementioned factors differed between colonized and infected patients in the present study, suggesting that induction of BSI by translocation may be mostly influenced by bacteria's than patients' characteristics.

The present study has limitations; it was conducted in two Greek ICUs with high colonization rates and results might not be generalized for other institutions. Another limitation is its retrospective nature and the small number of patients included.

To conclude, a high percentage of ICU patients developed bacteremia by KPC-Kp within two days from colonization, a finding more common for colistin-resistant isolates.

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SHORT COMMUNICATION

An Improve Protocol for PCR Using LM1 and LM2 Primers for *Listeria monocytogenes* Detection in Food Matrices

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Abstract

Several studies have observed that use of a conventional PCR protocol with primers LM1 and LM2 for the identification of the *hly*A gene of *Listeria monocytogenes* generates non-specific PCR amplifications and false positives. For this reason, in this study we provide a modified PCR protocol that improves the specificity of the results obtained with LM1 and LM2 primers.

Key words: *Listeria monocytogenes*, *hly*A, PCR

The *hly*A gene encoding for listeriolysin O, a secreted pore-forming toxin, is a conventional genetic marker for the identification of *Listeria monocytogenes* in foods. The LM1 and LM2 primers, designed by Border *et al*. (1990) are among the most commonly used primer sets for the detection of this pathogen as cited in at least 51 publications (Lawrence and Gilmour, 1994; Karpiskova *et al*., 2000; Van Coillie *et al*., 2004; Kawasaki *et al*., 2005; Marian *et al*., 2012; Jamali *et al*., 2013; Kuan *et al*., 2013). A conventional PCR protocol using these primers was originally designed by Aznar and Alarcón (2002) and has been commonly used in some investigations (Aznar and Alarcón, 2003; Aznar and Solis, 2006; Aznar and Elizaquível, 2008; Brankica *et al*., 2010; Peres *et al*., 2010; Elizaquível *et al*., 2011). However, the use of this PCR protocol results in non-specific bands after detection of *L. monocytogenes* and other *Listeria* species from pure cultures (Aznar and Alarcón, 2002). Moreover, during in-house validation, we also observed non-specific bands using DNA extracted from *Salmonella enterica* and *Escherichia coli*. For this reason, the goal of the present study was to improve the specificity of the LM1 and LM2 primers used for the amplification of the *hly*A gene from *L. monocytogenes*.

To validate the specificity of the Aznar and Alarcon (2002) PCR protocol, we used a collection of laboratory strains: *L. monocytogenes* ATCC 19115, *L. monocytogenes* CDC, *L. monocytogenes* Scott A, *Listeria innocua* ATCC 33090, *L. innocua*, *Listeria seeligeri*, *E. coli* ATCC H10407, *S. enterica*, and *Salmonella* Thompson ATCC 8391. The bacterial strains were cultivated in Trypticase Soy Broth (Dibico, México) and incubated at 35°C for 19 h. One milliliter of these cultures was used for DNA extraction using the DNeasy® Blood & Tissue Kit (QIAGEN, USA) with a minor modification. Briefly, before addition of AL buffer, 200 µl of ASL buffer was added, and the mix was incubated at 95°C for 15 min. In addition to pure cultures, DNA was extracted from enriched samples (Universal Preenrichment Broth, Difco, USA) of jalapeno peppers and tomatoes inoculated previously with a mixture of *S. enterica*, *Shigella* spp., *E. coli*, and *L. monocytogenes* cultures. One milliliter of enrichment broth was used to extract DNA using the QIAamp® DNA Stool kit (QIAGEN, USA). In all cases, the extracted DNA was quantified, and the purity was determined using a nanodrop (Thermo Scientific 2000c, USA).

DNA was mixed independently with two types of master mix: mic with TaKaRa Taq™ DNA Polymerase (Takara Shuzo Co., Japan) or with Bio-Rad DNA Polymerase (Bio-Rad, USA). PCR amplifications were performed at two laboratories from the Universidad Autónoma de Querétaro, México: Food Safety Laboratory, Chemistry Faculty and Animal Nutrition Laboratory, Natural Sciences Faculty. At the Food Safety Laboratory, the PCR reactions were conducted using

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a Techne TC-512 Thermal Cycler (Techne, UK), whereas a T100 Thermal Cycler (Bio-Rad, USA) was used at the Animal Nutrition Laboratory. PCR was performed using the conditions established by Aznar and Alarcon (2002). To optimize the PCR protocol, gradients of DNA concentrations (40 to 0.0625 ng/µl) and annealing temperatures (55–64.7°C) were evaluated; additionally, the annealing and extension times and the number of cycles were reduced to 25 s and 25 cycles, respectively. The electrophoresis gel was prepared with 2% agarose and 0.0028% ethidium bromide (10 mg/ml) in 1X TBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA). The lanes were loaded with 7 µl of the PCR products and 3 µl of 6X gel-loading buffer (30% glycerol, 0.25% bromophenol blue in 10 ml dH_2O), and gel images were taken using a MiniBis Pro photo documenter (Bio-Imaging Systems, Israel).

The electrophoresis gel obtained after running a PCR using the conditions from Aznar and Alarcon (2002) showed non-specific amplification products from pure cultures of all *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *E. coli*, and *Salmonella* strains and from DNA obtained from inoculated vegetables, when the expected product was a single band of 702 bp corresponding to *L. monocytogenes* (Fig. 1A). In DNA from *L. innocua*, *L. seeligeri* and *Salmonella*, a similar expected fragment (720 bp) was observed. The presence of atypical bands can lead to false-positive results. The observation of artifacts in *L. monocytogenes* and other *Listeria* species was also reported by the authors that established this PCR protocol.

The original PCR protocol was modified, and the optimized conditions were an initial denaturation of 5 min at 94°C, 25 cycles of 30 s at 94°C, 25 s at 60.5°C

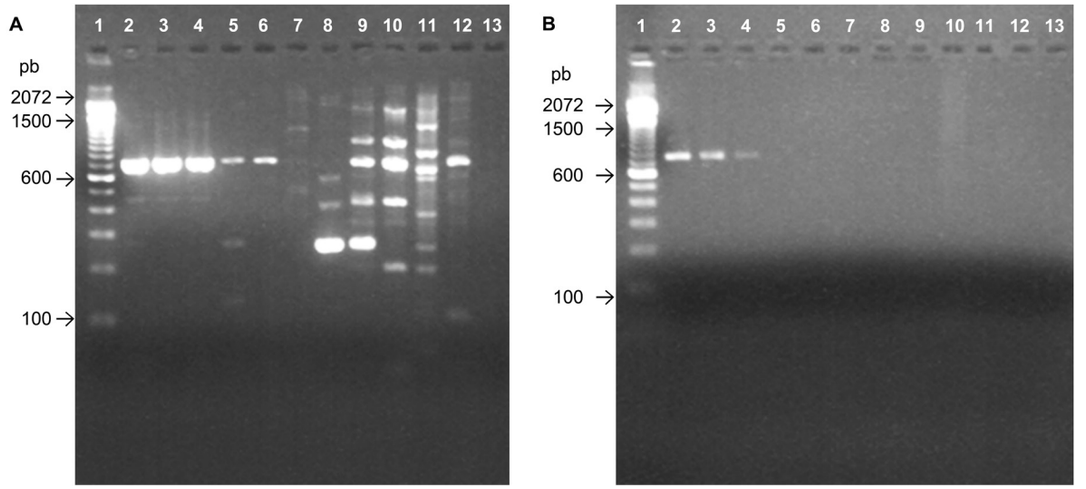

Fig. 1. PCR identification of *L. monocytogenes* strains using LM1 and LM2 primers.
A: PCR protocol established by Aznar and Alarcón (2002); B: Modified PCR protocol. Lane 1, DNA ladder; lane 2, *L. monocytogenes* Scott A; lane 3, *L. monocytogenes* CDC; lane 4, *L. monocytogenes* ATCC 19115; lane 5, *L. seeligeri*; lane 6, *L. innocua* ATCC 33090; lane 7, *L. innocua*; lane 8, *E. coli* ATCC H10410; lane 9, *S.* Thompson ATCC 8391; lane 10, *Salmonella* spp.; lane 11, inoculated jalapeno pepper; lane 12, inoculated tomato; lane 13, negative control.

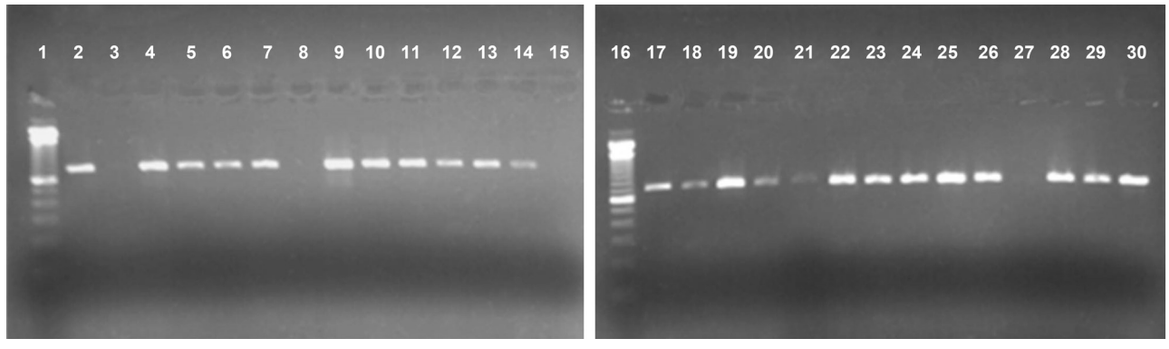

Fig. 2. Identification of *L. monocytogenes* strains isolated from a frozen vegetable processing plant using an optimized PCR protocol.
Lane 1 and 16, DNA ladder; lane 2 and 17, *L. monocytogenes* Scott A; lanes 3 to 15 and 18 to 30; environmental samples.

and 25 s at 72°C, and a final extension of 5 min at 72°C. The optimal concentration of DNA was set from 0.125 to 0.8 ng/µl. Using the optimized protocol, the expected fragment of 702 bp corresponding to the *hly*A gene only appeared in all *L. monocytogenes* strains, and no atypical bands were present in any sample (Fig. 1B). No amplifications were observed in inoculated vegetables and pure cultures of microorganisms different from *L. monocytogenes*. The absence of the expected fragment in DNA extracted from inoculated vegetables with the pathogens, including *L. monocytogenes* strains, may be due to the presence of PCR inhibitors in the food matrix.

Xiacyum *et al*. (2001) indicated that artifacts and chimeras increment significantly as the number of cycles is increased, reported that DNA template concentration affected PCR amplification kinetics, and demonstrated that PCR artifacts disappeared as the template concentration of each strain decreased. In this study, similar results were obtained; atypical bands disappeared as the number of cycles decreased and DNA concentration was reduced.

Finally, to validate the proposed optimized PCR protocol, 26 isolates presumptively identified as *L. monocytogenes*, were confirmed. The isolated strains were cultivated in Trypticase Soy Broth (Dibico, México) and incubated at 35°C for 19 h. The DNA was extracted as described above for pure cultures. In 22 samples (85%), the expected fragment and the absence of atypical bands were observed (Fig. 2).

In conclusion, the standardized PCR protocol can be used to confirm *L. monocytogenes* strains without producing false positives.

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SHORT COMMUNICATION

Bactericidal Activity of Octenidine to Various Genospecies of *Borrelia burgdorferi*, Sensu Lato Spirochetes *in Vitro* and *in Vivo*

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Abstract

The aim of our studies was to invent a reliable method for detection of the bactericidal activity of disinfectants against *Borrelia burgdorferi* in suspension (*in vitro*) and in cell line cultures (*in vivo*). In the suspension method, 0.01% octenidine at 20°C and 35°C was bactericidal to *Borrelia afzeli*; *Borrelia garini*, *B. burgdorferi* sensu stricto after 5 minutes treatment. Increase of the temperature to 35°C speed up the bactericidal effect to 1 minute. The bactericidal action of octenidine towards *B. burgdorferi* spirochetes growing in fibroblasts was less effective and needed a longer time to kill them than in the suspension.

Key words: *Borrelia burgdorferi*, octenidine hydrochloride, bactericidal activity

Lyme borreliosis is a zoonosis which can be transferred to humans exclusively by a vector – infected *Ixodes* spp. ticks. Bites by infected ticks introduce *Borrelia burgdorferi* sensu lato spirochetes into the skin (Stanek *et al*., 2012).

B. burgdorferi spirochetes are very fastidious bacteria and do not grow on any known solid and standard liquid bacteriological media. They can be cultivated only in liquid BSK medium supplemented with rabbit serum. They grow on this medium very slowly and results can be obtained after three months of incubation (Stanek *et al*., 2012). Consequently, classical methods for the evaluation of bactericidal activity of chemical disinfectants and antiseptics are not suitable. A method for testing the bactericidal activity of disinfectants against *B. burgdorferi* and other spirochetes has not yet been established. Previously, *in vivo* methods for susceptibility testing of *B. burgdorferi* towards antibiotics in mice, dogs and other mammals were proposed (Embers *et al*., 2012; Hodzic *et al*., 2008; Strabinger *et al*., 1997). However, the obtained results are not clear-cut and rewarding (Embers *et al*., 2012). Recently, the first *in vitro* method for testing the bactericidal activity of some leaf extracts against *B. burgdorferi* has been presented (Theophilus *et al*., 2015).

Reports have also described the *in vivo* and *in vitro* interaction of *B. burgdorferi*, sensu lato spirochetes with different host cells. It has been found that pathogenic strains penetrate mammalian cells (fibroblasts) and can multiply in them (Chmielewski and Tylewska-Wierzbanowska, 2010; Tylewska-Wierzbanowska and Chmielewski, 1997). According to these data, the bactericidal activity of octenidine hydrochloride against spirochetes should be also tested in culture of *B. burgdorferi* in HEL-299 cell line (fibroblasts).

The aim of our studies was to invent a reliable method for detection of bactericidal activity of disinfectants against *B. burgdorferi* spirochetes in suspension, in *in vitro* conditions as well as *in vivo*, in cell line cultures. Moreover, we have examined whether octenidine hydrochloride meets these conditions as the study was conducted in reference to a development of a new antiseptic pharmaceutical product (Octynix®).

Strains *B. burgdorferi* sensu stricto B31 (ATCC 35210), and *B. garinii* 20047 (ATCC 51383), *B. afzelii* VS461 (ATCC 51567) were cultured in BSK-H Medium Complete, supplemented with 6% of rabbit serum (SIGMA-ALDRICH Chemie GmbH, USA). In all tests, suspensions of 1×10^7 bacterial cells/ml (counted in Thoma cell counting chamber) were used. HEL-299 (ATCC-CCL-137) cell line derived from human fibroblasts was cultured in 2 ml Eagle's Minimum Essential Medium (EMEM) with Earle's BSS, 1 mM sodium pyruvate and 2 mM L-glutamine (ATCC 30–2003) (ATCC, Manassas, Canada) supplemented with 5% of fetal calf serum (ATCC), in shell-vials (Bibby Sterilin, Great Britain).

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Octenidine hydrochloride dissolved in methanol was added to tubes containing 990 µl *B. burgdorferi* sensu stricto spirochetes culture in BSK-H Medium Complete to obtain 0.1%, 0.01%, 0.005% and 0.0025% final concentrations in culture medium. The mixtures were incubated obligatorily at 20 ± 1°C for 5 min ± 60 sec., and additionally at 20 ± 1°C and 35°C for 1 min ± 30 sec.; 10 min ± 60 sec; 15 min ± 60 sec., and at 35°C for 5 min ± 60 sec.

Shell-vials with HEL-299 cell line confluent monolayer in 2 ml EMEM, supplemented with 5% of fetal calf serum (ATCC), were incubated at 35°C with 5% CO_2 atmosphere for 2 days. Next, EMEM medium was removed and replaced with 1.9 ml of BSK-H medium. After that, cell line cultures were inoculated with 100 µl of spirochetes cultured in BSK-H. Infected cell lines were incubated for 24 hrs. After that time, 20 µl of consecutive dilutions of octenidine hydrochloride in methanol to obtain 0.01%, 0.005%, 0.0025% final concentrations, were added for 5, 10, 15 minutes. Next, the cell lines were washed with fresh BSK-H medium to remove octenidine hydrochloride. Washed cell lines were incubated in 5% CO_2 at 35°C for 72 hours. Every 24 hours, the number or healthy and dead bacterial cells in the medium, outside the cell line, were counted.

Immediately, when incubations were terminated, slides were prepared and evaluated under dark field microscope (10 × 25). The evaluation of bactericidal activity of the tested substance was based on the observation of motility and the loss of motility of the examined spirochetes. One hundred cells were counted and the numbers of mobile (live) and motionless (dead) spirochetes were estimated (in triplicate). The influence of octenidine hydrochloride diluents (methanol, 2-phenoxyethanol) and octenidine hydrochloride neutralizer on the viability of spirochetes and HEL-299 cells was tested to exclude their side effects.

Decimal logarithm reduction for each product concentration and each experimental condition was calculated and recorded according to the formula: $lgR = lgN_o - lgN_a$ (lgR – decimal logarithm reduction; N_o – number

Table I
Bactericidal activity of octenidine to *B. burgdorferi*, sensu lato strains at 20°C

Genospecies of *B. burgdorferi*, sensu lato	Octenidine concentration*	Reduction** after treatment with octenidine for the following time				
		1 min	5 min	10 min	15 min	60 min
B. afzelii	0.1	0	0	0	0	0
		>7	(>7)	(>7)	(>7)	(>7)
	0.01	84	0	0	0	0
		(0.08)	(>7)	(>7)	(>7)	(>7)
	0.005	83	4	0	0	0
		(0.08)	(1.4)	(>7)	(>7)	(>7)
	0.0025	89	71	5	5	0
		(0.05)	(0.15)	(1.3)	(1.3)	(>7)
B. garinii	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	5	0	0	0	0
		(1.3)	(>7)	(>7)	(>7)	(>7)
	0.005	30	0	0	0	0
		(0.52)	(>7)	(>7)	(>7)	(>7)
	0.0025	79	72	68	56	6
		(0.1)	(0.16)	(0.17)	(0.25)	(1.22)
B. burgdorferi, sensu stricto	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	5	0	0	0	0
		(1.3)	(>7)	(>7)	(>7)	(>7)
	0.005	83	59	52	52	0
		(0.08)	(1.69)	(0.28)	(0.28)	(>7)
	0.0025	90	75	70	61	6
		(0.05)	(0.12)	(0.15)	(0.41)	(1.22)

* minimum bactericidal concentration (%) of octenidine in bacterial suspension in BSK-H medium
** expressed as percentage % of viable bacterial cells and in decimal logarithm (lgR)

Table II
Bactericidal activity of octenidine to *B. burgdorferi*, sensu lato strains at 35°C

Genospecies of *B. burgdorferi*, sensu lato	Concentration (%) of octenidine	Reduction of viable bacteria after treatment with octenidine for the following time**				
		1 min	5 min	10 min	15 min	60 min
B. afzelii	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	16	0	0	0	0
		(0.8)	(>7)	(>7)	(>7)	(>7)
	0.005	70	0	0	0	0
		(0.15)	(>7)	(>7)	(>7)	(>7)
	0.0025	86	7	0	0	0
		(0.06)	(1.15)	(>7)	(>7)	(>7)
B. garinii	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.005	12	0	0	0	0
		(0.92)	(>7)	(>7)	(>7)	(>7)
	0.0025	87	49	29	20	0
		(0.06)	(0.31)	(0.54)	(0.7)	(>7)
B. burgdorferi, sensu stricto	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	5	0	0	0	0
		(1.3)	(>7)	(>7)	(>7)	(>7)
	0.005	81	28	0	0	0
		(0.09)	(0.55)	(>7)	(>7)	(>7)
	0.0025	94	80	63	56	8
		(0.03)	(0.1)	(0.2)	(0.25)	(1.1)

* minimum bactericidal concentration (%) in bacterial suspension in BSK-H
** expressed as percentage of viable bacterial cells and in decimal logarithm (lgR)

of cells in control test suspension/ml; N_a – number of survived bacterial cells in the test mixture/ml). At least one concentration of octenidine hydrochloride per test shall demonstrate a 5 lg or more reduction (lgR ≥ 5) in motility (viability) of spirochetes and at least one concentration shall demonstrate a lg reduction of less than 5. Equivalence of lgR ≥ 5: lack of live spirochetes treated with octenidine hydrochloride in fresh medium after 5 days of incubation. The above criteria are in accordance with EN 1276/2009.

Octenidine hydrochloride activity toward *B. burgdorferi* spirochetes was dependent on time of exposure and to some extent on temperature. It was found that at 20°C 0.01% octenidine hydrochloride kills all three tested genospecies of *B. burgdorferi*, sensu lato within 5 minutes (Table I). Increase of the temperature to 35°C speed up the bactericidal effect to 1 minute (Table II). Generally, in the suspension method, octenidine hydrochloride in concentration 0.01% was bactericidal to all three tested genospecies of *B. burgdorferi* sensu lato (*B. afzelii*; *B. garinii*, *B. burgdorferi* sensu stricto) after 5 minutes treatment.

Octenidine hydrochloride in concentration 0.01% was bactericidal after 5 minutes treatment for *B. afzelii* and *B. garinii* spirochetes growing in eukaryotic HEL-299 cell line.

At this time, cells of the *B. burgdorferi* sensu stricto strain treated for 5 min. with 0.02% octenidine hydrochloride, were still weakly motile. Cells of *B. burgdorferi,* sensu stricto strain lost their motility (viability) totally within 24 hours after treatment with 0.02% octenidine hydrochloride for 15 minutes. They were motionless (dead) 48 hours later meaning that under these conditions they are not able recuperate and to grow and multiply. These results indicate that the bactericidal concentration of octenidine hydrochloride against *B. burgdorferi*, sensu lato spirochetes may vary depending on genospecies and strains (Table III).

A method for testing the bactericidal activity of disinfectants against *B. burgdorferi* and other spirochetes

Table III
Bactericidal activity of octenidine to *B. burgdorferi*, sensu lato strains growing in mouse fibroblasts at 35°C

Genospecies of *B. burgdorferi*, sensu lato	Concentration of octenidine (%) in culture medium*	Percentage of viable bacteria treated with octenidine for the following minutes** after 24 hrs			Percentage of viable bacteria treated with octenidine for the following minutes** after 48 hrs			Percentage of viable bacteria treated with octenidine for the following minutes** after 72 hrs		
		5	10	15	5	10	15	5	10	15
B. afzelii	0.0025	4	4	0	10	5	1	6	5	0
		(1.4)	(1.4)	(>7)	(1.0)	(1.3)	(2.0)	(1.33)	(1.3)	(>7)
	0.005	2	0	0	0	0	0	5	0	0
		(1.7)	(>7)	(>7)	(>7)	(>7)	(>7)	(1.3)	(>7)	(>7)
	0.01	0	0	0	0	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)
B. garinii	0.0025	3	0	0	6	1	0	2	5	0
		(1.52)	(>7)	(>7)	(1.22)	(2.0)	(>7)	(1.7)	(1.3)	(>7)
	0.005	0	0	0	1	0	0	1	0	0
		(>7)	(>7)	(>7)	(2.0)	(>7)	(>7)	(2.0)	(>7)	(>7)
	0.01	0	0	0	0	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)
B. burgdorferi, sensu stricto	0.005	29	18	6	48	41	68	41	4	4
		(90.54)	(0.74)	(1.22)	(0.32)	(0.39)	(0.17)	(0.39)	(1.4)	(1.4)
	0.01	8	2	1	23	0	0	10	0	0
		(1.1)	(1.7)	(2.0)	(0.64)	(>7)	(>7)	(1.0)	(>7)	(>7)
	0.02	3	2	0	0	0	0	0	0	0
		(1.52)	(1.7)	(>7)	(>7)	(>7)	(>7)	**(>7)**	(>7)	(>7)

* Treated with octenidine spirochetes were culture in HEL-299 cell line for 72 hours
** minimum bactericidal concentration against *B. burgdorferi* in cell cultures expressed as percentage (%) of viable bacterial cells and in decimal logarithm (lgR) – bold numbers

has not yet been established (no publication on this topic has been found in PubMed).

In our method, evaluation of the bactericidal activity of tested substance is based on observation of motility and loss of motility of the examined spirochetes. In the proposed bactericidal activity evaluation method of antiseptics and disinfectants against spirochetes, the test conditions such as temperatures of incubation, time of exposure to the tested substance, reduction of number of alive bacterial cells, *etc.* followed the rules included in European Standard EN 1276/2009. These regulations have applied to chemical disinfectants and antiseptics and characterize a quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. The obligatory test conditions have been set at: 20 ± 1°C for 5 min ± 60 sec.

According to PN-EN 1276/2009, the mixtures were incubated obligatorily at 20 ± 1°C for 5 min ± 60 sec. and additionally, at 20 ± 1°C for 1 min ± 30 sec., 10 min ± 60 sec., 15 min ± 60 sec., and at 35°C for 1 min ± 30 sec., 5 min ± 60 sec., 10 min ± 60 sec. and 15 min ± 60 sec.

It has been assumed that observed motile/motionless spirochetes mean live/dead spirochetes. *B. burgdorferi* are highly motile and invasive microorganisms that disseminate widely throughout their arthropod and vertebrate hosts (Groshong and Blevins, 2014; Wolgemuth, 2015). It has been discovered that motility and chemotaxis are critical for multiple stages of infection. There is an absolute requirement for cell motility during any stage of the *B. burgdorferi* life cycle (Motaleb *et al.*, 2015).

Motion ability of spirochetes is a necessary condition to survive in both vector as well as in consecutive reservoirs. Loss of ability to move leads to the death of the bacterial cell (Coburn *et al.*, 2013; Harman *et al.*, 2012).

It has been shown previously that *B. burgdorferi* spirochetes are able to enter and multiply in Vero cell and mouse fibroblasts (HELL-299) (Chmielewski and Tylewska-Wierzbanowska, 2010; Tylewska-Wierzbanowska and Chmielewski, 1997). It has been observed that *B. burgdorferi* can attach to the vascular endothelium and actively migrate between these cells to disseminate into other tissues (Coburn *et al.*, 2013; Harman *et al.*, 2012). Ticks as a vector inject spirochetes directly into the skin of animals and humans. This means that the skin protects the bacteria to some extent against disinfectants applied on its surface. The results of our *in vivo* bactericidal test suggest this possibility. *B. burgdorferi* growing in mouse fibroblasts were

less sensitive to octenididine. The bactericidal action of this substance towards *B. burgdorferi* spirochetes growing in fibroblasts was less effective and needed a longer time to kill them than in the suspension.

In conclusion: in the proposed suspension method, octenidine hydrochloride in concentration 0.01% has shown bactericidal activity to all three tested genospecies of *B. burgdorferi*, sensu lato (*B. afzelii*; *B. garinii*, *B. burgdorferi*, sensu stricto) after 5 minutes treatment.

Octenidine hydrochloride in concentration 0.01% was bactericidal after 5 min treatment for cells of *B. afzelii* and *B. garini* spirochetes growing in eukaryotic cell line whereas *B. burgdorferi*, sensu stricto strain was resistant to octenidine hydrochloride under these conditions. Moreover, cells of the *B. burgdorferi*, sensu stricto strain treated for 5 min with 0.02% octenidine hydrochloride, 24 hours later were still weakly motile. They lost motility (viability) 48 hours later meaning that under these conditions they were not able to grow and multiply. The cells of *B. burgdorferi*, sensu stricto strain totally lost their motility (viability) within 24 hours after 15 minutes treatment with 0.02% octenidine hydrochloride.

These results indicate that the method for detection of bactericidal activity of octenidine against spirochetes is sensitive and has potential usefulness for other disinfectants.

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SHORT COMMUNICATION

Erythromycin or Clindamycin – is it Still an Empirical Therapy against *Streptococcus agalactiae* in Patients Allergic to Penicillin?

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Abstract

Retrospective analysis of *Streptococcus agalactiae* antibiotic susceptibility isolated in 2010–2013 was performed. Penicillin was still the first-line antibiotic. Due to the high percentage of strains resistant to erythromycin and clindamycin empirical treatment with these antibiotics may not be effective. Lower resistance rate to erythromycin and clindamycin among strains isolated from infected pregnant women and newborns were observed than among strains isolated from samples from patients hospitalized in other departments (29% and 47% v. 46% and 63%). The increasing resistance rate might give a rise to a new epidemiological situation.

Key words: *Streptococcus agalactiae*, clindamycin, erythromycin, susceptibility on antibiotics

GBS colonizes the oral cavity, respiratory tract, gastrointestinal and genitourinary tract. The risk of transmission is up to 70%. Postpartum infections arise from other family members or from hospital environment. GBS colonization in pregnant women in full-term pregnancy is the most important predisposing factor that increases 25 fold the risk of developing early onset disease in newborn. It varies with ethnic groups, with equal numbers of women being colonized in a transitory, intermittent or persistent manner and depends on age, sexual activity, contraceptive methods used (Bigos *et al*., 2012).

Perinatal antibiotic prophylaxis should be given to pregnant women who carry GBS in the vagina and/or rectum, if in the course of pregnancy was bacteriuria diagnosed with GBS etiology, or if the neonatal GBS infection occurred in previous children born by this patient. Indication for antibiotic prophylaxis is also 35–37 week of pregnancy before labor before, if pregnant woman in labor was admitted to the hospital after more than 18 hours after disruption of fetal membranes and in case of intrapartum fever (Bigos *et al*., 2012).

An aim of our study was the comparison of antibiotic susceptibility of *Streptococcus agalactiae* strains isolated from various samples taken healthy or infected pregnant women, neonates (group A – wards: Gynecology, Obstetrics, Pregnancy Pathology, Neonatal, Neonatal Intensive Care Unit) and other adult patients hospitalized in various hospital wards group B – wards: Internal Diseases, Urology, Transplantology, Surgery, Orthopedics) during 2010–2013.

The retrospective analysis included isolates from vaginal/rectal swabs, blood, urine, swab, cervix, blood, external ear swabs. Swabs from vagina/rectum were transported in a transport Amies medium and submitted for culture within 24 hours, they were cultured onto the Todd – Hewitt's broth (24 hours incubation, 37°C, aerobic atmosphere). They were subcultured on the CHROMagar – Strep (bioMerieux) and blood agar. Other samples were cultured according to routinely used protocol. After incubation, the cultures were reviewed for the presence of characteristic for GBS colonies and latex agglutination test detecting polysaccharide C characteristic for group B was performed (bioMerieux). Susceptibility to erythromycin, clindamycin and vancomycin was performed in case of isolates from swabs from vagina/from patients allergic to penicillin – information was given on referral form. GBS isolates other patients had susceptibility tests done routinely. Retrospective

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Table I
Antibiotic resistance.

Year	Group A (Gynecology, Obstetrics, Pregnancy Pathology, Neonatal, Neonatal Intensive Care Unit)				Group B (Internal Diseases, Urology, Transplantology, Surgery, Orthopedics Hospital Wards)			
	Benzyl-penicillin	Erythro-mycin	Clindamycin	Vancomycin	Benzyl-penicillin	Erythro-mycin	Clindamycin	Vancomycin
2010	0/19	5/15	0/4	0/12	0/69	14/33	12/25	0/87
2011	0/15	0/3	4/8	0/41	0/43	15/27	35/59	0/77
2012	0/3	11/30	30/51	0/53	0/4	16/34	51/71	0/76
2013	0/3	10/41	19/50	0/52	0/10	14/34	36/58	0/73
Total	0/40 (0%)	26/89 (29%)	53/113 (47%)	0/158 (0%)	0/126 (0%)	59/128 (46%)	134/213 (63%)	0/313 (0%)

* based on data given on lab reports, not on microbiological test

analysis was made on the basis of information contained in Hospital IT System.

Incidence of colonization in consecutive years and number of examined vaginal/rectal swabs is 12.4% in 2010 (1409); 17% in 2011 (2125); 19.6% in 2012 (2348); 16.4% in 2013 (2401), respectively. All GBS strains were isolated from healthy pregnant women not allergic to penicillin and therefore no susceptibility test was performed.

Susceptibility analysis to benzylpenicillin, erythromycin, clindamycin and vancomycin was performed for GBS isolates from group B patients. All strains were susceptible to benzylpenicillin and vancomycin. Constitutive and inductive macrolide – lincosamide – streptogramin B resistance mechanisms were identified. Results of resistance rate are presented in Table I.

Sensitivity of bacterial cultures in detection of *S. agalactiae* varies from 50 to 84.3%. Underestimation of GBS neonatal disease can be related to non-hemolytic GBS isolates (5–8% of all isolates). Sensitivity of late antenatal cultures for identifying colonization status at delivery varies from 54.3–87%; specificity 96%; positive predictive value 87% and negative predictive value 95–97%. An important limitation of the detection of GBS in culture is the need for viable organisms and for an average culture period of 48–72 hours. Even if the swab would be taken according to procedure just before labour, it can give false negative result. Routine vaginal swab culture between 35 and 37 hbd has its limitations, because approximately 6% of pregnant women is colonized later (Edwards *et al.*, 2002; Verani *et al.*, 2010; Verani and Schrag, 2010; Brown *et al.*, 2013; Savini *et al.*, 2013; Szymusik *et al.*, 2014). Incidence of GBS colonization obtained by the gold standard – culture – reported by various authors is presented in Table II.

According to de-Paris *et al.* (2011) culture method was positive in 15.96% samples, while the PCR technique in 26.99%. Of the 221 culture-negative samples, 13% were positive with PCR Positive results of intrapartum PCR DNA were reported in 35 minutes and negative results confirmed in 50 minute. According to the studies performed by Abdelazim (2013) the sensitivity and specificity of intrapartum PCR test was 98.3% and 99%, respectively. Positive predictive value of intrapartum PCR test was 86.4% and negative predictive value – 97.4% (NIHCE, 2015).

Poncelet-Jasserand *et al.* (2013) stated that 70% of early-onset neonatal GBS infections were associated with mothers whose colonization status was either unknown or negative at the time of screening (35–37 hbd). The cost of reagents and labor was 13 times higher for PCR detection than for the use of chromogenic media. Helali *et al.* (2012) estimated that positive result of screening at 35–37 week's gestation resulted in unnecessary anti-

Table II
Literature review on incidence of GBS colonization in 1995–2014 years (literature is available from corresponding author)

Country	Year	Colonization incidence (%)
Italy	1995–2007	11.3–17.9
Ireland	1998–2004	11.8–25.6
The Netherlands	2000–2002	21
Greece	2003	6.6
Turkey	2003–2005	6.5–10,6
Scandinavia	2003–2008	24.3–36.0
Czech Republic	2004	29.3
Germany	2006	16.0
United Kingdom	2006	21.3
Pakistan	2007	18
Poland	2007–2008	11.4
	2008–2012	25–30
Nigeria	2010–2011	18
Ethiopia	2011–2012	11.3–15.4
Republic of Congo	2012–2013	20
Republic of South Africa	2014	30.9

Table III
Literature review on resistance rate to erythromycin and clindamycin among *S. agalactiae* strains in 1992–2014 years (literature is available from corresponding author).

Country	Year	Antibiotic resistance (number of resistant strains/ number of tested strains)	
		Erythromycin	Clindamycin
Spain	1992–2009	30/212	30/212
New Zealand	2002–2004	8/88	13/88
Italy	2002–2005	15/91	15/91
Poland	2006–2010	6/22	5/22
Australia	2002–2006	3/47	1/47
Portugal	2006–2011	9/43–22/95	7/120–17/97
Egypt	2008	5/38	9/38
Syria	2008	39/72	25/72
Myanmar	2009–2010	4/47	4/47
Israel	2010	15/88	15/88
Nigeria	2010–2011	5/58	5/58
Malesia	2010–2011	24/103	18/103
Ethiopia	2011–2012	2/17	3/17
Republic of South Africa	2012	27/128	22/128

biotic prophylaxis for 13,6% of pregnant women in the study compared to 4,5% for women using the intrapartum PCR test. This resulted in incremental costs of €36 and €173 to the health care system and hospital, respectively, for each mismanaged patient. Molecular tests have not identified susceptibility to antibiotics (penicillin, erythromycin, clindamycin) (Church *et al*., 2011; Kasahara *et al*., 2010).

Penicillin is still the first-line antibiotic effective against GBS. Recently non-penicillin-susceptible GBS isolates have been reported in Japan and the United States due to a Q557E mutation in *pbp2x* (Kasahara *et al*., 2010). The substantial increases of erythromycin-resistant GBS isolates were observed in England and Wales (< 3% in 1990s to 15% in 2010 (Clifford *et al*., 2011). Clifford *et al.* (2011) observed higher number of strain resistant to clindamycin than to erythromycin in isolates from New Zealand and Australia. Seo *et al.* (2010) have described GBS strains resistant to clindamycin and susceptible to erythromycin belonging to serotypes Ia, Ib, III and VIII. This mechanism was based on gene *lnu (B).* Arana *et al.* (2014), in 2014, reported the first human *S. agalactiae* isolate in Europe with new mechanism of resistance to clindamycin based on gene *lnu (B).* In 2014 in USA two vancomycin-resistant invasive *S. agalactiae* strains were isolated (both serotype II, multilocus sequence type 22). The strains were carrying van G elements (Srinivasan *et al*., 2014). The increasing resistance rate might give a rise to a new epidemiological situation. Furthermore, women arriving from various countries with high resistance rates to erythromycin or/and clindamycin might be admitted to Polish hospitals. They may constitute reservoir of multiresistant *S. agalactiae*. Lack of knowledge of local epidemiological data can contribute to ineffective empirical therapy. In the Table III are presented resistance rates to erythromycin and clindamycin in various countries in the world.

Experts had revised procedures that could improve current practices for prevention of perinatal GBS disease and facilitate consensus towards European guidelines and their implementation. If a woman is determined to be at high risk for anaphylaxis, a vaginal-rectal swab should be collected between 35–37 weeks gestation and susceptibility to clindamycin and erythromycin should performed by means of D-zone test. Erythromycin is not recommended because of high rates of resistance present in GBS and to subtherapeutic concentrations in amniotic fluid and fetal serum. Clindamycin could be a proper choice (Di Renzo *et al*., 2015).

The increasing resistance rate might give a rise to a new epidemiological situation. Annual resistance pattern analysis performed by local microbiological laboratory is required for an effective empiric therapy. Additionally, at the time of migrating refugee population knowledge of epidemiological data might contribute to better medical care.

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SHORT COMMUNICATION

Influence of *Pseudomonas* and *Bacillus* Strains Isolated from *Lolium perenne* Rhizospheric Soil in Vojvodina (Serbia) on Planth Growth and Soil Microbial Communities

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Abstract

The aim of this study was the isolation of *Pseudomonas* sp. and *Bacillus* sp. strains from rhizospheric soil and monitoring the impact of two isolates denoted as P12 (*Pseudomonas* sp.) and B1 (*Bacillus* sp.) on the parameters of English ryegrass (*Lolium perenne*) yield and activity of the soil microbial communities. During 2012–2014, a plot experiment was set up following the randomized block system. Better effect on the plant growth was recorded with the use of *Pseudomonas* sp. P12 isolate than with *Bacillus* sp. B1. Positive effect on the increase in the total number of microorganisms, aminoheterotrophs and azotobacter was also achieved. *Bacillus* sp. B1 increased only the number of actimycetes. Both isolates positively affected dehydrogenase activity (DHA).

Key words: *Bacillus* sp., *Lolium perenne*, *Pseudomonas* sp., DHA, plant growth, soil microbial community activity

The mechanisms by which plant-growth promoting rhizobacteria (PGPR) enhance plant growth are not fully understood, but it is believed that the PGPR promote plant growth and yield either by direct mechanisms such as: the ability to produce phytohormons like indolacetic acid, gibberellin, cytokinins and ethylene (Egamberdiyeva, 2007), asimbiotic N_2 fixation (Salantur *et al*., 2006), antagonism against phytopathogenic microorganisms by production of siderophores (Tian *et al*., 2009), and also solubilisation of mineral phosphates and other nutritients (Chen *et al*., 2006), or by indirect mechanisms: the extracellular production of antibiotics, synthesis of antifungal metabolites, production of fungal cell wall lysin enzymes, depletion of iron from the rhizosphere, competition for sites on roots and induced systemic resistance (Ahmad *et al*., 2006). To what extent this stimulative effect will affect the plant yield depends on the type of soil, effectivenes of the indigenous and introduced strains of microorganisms, plant species, agrotechnical measures *etc*. On the other hand, microbiological processes in the soil can additionally be stimulated by introducing PGPR. These microorganisms reproduce in soil and with their enzymatic activity raise and maintain the appropriate level of organic matter in soil (Dobbelaere *et al*., 2003). The effect of bacterial inoculation on the change of microbiological activity in soil depends on soil conditions, plant species, adaptability of introduced microorganisms *etc*. (Egamberdiyeva, 2007). Different bacteria have been reported as PGPR, but the most popular bacteria studied and exploited as biocontrol/promoter agents include the fluorescent species of *Pseudomonas* and *Bacillus* (Adesemoye *et al*., 2008).

The aim of this study was the isolation of *Pseudomonas* sp. and *Bacillus* sp. strains from rhizospheric soil and monitoring the impact of two isolates denoted as P12 (*Pseudomonas* sp.) and B1 (*Bacillus* sp.) on the parameters of the English ryegrass (*Lolium perenne*) yield and activity of the soil microbial communities.

Microorganisms were isolated from the roots and root-adhering soil of 30 days old plants (*L. perenne* L. Calibra) grown in carbonate chernozem type of soil (Stamenov *et al*., 2016). The 10 g samples of roots with tightly adhered soil were suspended in 90 ml of 0.1 M $MgSO_4 \cdot 7H_2O$ buffer and shaken for 10 min at 180 rpm on a rotary shaker. Hundred microliters of the suspension were spread onto King B agar (King *et al*., 1954) for *Pseudomonas* sp. isolates, and onto nutrient agar (Sambrook and Russell, 2001) for *Bacillus* sp. isolates, and incubated at 25°C for 48 h. Isolates

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which were established as gram-negative, rod-shaped cell and produce pigment fluorescein, were selected as *Pseudomonas* sp. isolates. Fluorescein production was tested on Pseudomonas Flo agar (Che *et al*., 1999). Isolates which were established as catalase-positive, gram-positive, rod-shaped cells that form endospores, were classified as *Bacillus* sp. isolates. The presence of catalase was detected by the release of oxygen bubbles in contact with dilute hydrogen peroxide solution (Sneath, 1986).

The plot experiment was set up following randomized block system, during 2012–2014. The size of the experimental plot was 5 m^2. Each variant was conducted in four repetitions. From each repetition (from each plot) it was taken one sample for analysis, *i.e.* four samples for each variant. In the lab, each of the samples was analyzed in three repetitions. The variants of the experiment were the following: 1. plots inoculated with *Pseudomonas* sp. P12, 2. plots inoculated with *Bacillus* sp. B1, 3. control – plots without inoculation. *Pseudomonas* sp. isolate was cultivated on King- B medium whereas *Bacillus* sp. isolate was cultivated on nutrient agar. Before sowing, 50 ml (10^8 CFU/ml) of *Bacillus* sp. as well as 50 ml (10^8 CFU/ml) of *Pseudomonas* sp. isolate cells was introduced into 5 l of tap water, respectively and then evenly sprayed on the plot surface. The sowing was performed manually with 20 kg of English ryegrass per hectare. The experiment was conducted in chernozem soil (FAO classification).

Three mowings (March, July and November) of the grass were performed yearly. The following parameters were determined: yield of fresh and dry mass (t/ha), stem and root length (cm). After the third mowing, the number of microorganisms was determined using the dillution method (Trolldenier, 1996) and dehydrogenase activity (DHA) was measured in accordance with Thalman (1968). Appropriate nutrient media were used (Hi Media Laboratories Pvt. Limited, Mumbai, India): nutrient agar for the total number of bacteria, synthetic agar for the number of actinomycetes, potato dextrose agar for the number of fungi, meat peptone agar for the number of aminoheterotrophs, and nitrogen free medium with manitol for the number of *Azotobacter* sp.

The data were statistically processed by Statistics12.0 programme. The significance of the difference between the applied treatments was determined using Fisher's LSD test.

Seventeen *Pseudomonas* sp. and 23 *Bacillus* sp. strains were isolated. This research was focused on examining the effect of isolate *Pseudomonas* sp. P12 which produces indole-3-acetic acid (IAA), siderophores, cellulase, lipase, urease and gelatinase and isolate *Bacillus* sp. B1, IAA producer and phosphorus solubilizing bacteria, on the growth of English ryegrass (Stamenov, 2014).

Table I
Plant yield (t/ha) and the length of stem and root (cm) of English reygrass

	plant	I year			II year			III year		
		Ø+	*P12*	*B1*	Ø	*P12*	*B1*	Ø	*P12*	*B1*
I	Fresh mass	2.3^{a}*	7.3^{b}	5.0^{b}	3.0^{a}	8.0^{b}	6.3^{bc}	22.0^{b}	40.9^{a}	27.2^{c}
	Dry mass	1.0^{a}	2.6^{b}	2.3^{b}	0.9^{c}	1.8^{a}	1.0^{c}	6.0^{b}	9.0^{a}	6.0^{b}
II	Fresh mass	3.0^{a}	3.7^{a}	3.3^{a}	5.3^{c}	8.0^{b}	8.0^{b}	15.7^{d}	19.9^{a}	14.3^{d}
	Dry mass	1.0^{b}	1.1^{b}	1.1^{b}	2.7^{a}	5.0^{cb}	4.3^{c}	4.2^{c}	7.0^{b}	5.5^{bc}
III	Fresh mass	12.0^{a}	13.0^{a}	15.0^{a}	8.0^{c}	8.6^{cb}	9.0^{cb}	22.5^{b}	36^{d}	44.0^{d}
	Dry mass	6.0^{a}	7.0^{a}	6.5^{a}	1.6^{b}	1.6^{b}	1.6^{b}	3.1^{d}	8.9^{b}	14.4^{a}
Total yield	Fresh mass	17.3^{b}	24.0^{a}	23.3^{a}	16^{b}	24.6^{a}	23.3^{a}	60.2^{d}	97.1^{a}	85.5^{b}
	Dry mass	8.03^{c}	10.7^{a}	9.9^{ab}	5.1^{b}	8.7^{a}	6.9^{a}	13.3^{c}	25^{b}	25.9^{b}
Total %	Fresh mass	–	38.5	34.6	–	51	63	–	61.3	42
	Dry mass	–	33.9	23.8	–	64	85.5	–	87.2	94.7
I	Stem	10.5^{b}	19.0^{a}	18.5^{a}	12.0^{a}	23.0^{c}	21.0^{cb}	24.0^{c}	34.5^{a}	28.5^{d}
	Root	3.0^{b}	5.0^{a}	4.5^{ab}	3.75^{a}	5.0^{a}	4.5^{a}	3.85^{b}	6.34^{a}	3.5^{b}
II	Stem	10.7^{a}	12.0^{a}	11.75^{a}	17.0^{a}	23.5^{b}	22.0^{cb}	26.7^{cb}	34^{b}	25.9^{c}
	Root	2.5^{a}	4.0^{a}	4.0^{a}	4.0^{a}	4.75^{a}	4.75^{a}	1.9^{ab}	2.7^{ab}	3.16^{b}
III	Stem	18.7^{b}	25.0^{a}	20.5^{b}	19.5^{a}	24.5^{cb}	26.0^{b}	32.0^{d}	39.2^{a}	28.7^{d}
	Root	4.7^{a}	5.5^{a}	5.0^{a}	4.75^{a}	5.0^{a}	5.5^{a}	4.0^{a}	4.5^{a}	3.75^{a}
Average	Stem	13.3	18.6	16.9	16.2	23.7	23.0	27.5	35.9	27.7
	Root	3.4	4.83	4.5	4.2	4.9	4.9	3.25	4.5	3.45

* Mean values with the same superscript(s) are not significantly different according to Fisher LSD test ($p < 0.05$);
+Ø – control; isolates *Pseudomonas* P12 and *Bacillus* B1.

In comparison with the control, a statistically significant increase in the yield of fresh and dry mass was recorded in plots inoculated with the microorganisms (Table I). During the first year, the total yield of fresh mass of the plants inoculated with isolate P12 was 38% higher than in the control, whereas the yield of dry mass was 33% higher. The yield of fresh and dry mass were also increased by the inoculation with B1 isolate for 34 and 23%, resectively. During the second year, the total yield of fresh mass of plants was 51 and 64% higher than in the control whereas the yield of dry mass was 64 and 85% higher in variant with *Pseudomonas* sp. and *Bacillus* sp. isolate, respectively. Results of the third year of experiment showed very similar results. In average, there was no significant statistical difference in the effect on the yield between the *Bacillus* sp. B1 and *Pseudomonas* sp. P12 isolates, except in the third year of experiment when the yield of fresh mass was statistically higher in the variant with *Pseudomonas* sp. P12 than in the variant with *Bacillus* sp. B1.

In comparison with the control, it was observed that isolates *Pseudomonas* sp. P12 *Bacillus* sp. B1 had a positive effect on the length of stem and root of English ryegrass. Better effect on the plant growth was recorded with the use of *Pseudomonas* sp. P12 isolate. Similarly to this study, Stamenov *et al.* (2012a) identified the positive effect of *Pseudomonas fluorescens* and *Bacillus subtilis* strains on the height and dry weight of English ryegrass. Garcia *et al.* (2004) noticed a positive effect of inoculation with *Bacillus lichenoformis* on plant yield. Stamenov *et al.* (2012b) investigated the effect of *P. fluorescens*, *B. subtilis*, *Streptomyces* sp. and *Trichoderma asperellum* on the yield of English ryegrass Esquire variety. On average, the best effect was achieved with *B. subtilis*, whereas the weakest effect was recorded in the variant with *T. asperellum*. Ratti *et al.* (2001) found that the combination of the arbuscular mycorrhizal fungus *Glomus aggregatum*, and the PGPR *Bacillus polymyxa* and *Azospirillum brasilense* maximized biomass and P content of the aromatic grass palmarosa (*Cymbopogon martinii*) when grown with an insoluble source of inorganic phosphate. Plant growth promoting activities include production of siderophores, phosphate solubilizing enzymes and auxin, strongly affect the environment, both because they inhibit growth of other deleterious microorganisms and because they increase nutrient availability for the plant (Suresh, 2010). In this study, isolates ability to produce enzymes and materia that promote plant growth (such as auxin and siderophores) could be the explanation of the isolates positive effect on the parameters of plants growth.

This investigation also included observing the quantitative changes in the microbial population in the rhizosphere of English ryegrass. Results of this research showed that the number of the investigated groups of microorganisms, apart from the number of fungi during the second year, increased in both variants in comparison with the control, (Table II).

In average, the use of *Pseudomonas* sp. isolate P12 had a better effect on the increase in the total number of microorganisms, aminoheterotrophs and *Azotobacter* sp. whereas the use of *Bacillus* sp. isolate B1 had a better effect on the increase in the number of Actinomycetes. According to the results of Schrader and Blevins (2001), biomass production of Actynomycetes was generally higher at the higher phosphorus concentrations than at the lower. These results suggest that the phosphor-solubilizing activities of isolate B1 directly influence the number of Actynomycetes. Dehydrogenase activity reflects the total range of oxidative activity of soil microorganisms (Liang *et al.*, 2014). In comparison with the control, introduction of both isolates positively affected the DHA. The highest DHA was recorded in the variant where isolate P12 was used. Our results are in agreement with the results of Nannipieri *et al.* (2003), who pointed out that the use of bacteria in plant production increases the number and enzymatic activity of microorganisms which enhaces the productive capability of soil. Our results are supported also by the research of Han *et al.* (2006).

Table II
The effect of inoculation on the number of soil bacteria (CFU/g dry soil) and DHA (TPF g^{-1} soil)

The number of microorganisms in	I year			II year			III year		
	Ø+	*P12*	*B1*	Ø	*P12*	*B1*	Ø	*P12*	*B1*
Total number, 10^6	25.74^{b}	76.71^{a}	34.68^{b}	15.77^{c}	90.61^{a}	30.51^{b}	$5{,}07^{c}$	$214{,}06^{a}$	$151{,}0^{b}$
Fungi, 10^4	5.3^{b}	5.68^{b}	14.90^{a}	20.1^{ab}	14.1^{bc}	10.7^{bc}	$10{,}1^{bc}$	$42{,}46^{a}$	$20{,}24^{b}$
Actinomycetes,10^5	7.8^{a}	11.37^{a}	15.9^{a}	7.87^{a}	22.44^{a}	8.08^{a}	$10{,}99^{a}$	$21{,}22^{a}$	$28{,}03^{a}$
Aminoheterotrophs,10^6	4.54^{b}	67.47^{a}	6.38^{b}	30.59^{b}	32.42^{b}	54.75^{b}	$86{,}2^{bc}$	$213{,}17^{a}$	151^{ab}
Azotobacter, 10^2	162.8^{b}	177.57^{b}	223.45^{a}	54.6^{bc}	151.7^{a}	91.9^{ab}	$86{,}6^{ab}$	$163{,}6^{ac}$	$73{,}96^{b}$
DHA	1118	1713.3	1219.4	768.2	896.7	871.3	680.9	974.3	951.9

* Mean values with the same superscript(s) are not significantly different according to Fisher LSD test ($p < 0.05$);
+Ø – control; isolates *Pseudomonas* P12 and *Bacillus* B1.

They improved the biological properties of soil by introducing *Bacillus* sp.

In this study, the increase in the number of microorganisms in soil and the positive effect of inoculation on the plant suggest that the use of *Pseudomonas* sp. P12 and *Bacillus* sp. B1 can result in a better yield of forage crops, especially in organic production, where mineral fertilizers are not used. Introducing of isolate P12 had a better effect on the stem and root length, total number of microorganisms, aminoheterotrophs, *Azotobacter* sp. and DHA than isolate B1, which might be due to ability of isolate P12 to produce siderophores and enzymes. On the other hand, isolate B1 more influenced the number of Actinomycetes, which can be explained by the fact that *Bacillus* sp. isolate B1 has ability to solubilize phosphate. Further studies on the isolates will uncover the exactly mechanisms by which they promote plant growth. Isolates P12 and B1, with the ability to promote plant growth, may represent a biological alternative for chemical fertilizers application in agriculture. In order to achieve the best results, it is necessary to isolate as many microorganisms as possible from the rhizosphere of grasses, determine their effect on the plant growth and find the most adequate way of applying the inoculants.

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SHORT COMMUNICATION

Betaine Improves Polymer-Grade D-Lactic Acid Production by *Sporolactobacillus inulinus* Using Ammonia as Green Neutralizer

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Abstract

The traditional $CaCO_3$-based fermentation process generates huge amount of insoluble waste. To solve this problem, we have developed an efficient and green D-lactic acid fermentation process by using ammonia as neutralizer. The 106.7 g/l of D-lactic acid production and 0.89 g/g of consumed sugar were obtained by *Sporolactobacillus inulinus* CASD with a high optical purity of 99.7% by adding 100 mg/l betaine in the simple batch fermentation. The addition of betaine was experimentally proven to protect cells at high concentration of ammonium ion, increase D-lactate dehydrogenase specific activity and thus promote the production of D-lactic acid.

Key words: *Sporolactobacillus inulinus*, ammonia as neutralizer, betaine as osmoprotectant, D-lactic acid production

Poly-lactic acid (PLA), a biodegradable polymer produced by lactic acid monomer are considered as the most promising substitution for petroleum-derived plastics in future (Zhang and Vadlani, 2013). The application of poly L-lactic acid (PLLA) is limited by its low melting temperature (< 180°C) while this problem can be obviated by blending it with poly D-lactic acid (PDLA). The melting point of the stereocomplex polymer is approximately 50°C higher than that of the respective single polymers (Ikada *et al*., 1987). This finding has attracted great interest to the production of D-lactic acid. However, the technology for D-lactic acid production is not well established, compared to the L-lactic acid fermentation process (Wang *et al*., 2012; Li *et al*., 2013).

In addition, the accumulation of lactic acid causes the pH to continuously decrease and further influences the growth and performance of the strains. Thus, it is necessary to add some neutralization agent to the medium to keep the pH value at a stable level during the process of fermentation. To avoid growth inhibition by lactic acid, $CaCO_3$ is normally added during fermentation to neutralize lactic acid and maintain the pH within the range of 5.0–6.0. However, a considerable amount of calcium sulfate (gypsum) is produced during the conversion of calcium lactate to free lactic acid, causing extensive environmental burden (Vaidya *et al*., 2005). Ammonia is a fast and effective neutralizer. It can maintain the pH stability of the broth without producing any calcium sulfate during the process of extraction. Additionally, it can be recovered from the fermentation broth in the process of lactic acid purification, and subsequently recycled for continuous fermentation. Moreover, it can serve as nitrogen source in the fermentation process (Miura *et al*., 2004). However, the final acid concentration and cell concentration are hampered by the toxicity of ammonia to the microbial cells.

The present paper reports an efficient NH_4^+-based production of polymer-grade D-lactic acid by *Sporolactobacillus inulinus* CASD [China General Microbiological Culture Collection Center (CGMCC), No 2185] (Wang *et al*., 2011). The effect of betaine, N,N,N-trimethylglycine, on the activity of D-lactate dehydrogenase (EC 1.1.1.28) and D-lactic acid production under such condition was investigated. The enzyme activity was determined by measuring the initial rate of oxidation of NADH at 340 nm with pyruvate as a substrate. The lactate dehydrogenase specific activity was defined as U/mg total protein. The protein concentration was determined with Bradford reagent (Bradford, 1976).

Both cell growth and lactic acid production would be inhibited by high concentration of the substrates (initial glucose concentration), end-product (fermented lactic

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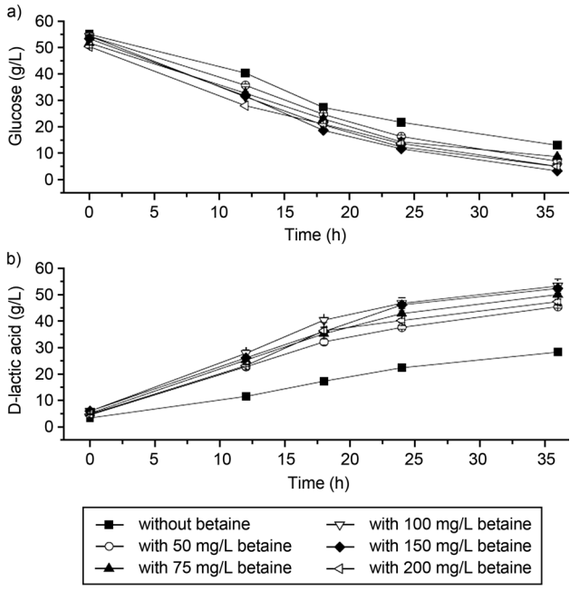

Fig. 1. The effects of betaine concentrations on glucose consumption and D-lactic acid production by strain *S. inulinus* CASD.
(a) the glucose consumption and (b) D-lactic acid production. Results are mean ± SD from three independent experiments.

acid) and ammonia (Ding and Tan, 2006). To relieve the substrate level-osmotic stress, betaine (0–200 mg/l) was added to the medium as osmoprotectant. As shown in Fig. 1, betaine exhibited a positive effect on D-lactic acid fermentation by *S. inulinus* CASD using ammonia as neutralizer. When betaine was added at 100 mg/l, the maximal D-lactic acid concentration was obtained. With further increase of the concentration of betaine, no obvious effect was observed on sugar consumption and lactic acid production. The addition of 100 mg/l betaine could provide enough intracellular accumulation of osmoprotectant to protect cells against the influence of high concentration of ammonium ion in broth, finally resulting in the improvement of D-lactic acid production. Thus, a betaine concentration of 100 mg/l was selected as the optimal concentration and was used in subsequent experiments.

The D-lactate dehydrogenase activity of *S. inulinus* CASD was also positively affected by betaine addition under the ammonium ion stress condition. The specific activity (2.36 U/mg) of lactate dehydrogenase in the crude extract of *S. inulinus* CASD was over two-fold than that without betaine supplementation (1.03 U/mg).

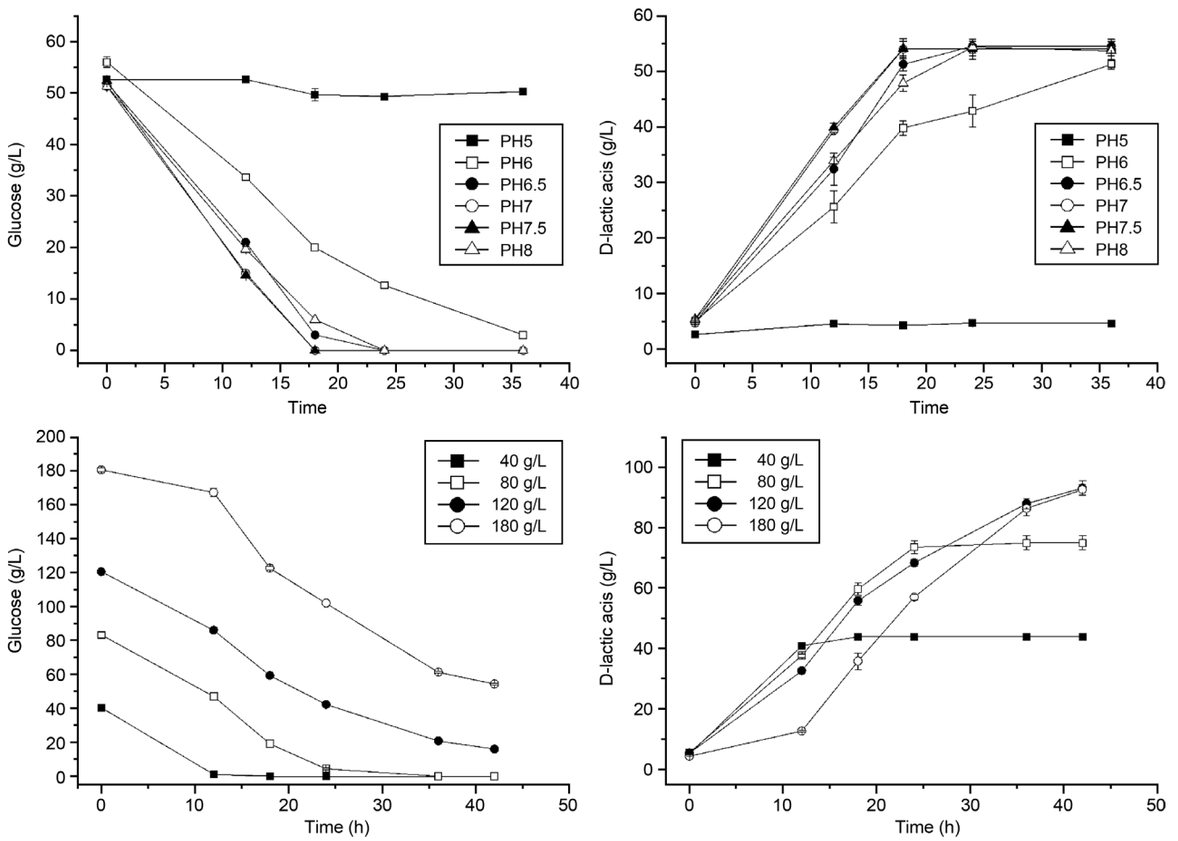

Fig. 2. Optimization of batch fermentation conditions for D-lactic acid production by *S. inulinus* CASD using ammonia as neutralizing agent.
Results are mean ±SD from three independent experiments. (a) the glucose consumption and (b) D-lactic acid production under different pH conditions; the effects of the initial glucose concentrations on (c) glucose consumption and (d) D-lactic acid production.

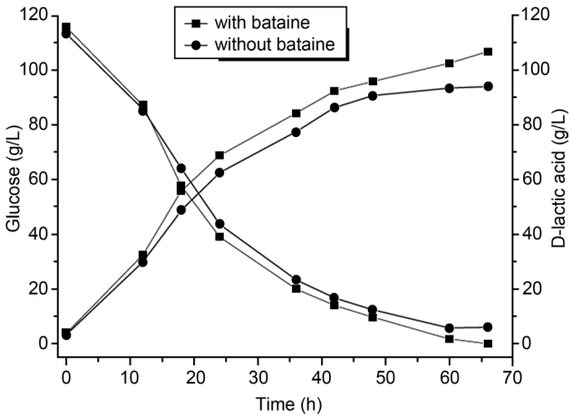

Fig. 3. Batch fermentation of D-lactic acid by *S. inulinus* CASD using ammonia as neutralizing agent with or without betaine.

Additionally, betaine was also found to improve the cell growth under the ammonium ion stress condition. The cell density (OD_{600}) reached 12.56 with betaine addition while it was only 9.42 without betaine supplementation. The increased D-lactate dehydrogenase activity and cell growth clearly indicated that betaine supplementation benefited the D-lactic acid fermentation under the ammonium ion stress conditions.

As ammonia was used as neutralizer, the optimum pH for D-lactic acid production was first investigated. The glucose consumption and lactic acid production were significantly inhibited when the pH of broth was maintained at 5.0 (Fig. 2a-b). The lactic acid concentration increased most rapidly and reached its highest values (54.3 g/l) after 18 h at pH 7.0. Although the concentrations of D-lactic acid reached the same values after 18 h at pH 7.5, pH 7.0 was finally chosen as the optimal pH since less of ammonia was used under such conditions.

To examine the substrate tolerance of strain CASD, different initial glucose concentrations were tested for D-lactic acid production (Fig. 2c-d). When glucose concentration was below 80 g/l, the concentration of D-lactic acid increased as the initial glucose concentration increased. The D-lactic acid concentration increased fast in 24 h with an initial glucose concentration of 80 g/l. However, the D-lactate productivity became much slower than others when the initial glucose concentration was higher than 180 g/l, indicating the inhibition of cell growth and D-lactic acid production by high concentration of glucose (over 180 g/l). As the glucose could almost consumed and a higher concentration of D-lactic acid obtained (93.2 g/l), 120 g/l was selected as the optimized initial glucose concentration for use in the following batch fermentations.

The fermentations were carried out at 42°C for 66 h in a 5 l bioreactor with working volume of 2 l of optimized medium (glucose 120 g/l, yeast extract 10 g/l and betaine 100 mg/l or without betaine, pH 7.0) with an agitation speed of 50 r/min without aeration. The glucose in the medium with betaine was completely exhausted and the final D-lactic acid concentration reached 106.7 g/l with a productivity of 1.62 g/l·h (Fig. 3). The fermentation with 100 mg/l betaine could produce 13.6% more lactic acid compared to the fermentation without betaine and raised the yield by 11.4%. Moreover, the optical purity of D-lactic acid was determined to be 99.7%, which meets the requirement for the lactic acid polymerization process.

Using ammonia as neutralizer is a green way to produce lactic acid, while the high concentration of ammonium ion in the broth will significantly affect the cell growth and performance, resulted in a rather low productivity. Okano reported a D-lactic acid fermentation strategy using ammonia solution as pH-controlling agent by a L-lactate dehydrogenase gene deficient *Lactobacillus plantarum* (Okano *et al*., 2009). However, the final concentration of D-lactic acid was 73.2 g/l and the yield was just 0.85 g/g initial glucose which almost ruled out the possibility of industrialization. Betaine has been applied in many fermentation processes as an efficient osmoprotectant (Sharma and Dubey, 2005; Robert *et al*., 2000), but its positive effects were not indicated on the protection inhibition caused by ammonium ion. In this study, betaine was proven to have positive effect on D-lactic acid production when ammonia was used as neutralizer. As a result, an efficient lactic acid production (106.7 g/l), with a small amount addition of betaine (100 mg/l), by the *S. inulinus* strain was developed, providing a good option for polymer-grade D-lactic acid production in a green fermentation process. Thus, an efficient and 'green' D-lactic acid fermentation strategy without producing recalcitrant wastes (*e.g.*, gypsum) for the industrial production of polymer-grade D-lactic acid was established.

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SHORT COMMUNICATION

The Serological Surveillance of *Hepatitis E virus* among Hunters and Foresters in Eastern Poland

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Abstract

The aim of the study was the analysis of the occurrence of specific antibodies against HEV among hunters and foresters who are at risk to be exposed. The study group consisted of 210 hunters (23–80 years old) and 60 foresters (22–64 years old). Anti-HEV IgG were present in 3.81% of the samples of the hunters and in 5% of the samples of the foresters. The statistical analysis shows no significant differences in the results anti-HEV IgG between the groups of hunters and foresters ($p = 0.5278$). Significantly higher anti-HEV IgG titers were found in the older age group (> 55 years old).

K e y w o r d s: anti-HEV IgG in hunters and foresters, HEV, risk of hepatitis E

Hepatitis E virus (HEV) is a ssRNA positive-strand virus, a member of the *Hepevirus* genus, *Hepeviridae* family and consists of 4 major genotypes (1, 2, 3 and 4). Genotypes 1 and 2 of HEV have been found only in humans and they are responsible for epidemics in endemic regions (HEV 1 in North America and Asia, HEV 2 in West Africa and Mexico). The transmission is primarily *via* the faecal-oral route through drinking water or food contaminated with human excreta. In developed countries those two genotypes cause travel associated infections, in Europe cases caused by HEV 1 are predominant. Genotypes 3 and 4 of HEV have been found in humans and animals (pigs, wild boars, shellfish and other), and are responsible for worldwide autochthonous infections. Zoonotic transmission to humans is the most important for HEV genotypes 3 and 4. Consumption of raw or undercooked wild-boar meat, offal or beef is significantly associated with autochthonous HEV infection. HEV infections caused by blood transfusions, mother-to-child, person-to-person, and sexual intercourses were also documented but uncommonly noted. In Europe, there is mostly genotype 3 in humans, but a few cases of HEV 4 were described (Wichmann *et al*., 2008; Lee *et al*., 2015; Pérez-Gracia *et al*., 2015). HEV-infected persons exhibit a wide clinical spectrum, ranging from asymptomatic infection through acute icteric hepatitis to fulminant hepatitis. Acute hepatitis E usually manifests with icterus, malaise, anorexia, fever, hepatomegaly, and occasionally pruritus. Certain population sub-groups are at a higher risk of severe disease following HEV infection. These include pregnant women, persons with pre-existing liver disease and persons with immunosuppression (WHO, 2014).

Contact exposure to infected animals leads to an elevated risk of HEV transmission in humans. Studies have shown that occupational groups *e.g.* swine breeders (Forgách *et al*., 2007), slaughterers (Krumbholz *et al*., 2012), veterinarians who come into close contact with pigs (Bouwknegt *et al*., 2008) run the risk of being infected with HEV. Swine workers in Spain were found to be 5.4 times more likely to be positive for anti-HEV IgG than those not exposed to swine (Galiana *et al*., 2008). Contact with swine is the most widely recognized route for occupational exposure to HEV; however, the multitude of novel strains of HEV in wildlife and other domestic animal species suggest additional mechanisms of transmission (Yugo and Meng, 2013). The aim of the study was the analysis of the occurrence of specific antibodies against HEV among hunters and foresters who are at risk to be exposed.

The study group consisted of 210 hunters aged 23–80 (54, SD 11.61): 208 men (99.05%) and 2 women (0.95%)

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Table I
The titers of anti-HEV IgG depending on the age of hunters and foresters

Group	The titers of anti-HEV IgG (RU/ml)						
	Age	Number of persons	Average	Median Me	MIN	MAX	SD
Hunters	≤ 35	17	0.68	0.26	0.20	4.00	0.96
	36–55	99	0.59	0.30	0.20	5.57	0.75
	> 55	94	0.89***	0.46	0.20	19.71	2.08
Foresters	≤ 35	13	1.61	0.22	0.20	16.73	4.55
	36–55	32	0.55	0.26	0.20	2.79	0.56
	> 55	15	2.13	0.47	0.20	24.00	6.07
All studied persons	≤ 35	30	1.08	0.25	0.20	16.73	3.05
	36–55	131	0.58	0.29	0.20	5.57	0.71
	> 55	109	1.06*/**	0.46	0.20	24.00	2.94
Total		270	0.83	0.35	0.20	24.00	2.19

Significant differences: * $p = 0.004$; ** $p = 0.0194$; *** $p = 0.0119$

and 60 male foresters, aged 22–64 (45.63, SD 11.81). The selection of the groups was purposeful because hunters and foresters have a diverse activity by being in the forest environment. Blood samples were collected in Lubelskie voivodship from 2014 to 2015. The groups of hunters and foresters were divided into three age groups: ≤ 35 years old, 36–55 years, > 55 years. The Bioethical Committee of Medical University of Lublin authorized the project (permission No. KE-0254/177/2014).

Serological analysis. The presence of anti-HEV IgG was detected by ELISA (Anti-*Hepatitis E virus* (HEV) ELISA IgG, Euroimmun). Microtiter wells were coated with mixed recombinant antigens of *Hepatitis E virus* genotypes 1 and 3. The results above or equal 2.2 relative units/ml (RU/ml) were considered as positive, below 1.6 RU/ml as negative, whereas borderline results were ≥ 1.60 and < 2.2 RU/ml. The test was carried out and the results were interpreted according to the manufacturer's instructions.

The obtained data were analysed statistically using Statistica v. 10 software. The Chi-square test was performed for nominal features in order to detect statistically significant dependence. For data expressed numerically nonparametric Mann-Whitney *U* and Kraskala-Wallisa test was performed. The assumptive level of significance was $p = 0.05$.

The results for anti-HEV IgG in hunters group were as follows: 8 positive samples (3.81%), 10 borderline (4.76%) and 192 negative (91.43%). Anti-HEV IgG in the foresters group were detected in 3 samples (5%) at positive level and in 1 sample (1.67%) at borderline level. Anti-HEV antibodies were not reported among 56 (93.33%) foresters. The statistical analysis shows no significant differences in the titers anti-HEV IgG between the groups of hunters and foresters ($p = 0.5278$). The titers of anti-HEV IgG were presented in Table I.

Significant statistical differences ($p = 0.004$) in titers of anti-HEV IgG between the age group 36–55 (0.58, SD 0.71), and the age group > 55 (1.06, SD 2.94) for all the tested subjects were found. There were also significant statistical differences ($p = 0.0194$) in IgG titers between the age group of ≤ 35 (1.08, SD 3.05) and the age group > 55 (1.06, SD 2.94) for all the tested subjects. Significant statistical differences ($p = 0.0119$) in titers of anti-HEV IgG between the age group 36–55 (0.59, SD 0.75) and the age group > 55 (0.89, SD 2.08) for the hunters were found.

In the study of Sadowska-Todys *et al.* (2015) performed in Poland among 1027 hunters (17 to 85 years of age), in 206 persons (25%) anti-HEV IgG were found by means of ELISA test (positive and borderline result). Anti-HEV IgM were confirmed in 3 persons. The study showed that the percentage of persons with anti-HEV IgG is the highest in the age group ≥ 70 and the difference is statistically significant comparing to other age groups. No relation between the percentage of seropositive persons and the duration of hunting activities was found.

In our study 210 hunters aged 23–80 were examined. At the beginning of the experiment we assumed that the forester group, without the direct contact with animals, would be the control group of the study. Positive results anti-HEV IgG were present in 3.81% of the samples of the hunters group and in 5% of the samples of the foresters. The statistical analysis in our study showed no significant differences in the results anti-HEV IgG between the groups of hunters and foresters. The higher titers anti-HEV IgG was in the older age groups, significantly higher antibody titers demonstrated in the age group > 55 years.

It has already been described by Carpentier *et al.* (2012) in France, which forestry workers, particularly woodcutters are group endangered with HEV infec-

tion. French forestry workers (n = 593) were tested for anti-HEV antibodies. One hundred eighty five subjects had anti-HEV antibodies (31.2%) with prevalence increasing steadily from 15.7% in 15–34-year-olds to 46.2 in subjects older than 55 years of age. Among the 135 controls with no direct contact with the forest environment (drivers, gardeners, traders) 26 (19.2%) had anti-HEV antibodies with the prevalence increasing from 4.5% in subjects aged 15 to 34 to 39% in elders. In the study of Montegnaro *et al*. (2015) performed in central Italy (Latium region) HEV seroprevelance was noted among 25% of the examined hunters. In Italy several authors have demonstrated an appreciable frequency of HEV infection, generally in travellers returning from endemic areas, however autochthonous cases have also been documented pointing to a possible autochthonous circulation of the virus in human population, sustained by the presence of an animal reservoir of HEV. In the study of Schielke *et al*. (2015) on 126 hunters (median age 55; 94% male) 21% tested were positive for anti-HEV IgG antibodies. Anti-HEV prevalence was the highest in the age group of the 70–79 (67%). Hepatitis E has been observed in Germany since 2001. Since then, the number of recorded cases has been increasing steadily each year. Men between 50–69 years of age are the most affected group with 0.9 cases per 100,000 populations. Underreporting is expected due to asymptomatic and underdiagnosed infections. In forest workers, anti-HEV prevalence of 18% in Germany was reported (Dremsek *et al*., 2012). Our study confirmed the higher exposure in older age groups, significantly higher anti-HEV titer demonstrated in the age group > 55.

In the study of Bura *et al*. (2015) 182 patients (101 men and 81 women), aged 19–85 (47.2 ± 14.2; half of the patients were under 48), hospitalized for different reasons in Wielkopolska Region (infectious and non-infectious liver diseases, diarrheal illnesses, herpes zoster, HIV infection, meningitis and erysipelas) were examined. Anti-HEV were found in 29 patients (15.9%).

In conclusion, the zoonotic risk of HEV is well established but seroprevelance in humans varies drastically between studies and countries. Numerous animal species seropositive for IgG anti-HEV, contaminated food, water and environment must be considered as potential sources of HEV infections in humans.

This study was financed thanks to the internal research funds of Pope John Paul II State School of Higher Education in Biala Podlaska. Title of grant: Assessment of the level of antibodies against hepatitis E (HEV) in the sera of animals and people at risk of hepatitis E. The principal investigator: Marcin Weiner, PhD, MsC.

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SHORT COMMUNICATION

Morphological and Molecular Characterization of *Phoma complanata*, a New Causal Agent of *Archangelica officinalis* Hoffm. in Poland

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Abstract

The paper concerns the fungus *Phoma complanata*, isolated for the first time in Poland, from the roots and umbels of angelica (*Archangelica officinalis*) in 2009. The morphology of fungal isolates was tested on standard culture media. Moreover, the sequence analysis of ITS regions was conducted. Morphological similarity of *P. complanata* Polish isolates to the reference isolate obtained from CBS culture collection was determined and together with the molecular analysis confirmed the affiliation of the fungus to the species.

Key words: *Phoma complanata*, *fungus Phoma sensu lato from* angelica, ITS rDNA sequences, SEM identification

Phoma sensu lato is a highly polyphyletic genus with its unclear species boundaries (Aveskamp *et al*., 2008; 2010; Rai *et al.,* 2014; Chen *et al.*, 2015). The conventional system of identification based on morphological features in *in vitro* conditions is still valid but insufficient. Increasingly, in order to achieve the correct identification of *Phoma sensu lato*, secondary metabolites, the protein profile and nucleotide sequences using modern molecular techniques have been examined (Aveskamp *et al*., 2008; 2010; Frisvad *et al*., 2008; Rai *et al*., 2014).

P. complanata according to the current rules of taxonomy, belongs to the family *Didymellaceae*, which according to the old system included species of the section *Phoma*, *Peyronella*, *Heterospora* oraz *ParaPhoma* (Aveskamp *et al*., 2010).

Farr *et al*. (1995) reported occurrence of *P. complanata* isolates on angelica stem in the USA. On the other hand, according to Boerema *et al*. (2004) the species *P. complanata* is commonly transferred by the seeds of parsnip (*Pastinaca sativa)*, parsley (*Petroselinm crispum*) and carrots (*Daucus carota*), and damaged petioles, leaves and roots of these plants.

P. complanata was isolated for the first time in Poland from the roots and umbels of angelica (*Archangelica officinalis*) in 2009 (Zalewska *et al*., 2013). The isolation of *P. complanata* was repeated in recent years.

The accessible literature provides information on disease symptoms caused by *P. complanata* (Farr *et al*., 1995; Zalewska *et al*., 2013), pathogenicity and the mode of penetration of angelica leave and stem tissue (oral communication). The present research undertakes identification with morphological features Polish isolates of *P. complanata*. Moreover, the sequence analysis of the ITS regions was carried out – in order to confirm the accuracy of identification.

In the studies there were used single-cultures of *P. complanata* (Tode) Desm. from the collection of the Department of Phytopathology and Mycology of the University of Life Sciences in Lublin. These cultures were obtained from angelica leaves (Zalewska *et al*., 2013) and identified on standard media basing on a study Boerema *et al*. (2004), taking into account the up to date rules of taxonomy of *Phoma* genus, while reference isolate CBS 100311 was from the stems of hogweed (*Heracleum spondyllium* L.) in the Netherlands obtained from Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Three randomly selected isolates of *P. complanata*: A 103, A 233 and A 235 and reference isolate CBS 100311 were the subject of morphological and genetic characteristics.

The 3 mm discs of sporulating mycelium of the above mentioned isolates were placed on three solidified standard media, *i.e.* MA – maltose agar medium, OA – oat agar medium and CA – cherry agar medium (Boerema *et al*., 2004). The mode of the culture incubation and description is provided in Boerema *et al*. (2004). The measurements of 300 conidia (3 isolates × 100 conidia)

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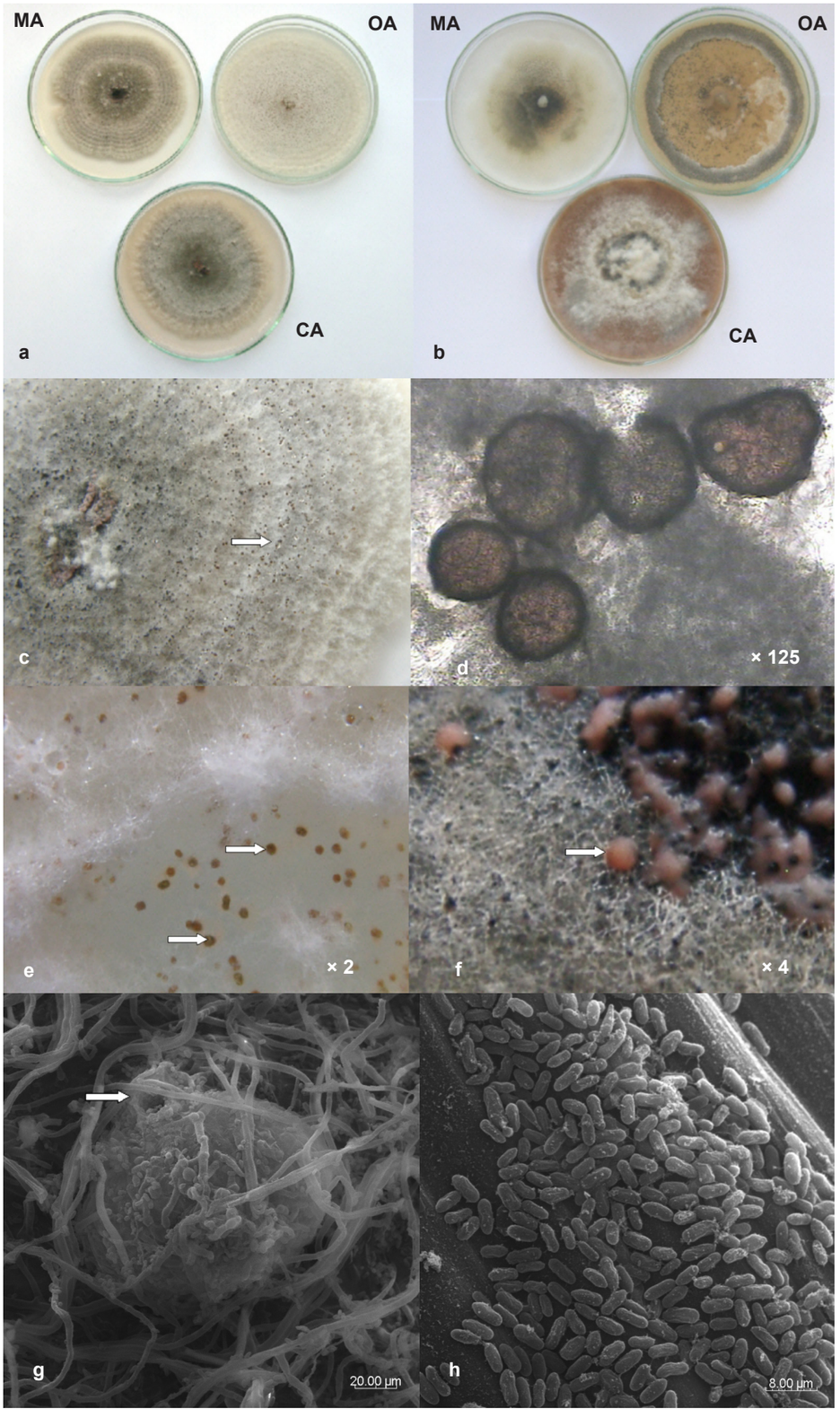

Fig. 1. *P. complanata* morphology.
(a) 7-day-old colonies on standard media. (b) 14 –day-old colonies on standard media. (c) pycnidia (arrow) in the aerial mycelium. (d) aggregate of pycnidia (×125). (e, f) drops of conidial exudate on OA (arrows). (g) Scanning electron micrograph of pycnidium with ostiole (arrow) (scale bar = 20.00 µm). (h) Scanning electron micrograph of conidia (scale bar = 8.00 µm).

and 150 pycnidia (3 isolates × 50 pycnidia) were performed after 2 weeks of culture on the oat agar medium (OA). The presence of chlamydospores was also detected. Documentation was made using the light and scanning electron microscopy (SEM).

Genetic identification was based on the differences in the nucleotide sequences of the PCR-amplified fragments of ITS regions of rDNA (ITS1, 5.8S r DNA gene, ITS2). ITS fragments were amplified with two sets of primers ITS1 and ITS4 (White *et al*., 1990).

Sequencing of the PCR products was made by the company Genomed S.A. Poland. The obtained nucleotide sequences were analysed with clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) software and compared with sequences collected in NCBI Gene Bank databases with Blast software (http://www.ncbi.nlm.nih.gov.BLAST/). The phylogenetic analysis were performed using the Phylogeny. fr program (http://www.phylogeny.fr/simple_phylogeny.cgi). The original DNA sequences obtained in this study have been deposited in GenBank.

Morphological studies showed that the growth of *P. complanata* isolates on MA was zoned. The colonies were cream-olive to grey floccose, aerial mycelium with a regular edge and a clear margin (Fig. 1a). The reverse of the colony was olive (Table I). After 14 days the mycelium formed a compact floccose to woolly structure more than after 7 days (Fig. 1b) . The diameter of the colony after 7 and 14 days was, 35–37 and 73–86 mm respectively (Table I). Colonies on OA after 7 and 14 days were white-gray with a bright-olive reverse and floccose to woolly aerial mycelium. The edge of the colonies was regular (Fig. 1a, b). The diameter of the colony after 7 and 14 days on OA was, 36 and 82–84 mm respectively (Table I). Colonies on CA after 7 days were gray-olive, dark in the oldest part of the colony, with a bright 1 cm margin. The reverse was dark-olive. The aerial mycelium was at the beginning floccose, but after 14 days it gradually became more compact and woolly (Fig. 1b). The diameter of the colony after 7 and 14 days on CA was, 34–36 and 76 mm respectively. The edge was regular (Table I). Application of a droplet of NaOH after 14 days did not have any effect. The crystals didn't form. The pycnidia were formed on all media after 7 days in the oldest part of the colonies, singly or in small aggregates (Fig. 1c, d) and secreted beige to rose exudate of conidia (Fig. 1e, f). The pycnidial walls were multilayer, thick, with one ostiole (Fig. 1g). The size of the pycnidia ranged from 86 to 288 µm (Table II). The conidia were differentiated in shape and size, usually oval, cylindrical, ellipsoidal, mostly aseptate, and 2.86 – 7.64 × 1.91 – 3.82 µm in dimension (Table II, Fig. 1h). Occasionally, 1-septate conidia with the dimension of 9.55–13.37 × 2.86–3.83 µm were observed in 14-old-days cultures. Similarly, in the case of isolate CBS 100311 1-septate conidia with the dimension of 14.21–18.23 × 4.33–6.11 µm constituted about 2% on 14-day-old cultures grown on OA medium (Table II).

Electrophoresis of PCR amplification products revealed a distinct band of approximately 550 bp. Nucleotide sequences of the ITS region from A 103, A 233 and A 235 of *P. complanata* isolates were identical. However, ITS sequences from these isolates slightly differed from the reference isolate by some substitutions and alignment gaps within ITS region. The amplified fragment showed 96% identity on the length of 434 bp for isolate A 103, 432 bp for isolate A 233 and 433 bp for isolate A 235 with nucleotide sequence of *P. complanata* collected in the CBS. A phylogenetic tree, based on the ITS sequence of three isolates of *P. complanata* and reference strain generated using the Phylogeny. fr analysis, indicated the segregation of all isolates into two main clusters. The first cluster grouped reference strain and our three native isolates of *P. complanata*: A 103, A 233 and A 235. The second cluster included *P. neerlandica* CBS 134.96 , the isolate which has been used the tree to be rooted (Fig. 2). Sequences of above isolates have been deposited in GenBank, respectively with the reference numbers MF062524, MF062525 and MF062524. Sequence-based identification was correlated with the identification by classical methods.

Genus *Phoma* discussed by Boerema *et al.* in 2004 and described in the 10th Edition, Dictionary of the Fungi "(Kirk *et al*., 2008), now should be considered as *sensu lato* because it involves a group of about 10 different genera, four already known and some new (Aveskamp *et al*., 2010; De Gruyter, 2012). The current taxonomical system based on phylogenetic analysis abolished the previous division into sections and made it necessary to reclassify *Phoma* (Aveskamp *et al.,* 2010; De Gruyter, 2012). Research conducted by Dutch scientists led to a division of genus of *Phoma sensu lato* into clades and groups that include species with a similar degree of relationship. Some of them are now raised to the level of genus.

P. complanata was classified as *Didymellaceae* family, which included the species of *Phoma* that previously belonged to the sections *Phoma*, *Phyllostictioides*, *Peyronellaea*, *Sclerophomella*, *Macrospora* and some phytopathologically similar species from the sections *Heterospora* and *ParaPhoma* are found in *Didymellaceae*

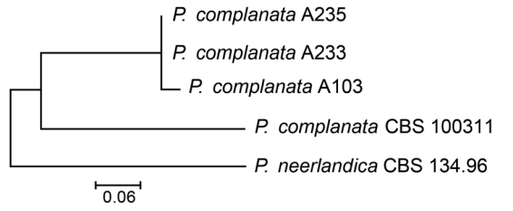

Fig. 2. Phylogenetic tree of native isolates of *P. complanata* and reference strain generated from Phylogeny. fr analysis of the ITS.

Table I
Features of *P. complanata* cultures on standard medium (mean for 3 isolates)

Medium	Malt Agar Medium (MA)		Oatmeal Agar medium (OA)		Cherry Agar Medium (CA)		According to Boerema *et al.* (2004)		
The studied features	After 7 days	After 14 days	After 7 days	After 14 days	After 7 days	After 14 days	MA	OA	CA
Diameter of colonies	35–37 mm	73–86 mm	36 mm	82–84 mm	34–36 mm	76 mm	59–79 mm	60 – 82 mm	49–79
Colour of averse	cream-olivaceous	cream-olivaceous	white-grey	white-grey	grey--olivaceous	grey olivaceous	colourless to primrose with citrine green to olivaceous tingers	colourless or buff to greenish olivaceous	colourless/saffron greenish olivaceous or olivaceous
Colour of reverse	olivaceous	pale-olivaceous	pale-olivaceous	pale-olivaceous	olivaceous	dark olivaceous	colourless to primrose with citrine green to olivaceous tingers	primrose to salmon or citrine green to olivaceous in centre	saffron/fulvous to olivaceous
Character of the growth of colonies margin	regular	regular	regular	regular	regular	regular	regular	regular	regular or slighty irregular
Structure of aerial mycelium	floccose	floccose to woolly compact	floccose	floccose to woolly	floccose	floccose to wolly compact	velvety to floccose woolly, compact	floccose to woolly, sometimes compact	woolly to floccose
Colour of cultures after reaction with 1N NaOH		negative		negative			negative	negative	

Table II
Features of pycnidia and conidia of *P. complanata* on oat medium (mean for 3 isolates)

Author	*Phoma complanata*		
	Pycnidia		Conidia
	a	b	a
Own data	globose to irregular without visible ostiole 86–288 µm	on the agar, partly submerged in the aerial hyphae of mycelium, olivaceous black with buff exudate of conidia	subglobose, cylindrical, ellipsoidal, mostly aseptate 2.86–7.64 × 1.91–3.82 µm and 1-septate conidia 9.55–13.37 × 2.86–3.82 µm with small guttules
References strain CBS 100311	globose to irregular, without visible ostiole, mostly 85–252 µm	glabrous, olivaceous-black with buff to salmon exudate of conidia, on the agar or partly submerged in the agar, solitary or sometimes aggregated	subglobos, ellipsoidal, cylindrical to fusiform, mostly aseptate 3.85–10.53 × 2.28–4.33 µm, 1-septate conidia 14.21–18.23 × 4.33 – 6.11 µm with small guttules
Boerema *et al.* 2004	globose to irregular, mostly 80–240 µm, with 1 non-papillate pore	glabrous, finally olivaceous black, solitary or confluent with buff to rosy exudate of conidia, walls made up of 2–6 layers of cells, outer layers pigmental	variable in shape and size, subglobose, ellipsoidal, cylindrical to fusiform, mostly aseptate 3–11 × 1.5–4 µm, usually 5–10 × 2–3 µm, sometimes in fresh culture 1-septate up to 16 × 4 µm, in oloder cultures large 22–34 × 6–10 µm, usually with several small guttules

a – shape and dimension in µm
b – arrangement and structure of wall surface

(Aveskamp *et al*., 2010). Despite the reclassification of species of *Phoma sensu lato*, the identification system based on constant macro-and microscopic features, physiological and biochemical characteristics observed *in vitro* in cultures developing in standard conditions is still valid (Aveskamp *et al*., 2010; De Gruyter, 2012). The study on the morphology and growth of Polish isolates of *P. complanata* was consistent with the description given by Boerema *et al*. (2004) and allowed to identify the species as *P. complanata*. In addition, the morphological and genetic similarity to the reference isolate from CBS has been proven. The Polish isolates of *P. complanata* on OA formed mainly aseptate conidia. In the case of isolate CBS 1-septate conidia were observed. Their share was about 2%. It is known from the literature that in the genus *Phoma sensu lato* the conidial septa formed secondarily, regardless of the conidiogenesis process, so a small percentage of spores may have secondary septa (Boerema and Bollen, 1975). It means that the morphological characteristics of conidia of these fungi are significant in secondary diagnostics. Literature reports the possibility of the occurrence of variation in morphological and physiological features between isolates obtained from different host plants, which may explain the absence or occasional presence of 1-septate conidia of native isolates of *P. complanata* (Koike *et al*., 2006).

Demonstrated in the present study close similarity in ITS sequence within our isolates demonstrated in this study confirms that they represent the same species of fungus. Moreover morphological characteristics and analysis of ITS1, ITS2 nucleotide sequence leads to the conclusion that isolates belong to *P. complanata* species. It seems that small differences between the three studied isolates and the reference isolate of *P. complanata* are possible, as in the case of species belonging to other taxa (Uddin *et al*., 1998). However, according to some authors ITS sequence did not provide unambiguous identification and additional sequencing of other gene fragments is required (Balmas *et al*., 2005; Woudenberg *et al*., 2009; Błaszczak *et al*., 2011). And so, the current results suggested that further research is needed to differentiation within of *P. complanata* isolates.

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POLSKIE TOWARZYSTWO MIKROBIOLOGÓW

Warszawa, 08.06.2017 r.

Szanowni Państwo Członkowie PTM

Poniżej przekazujemy szereg informacji o naszej aktualnej działalności.

1. Został uruchomiony system rejestracji uczestników Konferencji „90 lat PTM".
   Zmianie uległy niektóre terminy związane z Konferencją.
   Do 19.06.br. jest ustalony nowy termin nadsyłania abstraktów na naszą Konferencję.
   Zniżka na opłatę rejestracyjną obowiązuje do 07 lipca br.
   Zależy nam, aby w konferencji uczestniczyły zwłaszcza osoby wybrane w Oddziałach jako delegaci na Walne Zgromadzenie Delegatów PTM w Bydgoszczy. Zgodnie z obowiązującym Statutem PTM osoby, które otrzymały mandat delegata pełnią swoją funkcję przez 4 lata, czyli cały okres trwania kadencji ZG PTM. Osoby te zachowują więc swój mandat na Nadzwyczajne Walne Zgromadzenie Delegatów w Krakowie, na którym będziemy zmieniać Statut PTM. Bardzo zachęcamy do udziału w Konferencji
2. Zwróciliśmy się do firmy EthicalMedTech – Conference Vetting System (www.ethicalmedtech.eu) o umieszczenie naszej Konferencji na ich platformie, co miało świadczyć o transparentności i etyczności postępowania podczas organizacji naszej Konferencji. Podnosiły tę sprawę firmy BioMériux oraz Izba Producentów i Dystrybutorów Diagnostyki Laboratoryjnej Związek Pracodawców. Z otrzymanej odpowiedzi wynika jednak, że na razie do systemu nie są wprowadzane konferencje krajowe i tym samym sprawa jest nieaktualna. Wobec tego ponownie poprosiliśmy powyższe firmy o rozważenie możliwości wsparcia finansowego naszego Towarzystwa, poprzez zostanie Członkiem Wspierającym PTM, zamieszczenie reklam związanych z mikrobiologią w czasopismach PTM oraz udział jako sponsorzy lub wystawcy na naszej Konferencji.
3. Pani dr hab. Jolanta Solecka prof. NIZP – PZH wyraziła zgodę na objęcie funkcji Redaktora Naczelnego Polish Journal of Microbiology (PJM) (Editor-in-Chief). Pani prof. dr hab. Elżbieta Anna Trafny – członek Prezydium ZG PTM, zgodziła się działać w Redakcji PJM jako Zastępca Redaktora Naczelnego (Deputy Editor). Obsługa redakcji PJM będzie prowadzona przez zespół prof. J. Soleckiej w NIZP – PZH. Przekazanie spraw przez dotychczasowy zespół i objęcie obowiązków redakcji PJM przez nowy zespół osób odbywać się będzie stopniowo w ciągu kilku miesięcy br.
   Prosimy o przesyłanie do biura PTM propozycji osób ze stopniem naukowym doktora z Państwa Oddziałów, którzy mogliby podjąć się recenzowania manuskryptów przesyłanych do działów PJM: Environmental Microbiology, Medical Microbiology, Microscopic Fungi, Molecular Microbiology, Virology.
4. W czerwcu br. zacznie działać nowa strona internetowa kwartalnika Postępy Mikrobiologii, po czym przystąpimy do porządkowania strony internetowej PJM.
5. Główna Komisja Rewizyjna, po otrzymaniu wyjaśnień, przyjęła raport finansowy PTM za 2016 r. przygotowany przez firmę Unirach z Bydgoszczy.
6. W dniu 07.06.br. zorganizowaliśmy zebranie Prezydium ZG PTM w trybie korespondencyjnym, aby przyjąć szereg uchwał, w tym o przyjęciu 20 nowych członków zwyczajnych PTM, Srebrnego Członka Wspierającego PTM oraz o objęciem patronatem PTM II Ogólnopolską Konferencję „Działania przeciwdrobnoustrojowe".
7. Trzeba rozważyć problem obniżenia kosztów wydawania czasopism PM i PJM, wydaje się celowe zrezygnowanie z obligatoryjnego rozsyłania zeszytów czasopism do członków PTM. Dysponujemy internetowymi, aktualnie poprawianymi, wydaniami PM i PJM, do których wszyscy mają dostęp za darmo. W przypadku wersji papierowych zeszytów czasopism – ich druk i wysyłka są bardzo kosztowne. Zmniejszenie nakładu do 120 drukowanych egzemplarzy rozsyłanych do bibliotek oraz członków Rad Redakcyjnych i Redakcji czasopism pozwoli na zmianę formy druku na cyfrowy – tańszy. Zmiany te można by wprowadzić od nowego roku. Jeżeli ktoś chciałby otrzymywać wersję papierową zeszytów, już nie w ramach rocznej składki PTM, lecz w drodze prenumeraty, wnosiłby dodatkowa opłatę, która obniżona byłaby dla członków PTM. Sprawa będzie omawiana na Nadzwyczajnym Walnym Zgromadzeniu Delegatów, na razie jest sygnalizowana członkom ZG PTM do przemyślenia.

Nieopisana w Statucie zasada darmowego rozsyłania zeszytów czasopism do członków PTM obowiązywała od dawna i była dużym, ale kosztownym przywilejem. Obecnie Towarzystwa nie stać na tę formę, a w dobie dostępu do wersji internetowej, traci również sens drukowania zeszytów. Wiele czasopism przechodzi obecnie na ogólnie dostępne wersje elektroniczne.

8. Będziemy się kontaktować z Institute of Scientific Information (ISI) w Filadelfii (USA) zawiadującą przyznawaniem Impact Factor, aby uzyskać informacje, co możemy zmienić w formie naszych czasopism, aby nie utracić aktualnego współczynnika IF, a wprowadzić zmiany zmierzające do zmniejszenia kosztów wydawniczych, np. wydania tylko internetowe lub zmniejszenia nakładu tylko do 120 egzemplarzy (tańszy druk cyfrowy).
9. Rozważany jest pomysł, aby w *Postępach Mikrobiologii* zamieszczać artykuły w języku angielskim, autorów krajowych i zagranicznych, co powinno poprawić cytowalność tego czasopisma.
10. Rozważany jest pomysł, aby podczas wszystkich konferencji współorganizowanych przez PTM, członkowie naszego Towarzystwa otrzymywali zniżki na opłaty rejestracyjne. Obecnie ma to miejsce w przypadku konferencji „90 lat PTM" i konferencji „Mikrobiologia Farmaceutyczna". Organizatorzy przyszłych konferencji z udziałem PTM proszeni są o przemyślenie przedstawionej propozycji.
11. Rozważany jest pomysł, aby w przypadku pozyskania Członka Wspierającego PTM czy sponsora, przez dany Oddział Terenowy PTM, połowa środków finansowych przekazana przez Członka Wspierającego PTM lub sponsora mogła być wykorzystana na potrzeby statutowe przez Oddział PTM, który pozyskał dodatkowe środki finansowe.
12. Bardzo się cieszymy, że firma Aesculap Chifa Sp. z o.o., ul. Tysiąclecia 14, 64-300 Nowy Tomyśl, przystąpiła do PTM jako Srebrny Członek Wspierający PTM.
13. Zauważalny jest w ostatnim czasie wzrost płacenia zaległych składek PTM, za co bardzo dziękujemy.
    Liczby osób płacących składkę PTM za dany rok: do 24.03.br. wynosiły: 2015 r. – 646, 2016 r. – 674, 2017 r. – 179. a na dzień 05.06.br. wynoszą – i wzrosły odpowiednio: 2015 r. – 669 (+ 23), 2016 r. – 735 (+ 61), 2017 r. – 524 (+ 345).
14. Informujemy, że laureatem tegorocznej nagrody FEMS-Lwoff Award został Pan prof. Jeff Errington, Dyrektor The Centre for Bacterial Cell Biology w Newcastle University.
15. Złożyliśmy wniosek do FEMS o dofinansowanie Konferencji „90 lat PTM" w ramach FEMS Meeting Organizer Grant.
16. Pani prof. Beata Sadowska poinformowała, iż przygotowała wstępny dokument zawierający stanowisko PTM oraz propozycję rozwiązań kwalifikacyjnych dla absolwentów kierunku Mikrobiologia ubiegających się o wpis na Listę Diagnostów Laboratoryjnych, który został przesłany Pani dr J. Jursie-Kulaszy oraz Pani dr E. Stefaniuk z prośbą o przeanalizowanie.
17. Na naszą prośbę Redaktorzy Naczelni czasopism Postępy Higieny i Medycyny Doświadczalnej oraz Przegląd Epidemiologiczny wyrazili zgodę na udostępnienie materiałów archiwalnych ze swoich czasopism, dotyczących zasłużonych polskich mikrobiologów. Materiały te zostaną zamieszczone na stronie internetowej PTM w zakładce Historia > Wspomnienia o Mikrobiologach, za co bardzo dziękujemy.
18. Na stronie internetowej PTM zamieszczane są informacje przekazywane przez Zarządy Oddziałów Terenowych PTM dotyczące lokalnej działalności. Prosimy o zapoznanie się ze stroną PTM, która jest na bieżąco aktualizowana.
19. Prężnie działa strona PTM na facebooku, liczba polubień przekroczyła wartość 300. Dziękujemy Paniom, które wprowadzają nowe informacje na stronę facebooka i stronę PTM.
20. Na przełomie maja i czerwca odbyła się VI Konferencja Naukowo-Szkoleniowa Mikrobiologia Farmaceutyczna, współorganizowana przez PTM, na której omówiono zasady funkcjonowania Sekcji Mikrobiologia Farmaceutyczna. Na naszej stronie internetowej w zakładce Struktura > Sekcje znajdują się propozycje utworzenia szeregu Sekcji zajmujących się szczególnymi obszarami mikrobiologii. Osoby zainteresowane współpracą, wymianą informacji, doświadczeń i wiedzy w danych obszarach mikrobiologii mogą przystępować do danych sekcji – jednej lub kilku. Zachęcamy do włączania się w działalność tych Sekcji.

Pozdrawiamy serdecznie,

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

PREZES
Polskiego Towarzystwa Mikrobiologów
prof. dr hab. Stefan Tyski

INFORMACJA O KONFERENCJACH WSPÓŁORGANIZOWANYCH PRZEZ POLSKIE TOWARZYSTWO MIKROBIOLOGÓW ORAZ Z PATRONATEM PTM W 2017 r.

Konferencja Jubileuszowa 90 lat Polskiego Towarzystwa Mikrobiologów, PTM wczoraj – dziś – jutro Kraków, 22–23 września 2017 r.

Komunikat I

Konferencja Jubileuszowa Polskiego Towarzystwa Mikrobiologów organizowana jest przede wszystkim z okazji 90-tej rocznicy powołania naszego Stowarzyszenia. Jednocześnie w 2017 roku przypada rocznica 160-lecia urodzin Ojca mikrobiologii polskiej profesora Odona Bujwida oraz 130-lecia wygłoszenia przez niego w Krakowie słynnych pięciu „odczytów o bakteryjach". W 2017 roku upływa także 90 lat od powołania ogólnoświatowego stowarzyszenia towarzystw mikrobiologicznych International Society of Microbiology, obecnie International Union of Microbiological Societies (IUMS), którego współzałożycielem było PTM.

Konferencji towarzyszyć będzie Nadzwyczajne Walne Zgromadzenie Delegatów Polskiego Towarzystwa Mikrobiologów, które podejmie uchwały w sprawie wprowadzenia zmian do Statutu PTM.

Tematyka konferencji, poza częścią poświęconą historii polskiej mikrobiologii ze szczególnym uwzględnieniem roli prof. O. Bujwida oraz działalności PTM, będzie również dotyczyła przeglądu osiągnięć naukowych różnych dyscyplin mikrobiologii.

Planuje się zorganizowanie sesji wykładowej, na którą zostaną zaproszeni najwybitniejsi polscy naukowcy z różnych dyscyplin mikrobiologii oraz kilku sesji plakatowych, na których wszyscy mikrobiolodzy, a zwłaszcza Delegaci na Walne Zgromadzenie PTM będą mogli przedstawić wyniki swoich prac.

Planuje się również stworzenie warunków umożliwiających zorganizowanie stoisk wystawowych przez różne firmy działające w obszarze mikrobiologii.

Spotkanie członków Polskiego Towarzystwa Mikrobiologów będzie okazją do poznania początków polskiej mikrobiologii i serologii, do dyskusji, przeglądu sesji plakatowych, do integracji pokoleń mikrobiologów, do rekreacji w czasie spotkań koleżeńskich w uroczym klimacie dawnej stolicy Polski.

Konferencja organizowana jest przez Polskie Towarzystwo Mikrobiologów i Uniwersytet Jagielloński.

Termin nadsyłania streszczenia plakatu: 19.06.2017 r.

Termin powiadomienia o akceptacji streszczenia: 26.06.2017 r.

Opłaty zjazdowe:

Osoby do 35 roku życia (w tym studenci) członkowie PTM
W terminie do dnia 07.07.2017 r.: 100 zł; w terminie po 07.07.2017 r.: 150 zł

Osoby do 35 roku życia (w tym studenci) nie będący członkami PTM – udział czynny
W terminie do dnia 07.07.2017 r.: 200 zł; w terminie po 07.07.2017 r.: 250 zł

Członkowie PTM
W terminie do dnia 07.07.2017 r.: 300 zł; w terminie po 07.07.2017 r.: 400 zł

Osoby nie będące członkami PTM
W terminie do dnia 07.07.2017 r.: 400 zł; w terminie po 07.07.2017 r.: 500 zł

Konferencja BioMillenium 2017
„Trendy i rozwiązania w biotechnologii i mikrobiologii",
Gdańsk, 6–8 września 2017 r.

Szanowni Państwo

W imieniu Komitetu Organizacyjnego mamy zaszczyt zaprosić Państwa do udziału w Konferencji BioMillenium 2017 – „Trendy i rozwiązania w biotechnologii i mikrobiologii", która odbędzie się w **dniach 6–8 września 2017** na terenie Politechniki Gdańskiej w Gdańsku.

Głównym organizatorem Konferencji jest Katedra Biotechnologii Molekularnej i Mikrobiologii Politechniki Gdańskiej oraz Gdański Oddział Polskiego Towarzystwa Mikrobiologów. Współorganizatorami są również Katedra Mikrobiologii i Katedra Immunologii Wydziału Biologii Uniwersytetu Szczecińskiego oraz Gdański Oddział Polskiego Towarzystwa Epidemiologów i Chorób Zakaźnych.

Prezentowane zagadnienia będą podzielone na pięć tematycznych sesji:

- **Biotechnologia**: w medycynie, w przemyśle, w ochronie środowiska,
- **Mikrobiologia:** kliniczna, molekularna

Konferencja rozpocznie się wykładem przedstawiającym sylwetkę, zainteresowania i osiągnięcia naukowe zmarłego w tym roku prof. hab. dr Józefa Kur, założyciela Katedry Biotechnologii Molekularnej i Mikrobiologii Politechniki Gdańskiej oraz naukowca, który swoimi pracami nie tylko wniósł wiele do mikrobiologii i biotechnologii, ale był ich wielkim popularyzatorem. Przewidujemy wykłady plenarne, prezentacje ustne, sesję plakatową oraz warsztaty praktyczne prowadzone przez firmy.

Mamy nadzieję, że prezentacje wyników Państwa badań naukowych pokażą, że biotechnologia nie jest wciąż dziedziną „nauki przyszłości", ale już dziś przyniosła rozwiązania stosowane w wielu gałęziach przemysłu i w medycynie oraz że mikrobiologia nie jest „skostniałą dziedziną", ale obszarem nauki, w którym zarówno tworzy się, jak i wykorzystuje nowoczesne technologie. Wierzymy, że Konferencja będzie okazją do nawiązania kontaktów umożliwiających efektywną współpracę. Wszystkim, którzy wezmą udział w Konferencji, życzymy miłego pobytu w Gdańsku.

Szczegółowy program i informacje zostaną przesłane w następnym komunikacie.

Organizatorzy Konferencji BioMillenium 2017
Kontakt: 583 472 417 – sekretariat oraz 583 472 383; 583 476 412; 583 472 406; 583 472 302

Mikrobiologia w Ochronie Zdrowia i Środowiska
– MIKROBIOT 2017,
Łódź, 19–21 września 2017 r.

W imieniu Organizatorów, Instytutu Mikrobiologii, Biotechnologii i Immunologii Uniwersytetu Łódzkiego oraz Polskiego Towarzystwa Mikrobiologów mamy przyjemność serdecznie Państwa zaprosić do wzięcia udziału w **IV edycji konferencji naukowej „Mikrobiologia w Ochronie Zdrowia i Środowiska" – MIKROBIOT 2017**, która odbędzie się **w Łodzi w dniach 19–21 września 2017 r.**

Głównym celem Konferencji jest wymiana informacji naukowej w zakresie mikrobiologii, immunologii i biotechnologii, w tym badań czynników chorobotwórczości drobnoustrojów, reakcji organizmu człowieka na zakażenia, epidemiologii zakażeń, struktury i fizjologii mikroorganizmów środowiskowych, możliwości ich wykorzystania w procesach biotechnologicznych oraz w eliminacji skażeń środowiska, a także dotyczących interakcji drobnoustrojów z innymi organizmami w różnych mikroniszach środowiskowych.

Wzorem poprzednich edycji konferencji MIKROBIOT, które cieszyły się bardzo dużym zainteresowaniem środowisk naukowych z całej Polski i spotkały się z uznaniem gości z zagranicy, również podczas zbliżającej się IV edycji MIKROBIOT 2017 wykłady plenarne wygłoszą wybitni naukowcy, między innymi z Niemiec, Portugalii, Francji czy Włoch. Oficjalnym językiem konferencji będzie język angielski. W ramach konferencji MIKROBIOT 2017 odbędą się cztery sesje tematyczne:

1. Mikrobiologia kliniczna i immunologia
2. Biotechnologia mikrobiologiczna
3. Mikrobiologia ogólna i środowiskowa
4. Genetyka i genomika drobnoustrojów

Konferencja pod patronatem PTM

II Ogólnopolska Konferencja „Działania przeciwdrobnoustrojowe"
Bydgoszcz, 16.09.2017 r.

Katedra i Zakład Mikrobiologii Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy Uniwersytetu Mikołaja Kopernika w Toruniu wraz ze Stowarzyszeniem „Rozwój Mikrobiologii" organizują **II Ogólnopolską Konferencję „Działania przeciwdrobnoustrojowe.**

Pragniemy spotkać się w szerokim gronie specjalistów, aby poznawać nowe możliwości, jak również ograniczenia działań przeciwdrobnoustrojowych oraz zagrożenia wynikające z obecności drobnoustrojów.

Powyższe zagadnienia są wyzwaniem dla współczesnej medycyny, farmacji, kosmetologii, higieny, epidemiologii i mikrobiologii. W związku z tym, tematyka Konferencji adresowana jest nie tylko do osób zajmujących się dezynfekcją i sterylizacją, ale także do osób związanych z produkcją żywności, leków, kosmetyków, mikrobiologów różnych specjalności oraz diagnostów laboratoryjnych, immunologów, epidemiologów i lekarzy.

Powyższe zagadnienia będą omawiane w aspekcie klinicznym, diagnostycznym i prewencyjnym. Przewidzieliśmy dla Państwa trzy sesje naukowe:

1. Procesy dezynfekcji i sterylizacji w obszarze medycznym i przemysłowym
2. Działania przeciwdrobnoustrojowe w szpitalu i w warunkach polowych
3. Związki o działaniu przeciwdrobnoustrojowym oraz sesje plakatowe.

W ramach każdej sesji przewidziane są krótkie wystąpienia ustne uczestników.

INFORMACJE O KONFERENCJI:

Aule A i B budynku REKTORATU
Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytetu Mikołaja Kopernika w Toruniu
85-067 Bydgoszcz, ul. Jagiellońska 13–15

Planowane rozpoczęcie rejestracji od godz. 9^{00}

Katedra i Zakład Mikrobiologii Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
Uniwersytetu Mikołaja Kopernika w Toruniu
Stowarzyszenie „Rozwój Mikrobiologii"
85-094 Bydgoszcz, ul. Marii Skłodowskiej-Curie 9
Telefony: Informacje ogólne – tel. 52 585 35 01, 52 585 38 38

Płatności – tel. 52 585 36 73 e-mail: rozw_mikrob@cm.umk.pl
do 31.07.2017 r. – 90 zł/osoba
od 01.08.2017 r. – 100 zł/osoba
Organizatorzy nie zapewniają noclegu, ani nie pokrywają kosztów przejazdu

Konferencja pod patronatem PTM

IX OGÓLNOPOLSKA KONFERENCJA HYDROMIKROBIOLOGICZNA HYDROMICRO 2017: DROBNOUSTROJE – OSIĄGNIĘCIA I WYZWANIA 17–19 września 2017, Olsztyn

Szanowni Państwo!

Bardzo serdecznie zapraszamy na IX Ogólnopolską Konferencję Hydromikrobiologiczną, organizowaną w dniach od 17 do 19 września 2017 roku przez Katedrę Mikrobiologii Środowiskowej Wydziału Nauk o Środowisku Uniwersytetu Warmińsko Mazurskiego w Olsztynie. Będzie to dziewiąte ogólnopolskie spotkanie środowisk naukowych związanych z mikroorganizmami ekosystemów wodnych. Poprzednie spotkania odbywały się w: Słupsku-Ustce (2000), Toruniu (2002), Zielonej Górze-Łagowie (2004), Mikołajkach (2006), Warszawie-Wierzbie (2008), Gdańsku (2010), Wrocławiu (2013) oraz Gliwicach (2015).

Tematyka konferencji obejmuje: mikroorganizmy wód śródlądowych, podziemnych i morskich, mikroorganizmy w inżynierii środowiska, sanitarno-bakteriologiczne aspekty oczyszczania ścieków, transmisję mikroorganizmów patogennych drogą wodną, zanieczyszczenia biologiczne w systemach wodnych biobezpieczeństwo i bioremediację wód, diagnostykę organizmów wodnych, mikrobiologię przemysłową i biotechnologię.

Językiem konferencji jest język polski lub angielski Zgłoszenia (w postaci wypełnionej karty zgłoszenia uczestnictwa – do pobrania ze strony www.hydromicro2017.pl należy przesłać do 30 maja 2017 roku na adres: hydromicro@uwm.edu.pl Streszczenia w języku polskim lub angielskim przygotowane wg schematu zamieszczonego na stronie Konferencji prosimy przesyłać droga mailową do 30 czerwca 2017 roku. Konferencja odbędzie się w Olsztynie, w atrakcyjnie usytuowanym hotelu Omega.

W imieniu Komitetu Organizacyjnego
Dr hab. Zofia Filipkowska, prof. UWM

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 etiologicznym 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:
http://www.mikrobiologia.uni.wroc.pl

Organizator: Uniwersytet Wrocławski, Instytut Genetyki i Mikrobiologii
Miejsce: Uniwersytet Wrocławski, Instytut Genetyki i Mikrobiologii
ul. Przybyszewskiego 63/77, 51-148 Wrocław

Konferencja pod patronatem PTM

7 Międzynarodowa Konferencja Weiglowska
Lwów, 26–29 września 2017 r.
http://cellbiol.lviv.ua/2017/

Rudolf Weigl był wybitnym polskim mikrobiologiem austriackiego pochodzenia. Pracował w czasach międzywojennych we Lwowie (dzisiejsza Ukraina) w dziedzinach mikrobiologii lekarskiej, parazytologii, immunologii oraz biotechnologii, opracowując przy tym pierwszą skuteczną szczepionkę przeciwko durowi plamistemu.

Mikrobiolodzy polscy i ukraińscy od 2003 r. organizują naprzemiennie w Polsce i Ukrainie dwustronne (później międzynarodowe) konferencje Weiglowskie w dziedzinie mikrobiologii i dyscyplinach pokrewnych. Poprzednie konferencje Weigowskie odbywały się we Lwowie (2003), Warszawie (2007), Odessie (2009), Wrocławiu (2011), Czerniowcach (2013) oraz w Gdańsku (2015). Kolejną, 7 konferencję Weiglowską zaplanowano zorganizować we Lwowie, mieście, gdzie R. Weigl pracował.

Główne tematy konferencji to mikrobiologia ogólna, mikrobiologia lekarska, mikrobiologia środowiskowa, immunologia oraz biotechnologia. Konferencja odbędzie się w przepięknym Lwowskim Budynku Uczonych. Głównymi uczestnikami konferencji będą mikrobiolodzy ukraińscy i polscy, jednak przewiduje się także udział naukowców z innych krajów (Austria, Francja, Niemcy, Szwecja, Włochy, USA, Japonia). W konferencji weźmie udział około 250 uczestników. Oprócz programu naukowego, przewidziane są: wycieczka po Lwowie, koncert, recepcja oraz na życzenie – bankiet, spektakl w Operze Lwowskiej, a także wycieczka do pobliskich zamków. Konferencja przyczyni się do nawiązania bliższych kontaktów z naukowcami z Ukrainy oraz wzmocnieniu przyjacielskich stosunków między naszymi narodami.

Planuje się, ze opłata konferencyjna wyniesie 140 Euro oraz 110 Euro dla młodych naukowców. Koszty obejmą 3 obiady, recepcję, 5 poczęstunków podczas przerw na kawę, wycieczka po mieście oraz materiały konferencyjne. Dodatkowo płatne będą bankiet (około 40 Euro) oraz spektakl w operze Lwowskiej (10 Euro). Po konferencji można będzie odwiedzić zamek Oleski (1 dzień), Poczajow i Krzemieniec (1 dzień) lub Kamieniec Podolski (2 dni), wycieczki te są płatne dodatkowo.

Ukrainian Society of Cell Biology

For the attention of specialists in the field of microbiology, biotechnology, immunology.
Institute of Cell Biology National Academy of Sciences of Ukraine, All-Ukrainian Public Organization "Ukrainian Society of Cell Biology" inform you about:
7th International Weigl Conference that will be held on September 26–29, 2017 in Lviv, Ukraine, in the main building of Ivan Franko Lviv National University.

List of planned sessions:
1. Microbial cell biology.
2. Microbial biotechnology.
3. Environmental microbiology.
4. Metabolism and regulation.
5. Medical microbiology.
6. Immunology.
7. Microbe-host cell interaction.
8. Microbial genetics.

Participation of leading foreign and ukrainian scientists is expected in the Conference.
Working language – English.
weigl2017@gmail.com
Early bird registration & payment (till May 31, 2017): 110 € / 90 €.
Materials received after the deadline will not be accepted. Each person may submit up to 3 abstracts.
Invitation letters to the participants of the conference will be sent by email before 1st September 2017.
Complete information is presented on the website: http://www.cellbiol.lviv.ua/2017

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CZLONKOWIE WSPIERAJĄCY PTM

Złoty Członek Wspierający PTM
od 27.03.2017 r.

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Srebrny Członek Wspierający PTM
od 07.06.2017 r.

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