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MINIREVIEW

Microbial Glycosylation of Flavonoids

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Abstract

Flavonoids constitute a large group of polyphenolic compounds naturally found in plants, which have a wide range of biological activity. Although flavonoids are beneficial to human health, their application is limited by their low bioavailability and poor water-solubility. Therefore, recently there has been a particular interest in glycosylated forms of flavonoids, which usually are better soluble, more stable, and more functional compared to their aglycones. Microbial transformation of natural flavonoids may be an attractive way of receiving their glycosylated derivatives in amounts sufficient for the research on the effect of glycoside group on compound properties and for further application of these compounds as ingredients of dietary supplements and pharmaceuticals.

Key words: biotransformation, flavonoids, glycosides, microbial glycosylation

Introduction

Many natural bioactive compounds are found in nature in the form of glycosides and the sugar part is necessary for their biological activity. Several therapeutically important antibiotics (such as erythromycin), antifungal agents (amphotericin B) or anticancer drugs (doxorubicin) contain sugars attached to the aglycone core, which facilitate transport of the drugs to their targets in cells (Salas and Méndez, 2007). Also flavonoids which are plant secondary metabolites are found mostly in the form of O- or C-glycosides. However, the sugar moiety in compounds being in the centre of interest of scientific institutions and pharmaceutical industry plays different role in plant physiology than in the processes important for their application as medications. In the case of valuable flavonoids present in nature in the form of aglycones, such as xanthohumol found in hop cones, we can easily and efficiently transform them into glycosides, using microbial methods. Such biotransformations are usually highly regioselective.

In this review we discuss the prospect of microbial transformation of flavonoids as a cost-effective and environment protective tool for food supplements and drug designing. We summarize current knowledge regarding the glycosylation of flavonoids by means of various microbes and health benefits that may result from the use of such designed compounds.

Enzymes involved in glycosylation

Glycosylation is a common biochemical transformation which proceeds with the help of glycosyltransferases. These enzymes catalyse formation of glycoside bonds *via* transfer of a saccharide (usually monosaccharide) from a donor substrate to a nucleophilic glycosyl acceptor molecule, such as proteins, lipids, steroids, flavonoids or other small molecules.

In the case of Leloir-type glycosyltransferases the sugar residue is transferred from an activated donor, such as sugar-nucleotide derivatives (Palcic, 2011). Nine such derivatives used by mammals glycosyltransferases were identified: UDP-Glc, UDP-Gal, UDP-Glc-NAc, UDP-GalNAc, UDP-Xyl, UDP-GlcA, GDP-Man, GDP-Fuc and CMP-NeuAc. Whereas, non-Leloir glycosyltransferases use sugar mono- and diphosphates and glycosylated isoprenoid mono- and diphosphates as donors of a glycosyl residue. Acceptors of saccharide moieties in both cases may be carbohydrates, proteins, lipids, nucleic acids, natural compounds (e.g. antibiotics) or xenobiotics, precisely their nucleophilic oxygen atom from hydroxyl group or nucleophilic nitrogen, carbon or sulfur atoms (Lairson et al., 2008). Thus, we observe formation of O-glycosides, N-glycosides, C-glycosides and S-glycosides.

The reactions catalyzed by glycosyltransferases are stereoselective and regioselective. Transfer of a glycosyl

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group may proceed with either retention or inversion of configuration at the anomeric carbon atom of the substrate (donor), involved in the new glycosydic bond. Therefore, glycosyltransferases may be divided into retentive and invertive ones (Palcic, 2011). Glycosyltransferases have been classified by aminoacid sequence homology, substrate specificity and glycoside bond stereochemistry into 96 families (Gloster, 2014). One of them is GT1 comprising UDP-glucosyltransferases (UGTs), which were found in plants, animals, fungi and bacteria. These enzymes use UDP-activated sugar moiety as a saccharide donor and such molecules as flavonoids, alkaloids, antibiotics and plant hormones as the acceptors (Hyung et al., 2006). UDP-glucose and UDP-glucuronic acid are the most common donors used by GT1, less often UDP-rhamnose, UDP-xylose and UDP-galactose are observed (Paquette et al., 2003).

Physiological role of plant flavonoids

Flavonoids constitute the largest group of plant polyphenols. They exert considerable influence on growth and development of plants, protect them from UV radiation and bacterial and fungal infections and provide colour to fruits and flowers (Forkmann and Martens, 2001). A great diversity of flavonoids arises not only from their different carbon cores, but also from different substituents, being effects of hydroxylation, and hydroxyl group methylation and acylation. One of the common modifications is also glycosylation (Gachon *et al.*, 2005; Desmet *et al.*, 2012). For example, there are known about 300 different quercetin glycosides with potentially different biological properties (Wang, 2009).

Conjugation of plant secondary metabolites to saccharides enhances their stability by protection of reactive nucleophilic groups. For instance, glycosylation of anthocyanins at C-3 OH is crucial for the stability of their heteroaromatic ring (Chemler *et al.*, 2009; Gachon *et al.*, 2005). Moreover, it protects hydroxyl groups of secondary metabolites from autooxidation, enables their transport across cell membranes to the specific cellular compartments and augments their solubility in aqueous cell environment (Kumar and Pandey, 2013; Wang, 2009; Wang *et al.*, 2010; Zhao and Dixon, 2009).

By conjugation of a flavonoid molecule to a sugar moiety it is possible to store plant secondary metabolites in specific cellular compartments. It is believed that reactive aglycones are converted into stable and unreactive forms, which can be accumulated for example in vacuoles, and their interactions with other cell ingredients become inhibited. If necessary, the opposite process is possible, in which feeding deterrents stored in vacuoles in the form of conjugates, after cell decompartmentation due to its disintegration, are released and then cleaved by β -glycosidases to the more active forms (Kim *et al.*, 2006); Gachon *et al.*, 2005).

Recently, there has been growing interest in plant *C*-glycosides, which except for typical for phenols antioxidant activity may play different roles, for example being repellents or attractants. Stable and resistant to hydrolysis *C*-glycosides of flavones were also found in cereals, sweet corn, wheat and rice (Xiao *et al.*, 2014a).

Influence of glycosylation on human metabolism

Flavonoids present in food have beneficial effects on human health. They have antioxidant, antibacterial, antifungal, antiviral, anticancer, anti-inflammatory and antiallergenic properties (Forkmann and Martens, 2001; Hyung et al., 2006; Wang et al., 2010). Despite intensive studies on absorption and metabolism of flavonoids, several issues have not been fully elucidated, so far. It is not clear whether they are absorbed as aglycones, glycosides or in the both forms. It is known, however, that the type of sugar molecule in glycosides of flavonoids influences their absorption, distribution and to some degree - their metabolism (Xiao et al., 2014b). It was observed that after oral administration of quercetin glucoside, the maximal concentration of quercetin in plasma was 20 fold higher than after the intake of quercetin ruthoside. Therefore, it seems that quercetin glucoside is absorbed from small intestine in unchanged form, whereas absorption of quercetin ruthoside takes place after its deglycosylation (Hollman et al., 1999).

In order to be absorbed to the body, flavonoids must reach small intestine in the unchanged form. The majority of flavonoid glycosides retain their structures even after cooking processing. They are also resistant to low pH in stomach and to digestive enzymes found there. Absorption of flavonoids from food depends on their physicochemical properties, size of molecules, lipophilicity and solubility.

Aglycones of flavonoids may be transported by passive diffusion, whereas conjugation with saccharides increases hydrophilicity of the compound, which makes it better water-soluble and less capable of passive diffusion. Flavonoid glycosides may be actively transported by Na⁺/glucose transporters from intestinal lumen to enterocytes, where they are subsequently cleaved to aglycones by cytosolic glucosidases. Because the absorption of flavonoid glycosides in small intestinal is weak, it is suggested that flavonoid glycosides are hydrolysed by β -glucosidase, secreted by the brush border of human small intestine epithelial cells, known as LPH (lactase phloridzin hydrolase) (Kumar and Pandey, 2013). LPH is characterized by substrate specificity and the glycosides which are not substrates for this enzyme are transported to the large intestine, where they are hydrolysed by intestinal bacteria enzymes (*e.g.* α -arabinofuranosidase, α -fructosidase, α -rhamnosidase, β -fructosidase, β -glucosidase and β -glucuronidase). The aglycones are absorbed in colon (Kumar and Pandey, 2013; Xiao *et al.*, 2014b).

In intestinal cells flavonoids usually undergo phase II metabolism, *i.e.* they are conjugated with glucuronic acid in the reaction catalysed by UDP-glucuronyltransferase, conjugated with sulphuric acid with the help of sulphotransferase, and flavonoids which contain a catechol moiety are methylated by catechol-O-methyltransferase. From intestines flavonoids are transported with blood to liver, where they may be deconjugated and again conjugated with either glucuronic or sulphuric acid with the help of enzymes, such as β -glucuronidase and sulphatase, or they may be metabolised to smaller phenolic compounds (Kumar and Pandey, 2013). Next the flavonoid glucuronates and sulphates formed in liver are delivered with blood to tissues of the whole organism (Desmet et al., 2012). An excess of flavonoids delivered to the body is not accumulated. There are two ways of elimination of flavonoids from the organism: flavonoids absorbed in intestines are eliminated with urine, whereas the ones which were not absorbed are eliminated with faeces in the form of glycosides (Xiao et al., 2014b).

Microbial glycosylation of flavonoids

Due to their valuable biological properties, recently flavonoids have been being the subject of numerous researches in the field of food technology, biotechnology, medicine and pharmacy.

Application of flavonoids is limited by their poor water-solubility and short time spent in intestine, which result in low absorption. One of the method to overcome these drawbacks is conjugation of plant polyphenols with sugars (Hyung *et al.*, 2006; Kumar and Pandey, 2013; Tronina *et al.*, 2013). Glycosides of flavonoids are better water-soluble than their aglycones. For example, 5-O- α -D-glucopyranosyl-(+)-catechin is

> 1. R1=R2=H, daidzein 2. R1=OH. R2=H, genistein

3. R1=H. R2=Me. formononetin

4. R1=OH, R2=Me, biochanin A

at least 40-fold better soluble in water than its aglycone – (+)-catechin (Ochiai *et al.*, 2010). Some of flavonoid glycosides demonstrate also better thermal stability than their aglycones, which is in the case of the mentioned 4'-O- α -D-glucopyranosyl-(+)-catechin and its aglycone (+)-catechin (Ochiai *et al.*, 2010).

Synthesis of glycosylated flavonoids, especially with the use of biological catalysts, may be an attractive method of receiving these compounds in amounts sufficient for the research concerning influence of sugar residue in flavonoid molecules on their properties and subsequent application of these compounds as dietary supplements or pharmaceuticals (Alluis and Dangles, 1999).

An example of chemical synthesis of flavonoids may be the reaction of isoflavones, such as daidzein, genistein, formononetin or biochanin A with α -tetraacetylbromoglucose in the presence of tetrabutylammonium bromide as a catalyst, leading to *O*-glucosides of the mentioned aglycones (Lewis *et al.*, 1998) (Fig. 1). The catalyst used in this reaction is harmful for the environment. Another disadvantage of chemical glycosylation is the necessity of protection of these hydroxyl groups that are not meant to conjugate with saccharide.

Biotransformation processes, as opposed to the classic chemical synthesis, are environmentally friendly, proceed under mild conditions and allow regio- and stereoselective modifications of the substrates (Cao *et al.*, 2015; Wang *et al.*, 2010). Moreover, according to the European Union Law, the products obtained by biotransformation of natural compounds are classified as natural ones (EU Directive 88/388/EEC). Because the reaction of microbial transformation of flavonoid aglycones to their glycosides is similar to the mammal metabolic processes (Xiao *et al.*, 2014c), it may serve as a model of mammalian metabolism (Miyakoshi *et al.*, 2010). Selected examples of microorganisms with identified substrate specificities that have been described in the last years are listed in Table I.

Prenylated flavonoids naturally found in common hop (*Humulus lupulus*), such as xanthohumol, isoxanthohumol and 8-prenylnaringenin, display a wide range of biological activity and naturally occur in the



5. R1=R2=H, daidzein 7-O-ß-D-glucopyranoside

6. R1=OH, R2=H, genistein 7-O-ß-D-glucopyranoside

7. R1=H, R2=Me, formononetin 7-O-ß-D-glucopyranoside

8. R1=OH, R2=Me, biochanin A 7-O-ß-D-glucopyranoside

| | | | | Transfor- | | |
|--|--|--------------------|--|-----------------|--|---------------------------------|
| croorganism | Strain origin | Compound | Glycosylated form | mation yield | Comment | Reference |
| ia coerulea AM93 | Institute of Biology and Botany of the Medical Academy of Wrocław | xanthohumol | xanthohumol 4'-Ο-β-D-glucopyranoside | 29.0% | Biotransformation at 25°C for 9 days | Tronina <i>et al.</i> , 2013 |
| | | 8-prenylnaringenin | 8-prenylnaringenin 7-0-β-D-glucopyranoside | 49.3% | Biotransformation at 25°C for 5 days | Bartmańska <i>et al</i> ., 2012 |
| ia glauca AM177 | Institute of Biology and Botany of the Medical Academy of Wrocław | isoxanthohumol | isoxanthohumol 7-0-β-D-glucopyranoside | 61.6% | Biotransformation at 25°C for 7 days | Bartmańska <i>et al</i> ., 2009 |
| veria bassiana 46 | Institute of Biology and Botany of the Medical Academy of Wrocław | xanthohumol | xanthohumol 4'-O-β-D-(4"-O-methyl)- glucopyranoside | 23.0% | Biotransformation at 25°C for 3 days | Tronina <i>et al.</i> , 2013 |
| veria bassiana 78 | Institute of Biology and Botany of the Medical Academy of Wrocław | isoxanthohumol | isoxanthohumol 7- O - β -D- $(4$ ^m - O -methyl)- glucopyranoside | 50.2% | Biotransformation at 25°C for 12 days | Bartmańska <i>et al.</i> , 2009 |
| veria bassiana 78 | Institute of Biology and Botany of the Medical Academy of Wrocław | 8-prenylnaringenin | 8-prenylnaringenin 7-0- β -D-(4 ^m -0-methyl)- glucopyranoside | 32.9% | Biotransformation time: 6 days | Bartmańska <i>et al.</i> , 2012 |
| veria bassiana C 7159 | American Type Culture Collection | quercetin | quercetin 7-0-β-D-(4'-0-methyl)- glucopyranoside | 87.0% | ND | Zhan and Gunatilaka, 2006 |
| inghamella ılata AS 3.3400 | China General Microbiological Culture Collection Center | kurarinone | kurarinone-7-0-β-glucopyranoside | 2.2% | Biotransformation at 28°C for 5 days | Shi <i>et al.</i> , 2012 |
| inghamella | Centraalbureau voor | kaempferol | kaempferol 3- <i>O</i> -β-D-glucopyranoside | ND | Biotransformation | Miyakoshi <i>et al.</i> , 2010 |
| ulata | Schimmelcultures (Netherlands) | morin | morin 3-O-β-D-glucopyranoside | ND | at 28°C for 2-5 days | |
| 596.68 | | 3-hydroxyflavone | flavone 3-O-β-D-glucopyranoside | ND | | |
| inghamella | American Type Culture Collection | quercetin | 3-O-β-D-glucopyranoside | 55.7% | Biotransformation | Zi et al., 2011 |
| 18 | | | kaempferol 3- <i>O</i> -β-D-glucopyranoside | 2.9% | at 28°C for 4 days | |
| 0245 | | | isorhamnetin 3-0-β-D-glucopyranoside | 4.9% | | |
| inghamella elegans legans KCTC 6992 | Korean Collection for Type Cultures | xantohumol | isoxsanthohumol 7-0-β-D-glucopyranoside | 9.3% | Biotransformation at 25°C for 5 days | Kim and Lee, 2006 |
| erella isabellina 33 38063 | American Type Culture Collection | daidzein | daidzein 4'-O-rhamnopyranoside | 3.0% | Biotransformation time: 12 days | Maatooq and Rosazza, 2005 |
| erella mutabilis 04 | Institute of Biology and Botany of the Medical Academy of Wrocław | xantohumol | isoxanthohumol 7-0-β-glucopyranoside | 49.0% | Biotransformation time: 11 days | Tronina <i>et al.</i> , 2013 |

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| | | | | | Transfor- | | |
|-------|-------------------------------------|---|----------------|--|-----------------|--|------------------------------|
| | Microorganism | Strain origin | Compound | Glycosylated form | mation yield | Comment | Reference |
| | Mucor hiemalis | China General Microbiological | isoangustone A | isoangustone A 7-0-β-D-glucopyranoside | 3.6% | Biotransformation | Feng <i>at al</i> ., 2015 |
| | CGMCC 3.14114 | Culture Collection Center | | isoangustone A 7-O- β -D- | 1.0% | at 30°C for 48 h | |
| | | | | glucopyranosyl-4'-O-sulfate | | | |
| | | | | isoangustone A 7,3'-di-O-β-D | 0.5% | | |
| | | | | grucopyrarioside | | | |
| | Mucor spinosus | China General Microbiological | cardamonin | cardamonin 4- <i>O</i> -β-D-glucopyranoside | 0.4% | Biotransformation time: | Xu <i>et al.</i> , 2011 |
| 1 | CGMCC 3.3450 | Culture Collection Center | | cardamonin 6-0-β-D-glucopyranoside | 0.8% | 4 days | |
| รกฮิบ | Penicillium chrysogenum | Korean Collection for Type Cultures | xanthohumol | xanthohumol 4'-O-β-D-glucopyranoside | 5.5% | Biotransformation | Kim and Lee, 2006 |
| mJ | KTCC 6933 | | | xanthohumol 4,4'-O- β -D-diglucopyranoside | 1.0% | at 25°C for 6 days | |
| | Rhizopus nigricans UPF701 | Department of Plant Protection of Wrocław University of Environmental and Life Sciences | xanthohumol | xanthohumol 4'-O-β-D-glucopyranoside | 14.2% | Biotransformation at 25°C for 14 days | Tronina <i>et al.</i> , 2013 |
| | Trichoderma koningii | Korean Collection for Type Cultures | silybin A | silybin A 3-0-β-D-glucopyranoside | 18.0% | Biotransformation | Kim <i>et al</i> ., 2006a |
| | KCTC 6042 | | | silybin A 7-0- β -D-glucopyranoside | 7.5% | at 25°C for 96 h | |
| | | | silybin B | silybin B 3-O-β-D-glucopyranoside | 15.0% | | |
| | | | | silybin B 7-0-β-D-glucopyranoside | 4.5% | | |
| | Streptomyces M52104 | Wild strain isolated from soil | quercetin | quercetin 4'- <i>O</i> -β-D-glucuronide | 50.0% | Biotransformation | Marvalin and |
| | | | | quercetin 3-O-β-D-glucuronide | 19.0% | at 28°C for 48–192 h | Azerad, 2011 |
| | | | | quercetin 7-0-β-D- glucuronide | 9.0% | | |
| ei1 | | | | quercetin 3'- O - β -D- glucuronide | 5.0% | | |
| acte | | | naringenin | naringenin 7- <i>O</i> -β-D-glucuronide | 25.0% | | |
| я | | | | naringenin 4'-O- β -D-glucuronide | 5.0% | | |
| | Streptomyces rimosus | American Type Culture Collection | quercetin | quercetin 7-O-β-4'-deoxy-hex-4'- | ND | Biotransformation | Ma <i>et al.</i> , 2013 |
| | subsp. <i>rimosus</i> ATCC 10970 | | | enopyranosiduronic acid | | at 28°C for 96 h | |

Table I Continued

| رە | ner, |)14 | 08 | | 4 | | | | | | | | | | _ | | | |)6b |)6b | 16b |
|------------------------------|---|---|----------------------------------|-------------------------------|---|-----------------------------------|----------------------------------|---|--|-----------------------------------|--|--|--|-------------------------------------|-------------------------------------|-------------------------------------|--------------------------|---------------|--|---|--|
| Referenc | Rao and Weis 1981 | Wang <i>et al.</i> , 20 | Jiang <i>et al.</i> , 20 | | Chu et al., 201 | | | | | | | | | Shimoda and | Hamada, 2010 | | | | Kim <i>et al.</i> , 200 | Kim <i>et al.</i> , 200 | Kim <i>et al.</i> , 200 |
| Comment | Biotransformation at 30°C for 24–36 h | Biotransformation in aqueous hydrophilic media at 30°C for 48 h | Optimal biotransforma- | tion at 30°C for 12 h | Biotransformation in | aqueous hydro philic | media at 30°C for 60–96 h | | | | | | | Biotransformation | product were obtained by | incubation of lyophilized | cells with substrate and | a sugar donor | a sugar donor Complete conversion after | a sugar donor Complete conversion after 7 h of incubation at 30°C | a sugar donor Complete conversion after 7 h of incubation at 30°C Complete conversion after |
| Transfor- mation yield | ND | 91.6% | 40.0% | 5.0% | 86.0% | 96.0% | %0.06 | 86.0% | 82.0% | 84.0% | %0.66 | 98.0% | 82.0% | 12.0% | 10.0% | 15.0% | | | ND | ND | UN UN UN |
| Glycosylated form | quercetin 3-O-glucopyranoside (unspecified α or β) | puerarin 7-0-α-D-fructofuranoside | puerarin 7-0-α-D-glucopyranoside | puerarin 7-0-α-D-isomaltoside | naringenin 7- <i>O</i> -β-D-glucopyranoside | quercetin 7-0-β-D-glucopyranoside | daidzein 7-0-β-D-glucopyranoside | genistein 7-0- β -D-glucopyranoside | for mononet in -7-O- β -D-glucopyr anoside | baicalein-7-0-β-D-glucopyranoside | luteolin-7-0- β -D-glucopyranoside | apigenin-7-0- β -D-glucopyranoside | isosilybin-7- O - β -D-glucopyranoside | hesperetin 3'-O-α-D-glucopyranoside | hesperetin 5-0-α-D- glucopyranoside | hesperetin 7-0-α-D- glucopyranoside | | | quercetin 3-O-glucopyranoside | quercetin 3-0-glucopyranoside (unspecified α or β) | quercetin 3-O-glucopyranoside (unspecified α or β) kaempferol 3-O-glucopyranoside |
| Compound | quercetin | puerarin | puerarin | | naringenin | quercetin | daidzein | genistein | formononetin | baicalein | luteolin | apigenin | isosilybin | hesperetin | | 1 | | | quercetin | quercetin | quercetin kaempferol |
| Strain origin | Wild strain isolated from soil | China General Microbiological Culture Collection | China General Microbiological | Culture Collection | Organic solvent-tolerant bacteria | screened from soil | | | | | | | | Department of Chemistry | of Okayama University of Science | | | | A glucosyltransferase cDNA, RF5 | A glucosyltransferase cDNA, RF5 from <i>Oryza sativa</i> | A glucosyltransferase cDNA, RF5 from <i>Oryza sativa</i> |
| Microorganism | Bacillus cereus | Lysinibacillus fusiformis CGMCC 4913 | Microbacterium oxydans | CGMCC1788 | Staphylococcus | saprophyticus CQ16 | | | | 1010 | pg | | | Xanthomonas campestris | | | | | Escherichia coli (GMO) | Escherichia coli (GMO) | Escherichia coli (GMO) |

Table I Continued Sordon S. et al.

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xanthohumol 4'-O-ß-D-(4'''-O-methyl)-glucopyranoside



isoxanthohumol 7-O-ß-D-(4"'-O-methyl)-glucopyranoside

Fig. 3

form of aglycones. Biotransformation of xanthohumol in the cultures of filamentous fungi: *Absidia coerulea* AM93 and *Rhizopus nigricans* UPF701 led to xanthohumol 4'-O- β -D-glucopyranoside in 29.0% and 14.2% yields, respectively (Fig. 2). The identical product, but with a much less efficient conversion compared to these processes (5.5% yield) was previously observed by Kim and Lee (2006), who applied *Penicillium chrysogenum* KTCC 6933 as a biocatalyst. Whereas fungus *Beauveria bassiana* AM446 transformed xanthohumol to its 4'-O- β -D-(4'-O-methyl)-glucopyranoside in 23.0% yield (Tronina *et al.*, 2013) (Fig. 2).

Very efficient biotransformations were described for isoxanthohumol. They led to isoxanthohumol 7-O- β -D-glucopyranoside with 61.6% yield using the culture of *Absidia glauca* AM177 and isoxanthohumol 7-O- β -D-

(4^{*w*}-*O*-methyl)-glucopyranoside with 50.2% yield using the fungal strain *B. bassiana* AM278 (Bartmańska *et al.*, 2009) (Fig. 3). The analogous products were received when 8-prenylnaringenin was used as a substrate: in the culture of *A. coerulea* AM93 it was transformed to the 7-*O*-β-D-glucopyranoside and in the culture of *B. bassiana* AM278 to the 7-*O*-β-D-(4^{*w*}-*O*-methyl)glucopyranoside (Bartmańska *et al.*, 2012) (Fig. 4). The yields were 49.3% and 34.0%, respectively.

Quercetin is a strong antioxidant found in many plants and it is widely used in dietary supplements. Bacteria *Bacillus cereus* transformed quercetin to isoquercetin (quercetin 3-O-glucopyranoside) with 20.0% yield (Rao and Weisner, 1981). Whereas, fungus *B. bassiana* ATCC 7159 transformed quercetin to quercetin 7-O- β -D-(4^{***}-O-methyl)-glucopyranoside with 87.0% yield



2



8-prenylnaringenin 7-O-ß-D-(4"'-O-methyl)-glucopyranoside

Fig. 4



quercetin 7-O-ß-D-(4"'-O-methyl)-glucopyranoside

Fig. 5



Fig. 6

(Zhan and Gunatilaka, 2006), and *Cunninghamella elegans* ATCC 9245 to quercetin 3-O- β -D-glucopyranoside with 55.7% yield (Zi *et al.*, 2011) (Fig. 5).

Fungus *Cunninghamella echinulata* CBS 596.68 is also capable of glucosylation of kaempferol and morin. These transformations gave kaempferol $3-O-\beta$ -D-gluco-

pyranoside in 67.0% yield and morin 3-*O*-β-D-glucopyranoside in 20.0% yield (Miyakoshi *et al.*, 2010) (Fig. 6). It was proved that kaempferol 3-*O*-β-D-glucopyranoside has antibacterial properties (Mary and Merina, 2014), whereas morin 3-*O*-β-D-glucopyranoside has antifungal and anticancer activity (Hussain *et al.*, 2014).



kurarinone

kurarinone-7-Oß-D-glucopyranoside

Fig. 7





Mucor hiemalis CGMCC 3.14114





isoangustone A 7-O-ß-D-glucopyranoside

isoangustone A 7-O-ß-D-glupyranosyl-4'-O-sulfate



isoangustone A 7,3'-di-O-ß-D-glucopyranoside

Fig. 8

Strain *C. echinulata* AS 3.3400 is also capable to perform glucosylation of kurarinone to kurarinone 7-*O*- β -D-glucopyranoside (Fig. 7). In recent years, pharmacological research of kurarinone indicate that it had the significant cytotoxicity, tyrosinase inhibitors and glycosidase inhibitor. Microbial transformation of this lavandulyl flavonoid can improving the bioactivities or water solubility and therefore enhances the chances of this bioactive compound for their potential application in medicinal use (Shi *et al.*, 2012). Isoangustone A is a flavonoid isolated from licorice and exhibit various pharmacological properties, such as antimicrobial, antioxidative, anti-inflammatory and antitumor. Biotransformation of isoangustone A in the culture of *Mucor hiemalis* CGMCC 3.14114 at 30°C for 48 h affords three new derivatives: isoangustone A 7-O- β -D-glucopyranoside (3.6%), isoangustone A 7-O- β -D-glupyranosyl-4'-O-sulfate (1.0%) and isoangustone A 7,3'-di-O- β -D-glucopyranoside (0.5%) (Feng *et al.*, 2015) (Fig. 8).



Fig. 9

Fungus *Trichoderma koningii* KCTC 6042 transform two silybin diastereomers: silybin A and silybin B and gave two pairs of glucosylated derivatives, silybin 3-*O*- β -D-glucopyranosides and silybin 7-*O*- β -Dglucopyranosides (Fig. 9). Biotransformation of silybin by microbes can be useful methods to achieve selective conversions of compounds to derivatives which are difficult to produce synthetically. Silybin is a major active constituent of silymarin, which have hepatoprotective and antioxidant activity (Kim *et al.*, 2006a). Microbial transformations may also lead to unusual products, which we observed in the case of *Mortierella isabellina* ATCC 38063, which was able to metabolize daidzein, one of the main isoflavones found in soybean, to the untypical metabolite daidzein 4'-O-rhamnopyranoside (Maatooq and Rosazza, 2005) (Fig. 10).

Bacteria strains are also capable to glycosylation flavonoids compounds. For example bacteria *Microbacterium oxydans* CGMCC 1788 converted puerarin into two novel compounds, puerarin-7-O- α -D-



Fig. 11

glucopyranoside and puerarin-7-O- α -D-isomaltoside in 40.0% and 5.0% yields, respectively (Fig. 11). Puerarin is one of several known isoflavones, contains a unique *C*-glucoside moiety and is found in a number of plants and herbs, such as the root of *Pueraria* (*Radix puerariae*) and have anticancer and antioxidant activity (Jiang *et al.*, 2008). Wang *et al.* (2014) reported the efficient glucosylation of puerarin by an organic solvent-tolerant strain of *Lysinibacillus fusi-formis* CGMCC 4913 in aqueous hydrophilic media at 30°C for 48 h. Incubation of this strain with puerarin led to efficient production (91.6% conversion rate) of

Fig. 12



hesperetin 3'-O-α-D-glucopyranoside



hesperetin 5-O-a-D-glucopyranoside



hesperetin 7-O- α -D-glucopyranoside



puerarin-7-O- α -D-fructofuranoside and puerarin-7-O- α -D-isomaltoside (Fig. 11) with a conversion rate of less than 1% after 48 h reaction.

Chu *et al.* (2014) reported the example of highly regioselective glucosylation of various flavonoids catalysed by organic solvent-tolerant *Staphylococcus saprophyticus* CQ16. The efficient glucosylation of flavonoids was achieved in aqueous hydrophilic media. The addition of the polar solvent 15% DMSO significantly improved the glucosylation of flavonoids substrates (Table I).

Shimoda and Hamada (2010) investigated the production of hesperetin glycosides using glycosylation with bacteria *Xanthomonas campestris*. They obtained hesperetin 3'-O- α -D-glucopyranoside (12.0%), hesperetin 5-O- α -D-glucopyranoside (10.0%) and hesperetin 7-O- α -D-glucopyranoside (15.0%) (Fig. 12). Biotransformation products were obtained by incubation of lyophilized cells with substrate and a sugar donor.

Microbial transformations can be also employed to receive mammalian flavonoid metabolites, such as the most common glucuronides. Glucuronides of quercetin and naringenin can be obtained with the help of bacteria *Streptomyces* M52104 (Marvalin and Azerad, 2011). Incubation of quercetin in the culture of these bacteria at 28°C for 65 h affords four derivatives: quercetin 4'-O- β -D-glucuronide (50.0%), quercetin 3-O- β -D-glucuronide (19.0%), quercetin 7-O- β -D-glucuronide (5.0%) (Fig. 13).

This strain is also capable to perform glucuronidation of naringenin to give naringenin 7-*O*- β -D-glucuronide in 25% yield and naringenin 4'-*O*- β -D-glucuronide in 5.0% yield (Marvalin and Azerad, 2011) (Fig. 14).

Another way of economic production of glycosylated flavonoids may be employment of genetically modified microorganisms (Simkhada *et al.*, 2010). Biotransformation of kaempferol and quercetin in the culture of *Escherichia coli*, expressing glucosyltransferase gene cloned from rice (*Oryza sativa*), afforded 3-*O*-glycosides of both flavonoid substrates. Complete conversion of quercetin to its 3-*O*-glucopyranoside took place after seven hours of the incubation, whereas complete conversion of kaempferol to the 3-*O*-glucopyranoside after 24 h of the incubation (Kim *et al.*, 2006b).

ОН

.OH





quercetin 3-O-ß-D-glucuronide



quercetin 7-O-ß-D- glucuronide

Fig. 13



OF

č ÓН

quercetin 3'-O-ß-D- glucuronide



naringenin 7-O-ß-D-glucuronide



naringenin 4'-O-ß-D-glucuronide

Fig. 14

Summary

Flavonoid glycosides are widespread in the plant kingdom and thus common in human diet. Pro-healthy properties of flavonoids, including their sugar derivatives, make them promising ingredients of dietary supplements and biomedical preparations. Employment of microbial transformations to obtain glycosides of the flavonoids found in nature in the form of aglycones or to obtain new derivatives of natural glycosides has many advantages. Such processes have potential industrial application, due to their relatively low costs and mild reaction conditions. Noteworthy is that glycosides obtained by microbial transformation of flavonoids

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are classified as natural compounds, which facilitates their potential application as food supplements or ingredients of cosmetics and pharmaceuticals.

The achievements of researchers described in this paper indicate that an microbial transformation is a powerful approach to modify the structures of bioactive natural flavonoids to glycosylated derivatives. The use of the mentioned methods offers the possibility to receive new or known glycosides with the high regioselectivity (Chu *et al.*, 2014) and yield (Zhan and Gunatilaka, 2006; Wang *et al.*, 2014; Chu *et al.*, 2014). In most cases microorganisms convert flavanones into the corresponding 7-O-glycosides, chalcones into 4'-O-glycosides and flavonols to 3-O-glycosides (Table I). In these reactions, aglycones are predominantly conjugated with the glucose moiety.

Microbial glycosylation of flavonoids leads to improvement of their water-solubility and therefore enhances the chances of bioactive compounds for their potential application in large scale (Jiang *et al.*, 2008; Chu *et al.*, 2014). Better solubility of flavonoids may result in their better absorption by the human body (Jiang *et al.*, 2008).

The results described in this work clearly demonstrate the biotechnological potential of microbial glycosylation as a method for the preparation of the highvalue flavonoids with medical applications. However, there is still too little *in vivo* data on the biological benefits of most flavonoids glycosides. Further research is also needed to develop biotransformation technologies that can be competitive alternative to the current methods that involve plant extraction or chemical synthesis.

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ORIGINAL PAPER

Relationship between *ure*B Sequence Diversity, Urease Activity and Genotypic Variations of Different *Helicobacter pylori* Strains in Patients with Gastric Disorders

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Abstract

Association of the severity of *Helicobacter pylori* induced diseases with virulence entity of the colonized strains was proven in some studies. Urease has been demonstrated as a potent virulence factor for *H. pylori*. The main aim of this study was investigation of the relationships of *ureB* sequence diversity, urease activity and virulence genotypes of different *H. pylori* strains with histopathological changes of gastric tissue in infected patients suffering from different gastric disorders. Analysis of the virulence genotypes in the isolated strains indicated significant associations between the presence of severe active gastritis and *cag*A⁺ (P=0.039) or *cag*A*/ice*A1 genotypes (P=0.026), and intestinal metaplasia and *vac*A m1 (P=0.008) or *vac*A s1/m2 (P=0.001) genotypes. Our results showed a 2.4-fold increased risk of peptic ulcer (95% CI: 0.483–11.93), compared with gastritis, in the infected patients who had *dup*A positive strains; however this association was not statistically significant. The results of urease activity showed a significant mean difference between the isolated strains from patients with PUD and NUD (P=0.034). This activity was relatively higher among patients with intestinal metaplasia. Also a significant association was found between the lack of *cag*A and increased urease activity among the isolated strains (P=0.036). While the greatest sequence variation of *ureB* was detected in a strain from a patient with intestinal metaplasia, the sole determined amino acid change in UreB sequence (Ala201Thr, 30%), showed no influence on urease activity. In conclusion, the supposed role of *H. pylori* strains that present specific virulence factors was indicated as a risk factor for promotion of histopathological changes of gastric tissue that advance gastric malignancy.

K e y w o r d s: *Helicobacter pylori*, virulence, factor urease activity, histopathological changes

Introduction

Helicobacter pylori is a Gram-negative spiral bacterium that infects at least half of the world's population and is a known carcinogen (WHO, 1994). This bacterium is responsible for different gastrointestinal diseases, including duodenal and gastric ulcer diseases (Ribeiro *et al.*, 2003), gastritis, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer (Kusters *et al.*, 2006). Infiltration of immune cells, particularly polymorphonuclear leukocytes, commonly occurs after colonization of *H. pylori* strains in the gastric tissue (D'Elios *et al.*, 2007). Association of the severity of *H. pylori* induced diseases with virulence entity of the colonized strains was reported in several studies. CagA, VacA, IceA, DupA and urease are among the most important virulence factors whose involvement in the progression of these diseases has been established (Rathbone and Rathbone, 2011). Early colonization of *H. pylori* strains in childhood, expression

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of more virulent allelic variants of the virulence factors, and chronic induction of the inflammatory responses caused histopathological changes that are associated with poor clinical outcomes (Kusters *et al.*, 2006; Yahav *et al.*, 2000). Urease of *H. pylori* accounts for about 10% of the total cell protein that is expressed in most of the strains (Suzuki *et al.*, 2007). This enzyme, a nickelrequiring metalloenzyme, consists of two subunits, UreA and UreB (Hu *et al.*, 1992), which hydrolyze urea to ammonia and carbon dioxide within the gastric mucus layer to facilitate its initial interaction in this acidic environment (McGee and Mobley, 1999).

There are some other proposed roles for *H. pylori* urease, including its involvement in colonization of the gastric tissue, chemotactic activity for human monocytes, inhibition of the phagocytosis, intracellular survival of the bacterium, induction of inflammatory cytokines and inducible NO synthase (iNOS) (Shimoyama et al., 2003). Urease can bind to class II MHC on gastric epithelial cells and induces apoptosis, a phenomenon that may explain its involvement in ulcer formation (Fan et al., 2000). Beyond the proposed roles for *H. pylori* urease, the association of its activity with clinical results and the pathological changes of the gastric tissue is not so clear. The activity of this enzyme has been shown to be dependent on nickel availability and the promoter of the ureA gene. However, little information is known about the effects of genetic variation of the enzyme subunits or its expression level on its catalytic activity. It is recognized that the active site of the enzyme is located in the B subunit that is involved in restoration of urease activity, induction Th17 cell response, induction of NF-κB and interleukin-8 production (Lee et al., 2001; Eaton et al., 2002; Beswick et al., 2006; Zhang et al., 2011). The aim of this study was to determine the relationships among ureB sequence diversity, urease activity of the collected strains and virulence property of *H. pylori* strains in patients with different histopathological changes.

Experimental

Materials and Methods

Patients and sampling. Isolation of *H. pylori* strains was performed from 75 patients suffering from gastric disorders that referred to an endoscopy unit in Taleghani Hospital in Tehran, Iran. The study received ethical approval from the ethics committee of Shahid Beheshti University of Medical Sciences. Informed consent forms were signed by all the patients. Gastric biopsy specimens from antrum and corpus were collected from each patient. The biopsies were used for both histopathological and microbiological studies. The

homogenized biopsies were cultured on supplemented Brucella agar medium supplemented with 7% sheep blood, 10% FBS and selective antimicrobials. The cultures were incubated up to 5 days in 37°C under microaerobic conditions. The entity of the grown colonies was characterized by both biochemical (urease activity, catalase, oxidase) and molecular tests (see below). The characterized strains were stored at -70°C for further examination.

Histopathological examination. The histological sections were evaluated and graded according to the features suggested by the updated Sydney Classification system (Dixon *et al.*, 1996). The formalin fixed paraffin embedded biopsy samples were cut in 5- μ m-thick sections on a microtome with a disposable blade. Patients were classified based on the determined pathological changes and clinical data into three following groups: chronic gastritis, sever active gastritis (SAG), and intestinal metaplasia (Nishiya *et al.*, 2000).

DNA extraction. Genomic DNA extraction of the freshly grown *H. pylori* colonies was performed using YTA Genomic DNA Extraction Mini Kit (Yektahtajhiz, Tehran, Iran) according to the manufacturer's instructors. The DNA samples were stored at –20°C until used for gene amplification.

Characterization of H. pylori isolates and genotyping. Genus and species specific primer pairs for 16srRNA and glmM were used to characterize the initially detected isolates (Table I). Therefore, a final reaction volume of 25 µl, including 2 µl of the template DNA, 0.01 µM of each primer, 1X PCR buffer, 200 mM deoxynucleoside triphosphates, 4 mM MgCl, and 0.5 unit U Taq DNA polymerase. The amplification were performed at following conditions: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at the indicated temperatures for each reaction in Table I for 45 s, extension at 72°C for 1 min, and then final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in agarose gel after staining with ethidium bromide.

Multiplex-PCR genotyping. Four different genes *cagA*, *vacA* (*s* and *m* alleles), *iceA* (A1/A2 alleles) and *dupA* were investigated for virulence genotyping of the collected strains. The used primers, length of PCR products, and annealing temperatures are shown in Table I. The PCR was performed in a multiplex assay as described by Farzi *et al.* (2014) under the following conditions: 35 cycles of 1 min at 94°C, 40 seconds at 57°C, and 1 min at 72°C.

ureB sequencing and sequence alignment. To study sequence diversity of *ureB* among *H. pylori* strains from patients presented different pathological changes, a pair of primers covering 1056 bp of the gene that encodes functionally important regions of

| Target | Primer sequence (5'3') | Amplicon size (bp) | Annealing temp. | References |
|--------------|--|-----------------------|--------------------|-------------------------------------|
| glmM | F: GGATAAGCTTTTAGGGGTGTTAGGGG R: GCTTACTTTCTAACACTAACGCGC | 269 | 58°C | (Kauser <i>et al.</i> , 2005) |
| iceA1 | F: TATTTCTGGAACTTGCGCAACCTGAT R: GGCCTACAACCGCATGGATAT | ~900 | 57°C | (Mukhopadhyay <i>et al.</i> , 2000) |
| iceA2 | F: CGGCTGTAGGCACTAAAGCTA R: TCAATCCTATGTGAAACAATGATCGTT | ~800 | 57°C | (Mukhopadhyay <i>et al.</i> , 2000) |
| vacA (s1/s2) | F: CTGCTTGAATGCGCCAAAC R: ATGGAAATACAACAAACACAC | 259/286 | 57°C | (Farzi <i>et al.</i> , 2014) |
| vacA (m1/m2) | F: CAATCTGTCCAATCAAGCGAG R: GCGTCAAAATAATTCCAAGG | 567-42 | 57°C | (Qiao <i>et al.</i> , 2003) |
| cagA | F: AACAGGACAAGTAGCTAGCC R: TATTAATGCGTGTGTGGGCTG | 500 | 57°C | (Russo <i>et al.</i> , 1999) |
| ureB seq | F: AGCGGATATTGGTATTAAAG R: TGAGCGAACATTTCTCTG | 1056 | 48°C | this study |
| dupA | F: ACGATTGAGCGATGGGAATA R: AAGCTGAAGCGTTTGTAACGA | 1598 | 57°C | this study |
| 16S rRNA | F: GGCTATGACGGGTATCCGGC R: GCCGTGCAGCACCTGTTTTC | 764 | 58°C | (Bohr <i>et al.</i> , 2002) |

Table I Specific primers used in this study

UreB subunit, including its active site, were designed (Table I). PCR was performed under the following conditions: 30 cycles of 1 min at 94°C, 45 seconds at 48°C, and 1 min at 72°C. The PCR products were electrophoresed in 1.8% agarose gel and finally bidirectional sequence analysis of the amplicons was performed using the same primers after purification. Diversity of the obtained sequences was determined after their alignment using the MEGA6.06 software in compare to reference sequence strain j99 (ATCC 700824).

Urease activity assay. In this study, the urease activity was determined among different strains of H. pylori by colorimetric assay according to the method of Onal Okyay and Frigi Rodrigues (2013) with some modifications. All the strains were cultured on Brucella agar medium supplemented with 5% defibrinated sheep blood and 7% fetal bovine serum and incubated for 5 days at microaerophilic conditions. The grown bacterial colonies were suspended in phosphate buffer solution (PBS, pH 7.4) and then adjusted to an optical density (Ribeiro et al., 2003) of 0.08 at 620 nm with an Elisa plate reader. Rapid urease broth medium was used for the proposed assay. Suspensions of 100 µl of each strain at determined OD were inoculated into 96-well microplate containing 300 µl of rapid urease broth medium in duplicate. Changes of color and absorbance during 20 min were recorded for all the strains. To estimate any significant difference in the obtained urease activity rates and for the grading the activities, a cutoff value was determined as follows: Mean OD + 2 SD (standard deviation).

Statistical analysis. The correlations between *H. pylori* genotypes, *ure*B sequence diversity, urease activity (color change rate) and the clinical and pathological findings were estimated using either the chi-square or fisher exact tests. Student's t-test and Man-Whitney test were used to analyze significant difference of the estimated mean activity of urease in *H. pylori* strains in comparison with severity of pathological changes and disease status (*p* value < 0.05).

Results

Out of 75 examined patients, H. pylori isolates were obtained from 30 patients with different gastric disorders (age range, 28 to 79 years). Among these patients, 12 patients presented peptic ulcer disease (Duodenal ulcer (DU)), 4/12, and gastric ulcer, (8/12), while non-ulcer diseases (NUD) was detected in 18 patients. The entity of all the isolates was confirmed by both biochemical and PCR methods. Analysis of the pathological findings showed the presence of CG, SAG, and IM in 11 (36.6%), 14 (46.6%), and 5 (16.6%) patients, respectively. No significant correlation between age, nutrition, smoking and the pathological changes was determined in the infected patients. Among the 30 isolated strains, different virulence genotypes were detected. In total, vacA, cagA, iceA and dupA were detected in 100%, 60%, 83.3%, and 63% of the strains, respectively. The vacA s1/m1 accounted for 36% of the strains, while the *s*2/*m*1, *s*1/*m*2, and *s*1/*m* – allelic

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Table II The relationship between variables and pathological outcomes

| variables | CG | SAG | IM | <i>p</i> value | OR | 95%CI |
|---------------------------------------|----|-----|----|----------------|-----|------------|
| cagA ⁺ | | * | | 0.034 | 8.8 | 0.9-38.35 |
| vacA | | | | | | |
| s1/m1 | | | | | | |
| s1/m2 | | | * | 0.001 | 9.3 | 2.4-57.4 |
| m1 | | | * | 0.08 | 13 | 1.36-24.2 |
| iceA1+ | | | | | | |
| iceA2+ | | | | | | |
| iceA1 ⁺ /A2 ⁺ | | | | | | |
| dupA ⁺ | | | | | | |
| iceA1 ⁺ /cagA ⁺ | | * | | 0.026 | 6.4 | 1.15-35.43 |
| variation Ala < Thr | | | | | | |
| Cutoff-point | | | | | | |

variants accounted for 33%, 23% and 6% of the strains. Among the *iceA* positive strains, different allelic types were detected in these patients, including *iceA*1⁺/*iceA*2⁺ (36%), *iceA*1⁺/*iceA*2⁻ (33%), *iceA*1⁻/*iceA*2⁺ (13%). Statistically significant associations were found between the presence of *cagA* and SAG, and also *vacA m1* and *vacA s1/m2* genotypes and IM. Also significant association was found between the *iceA*1⁺/*cagA*⁺ genotype and IM (Table II). However, no correlation was determined between ulcer formation (GU and DU) and virulence genotypes in these patients.

ureB sequence diversity. The *ureB* was detected in all the strains and the diversity in their sequences was



Fig. 1. Diagram of frequency distribution of urease activity with mean \pm S.E.M bars. Among clinical findngs isolated strains of *H. pylori* from peptic ulcer patients had higher urease activity than from gastritis patients (0.049 \pm 0.01 *vs.* 0.04 \pm 0.013). Comparing urease activity based on pathological outcomes showed isolated strains from patients with IM had highest urease activity than SG and CG patients respectively (0.046 \pm 0.006 vs. 0.042 \pm 0.015 and 0.042 \pm 0.012).

Abbrev: IM – intestinal metaplasia, SG – severe active gastritis, CG – chronic moderate active gastritis. Graphs depicted by GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA, USA). * = Statistically significant, — = mean bar, T = S.E.M bar. analyzed. The *ureB* sequence variants were assigned by GenBank (accession numbers: KP401951-KP401975). Based on the *ureB* reference sequence (Strain J99), different point mutations were found in the *ureB* sequence, with one of them being responsible for Ala→Thr amino acid change at position 201. This mutation was detected in 30% (9/30) of the strains. No correlation was detected between this mutation and the clinical or pathological data. The neighbor-joining method was used for investigation of relationships between the obtained sequences. Comparison of the nucleotide sequences with reference sequence J99 showed the highest diversity (3% difference) in a strain (HC452), which was isolated from a patient with IM.

Urease activity. Urease activity was evaluated in all the strains. Analysis of mean values of urease activity (Absorbance change/ Δt) showed diversity of this activity among different strains of H. pylori in different patients groups. The highest activity was detected among the strains isolated from patients with PUD compared with those presenting NUD (P=0.043)(Fig. 1). The strains isolated from patients with IM had higher urease activity than those from patients with other pathological changes. However, the obtained mean difference was not statistically significant. The results of our study showed significant association between the lack of cagA and increased urease activity (P = 0.031) (Fig. 2). No significant mean difference between urease activity of H. pylori strains carrying Ala>Thr amino acid mutation and wild type strains (P = 0.525) as detected (Fig. 2). A cut-off value of 0.056 was estimated for qualitative analysis of urease activity according to the obtained absorbance values. Considering the cut-off value, our data didn't show any significant relationship between urease activity and clinical or pathological data.



Fig. 2. Diagram of frequency distribution of urease activity with mean \pm S.E.M bars. The obtained results this study showed strains of positive *cagA* had higher urease activity than of negative cagA (0.039 \pm 0.01 *vs.* 0.05 \pm 0.013). The mean of urease activity of strains with aminoacid variations Ala \leftrightarrow Thr-201 had no statistical differences (0.043 \pm 0.014 *vs.* 0.045 \pm 0.007). Graphs depicted by GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA, USA).

* = Statistically significant, — = mean bar, T = S.E.M bar

Discussion

In this study, the relationships of ureB sequence diversity, urease activity and genotypes of different H. pylori strains were investigated in patients with different gastric disorders. Pathological changes of the infected stomach tissue could be in association with virulence entity of the colonizing strains in this organ. The results of our study showed an association between occurring IM and vacA m1 or s1m2 allelic variants. The higher expression VacA in H. pylori strains conferring s1/m1 genotype compared with those presenting *s1/m2* variant, could explain the observed pathological changes in patients with IM (Evans et al., 1998). In a study by Nogueira et al. (2001) it was shown that higher degrees of lymphocytic and neutrophilic infiltrates were seen in gastric biopsy specimens of patients infected with strains encoding distinct genotypes. They showed that vacA s1 and m1 allelic variants are significantly associated with atrophy and IM. While the association of vacA s1 allele with PUD in the infected patients was established in some studies (Atherton, 1997), this association was not supported by our results. The lack of an association between variability of the vacA s and m regions and PUD was similarly established by Aydin et al. (2004). The interplay between vacA and cagA signaling pathways may explain this incongruity (Argent et al., 2008). The association between cagA status and pathological changes was identified in our study. In this subject, SAG was dominantly found in patients infected with cagA positive strains that was agreed to in earlier reports (Plummer et al., 2007). Although the association of cagA with PUD was reported in some studies, our results didn't confirm such a relationship. In the case of *iceA*, the results was similar to other studies (Nogueira et al., 2001), its allelic variants were not significantly associated with neutrophil infiltration in the studied biopsy samples. However, cagA/iceA1 genotype was in correlation with the occurrence SAG. In a study by Nishiya et al. (2000) it was concluded that iceA1-positive strains can induce more increased active gastritis in cagA-positive and vacA s1/m1 strains. The association between inflammatory cell infiltration and H. pylori virulence factors was also detected for dupA (Wang et al., 2015). This virulence factor is in correlation with the occurrence of duodenal ulcer (Arachchi et al., 2007; Lu et al., 2005). Our results showed a 2.4-fold increased risk of peptic ulcer (95% CI: 0.483-11.93), compared with gastritis in the infected patients who had dupA positive strains; however, this association was not statistically significant. There are other controversial results for the noted association between gastrointestinal disorders and dupA status (Lu et al., 2005; Arachchi et al., 2007; Argent et al., 2007; Nguyen et al., 2010). Although an increased risk of DU was detected in our study, the lack of significance difference in these patients could be explained by the probable lack in the function of DupA or its secretion in the responsible strains (Jung et al., 2012). The H. pylori ureA-ureI genes play important role in urease production. Urease has two major subunits A and B and five accessory subunits E, F, G, I and D. There are six copies of UreA and UreB subunits in the holoenzyme, whose active site is located within the UreB subunit (at position 322) (Mobley et al., 1995). Urease can express on the bacterial surface or release into the gastric mucosa. UreB subunit is composed of 569 amino acids and is nearly conserved among different strains. In a study by Muller et al. (2002), they compared obtained ureB sequences and found more than 98% identity among the sequences, which is similar to our results. Theoretically, it seems that diverse urease activity affect survival of the bacterium and its pathogenesis in the gastric tissue. In our study, increased urease activity in the patients with peptic ulcer than those with gastritis was indicated. This diversity was not explained by the sole determined amino acid change (Ala>Thr) in the *H. pylori* strains isolated from the studied patients groups. This mutation is not located near the active site, which refuses its influence on urease activity. The lack of urease accessory proteins may explain the noted variations that were detected in these strains (Benoit et al., 2007; Fong et al., 2013). The inverse association that was found between cagA status and urease activity was a new finding in our experiments. It is well known that H. pylori possesses protondependent intrabacterial transportation systems that transport CagA and urease toward the type-IV secretion

machinery and UreI (Wu et al., 2014). Although UreI dependent translocation of CagA within the cytoplasm of *H. pylori* was established by Wu et al. (2005), it remained unclear how CagA interacts with UreI to modulate its activity. The development of ulcers in the antral mucosa caused by the urease of *H. pylori* due to apoptosis was suggested by several studies (Kohda et al., 1999; Fan et al., 2000). In our study, the highest activity was found among the strains isolated from patients with PUD compared with those presented NUD. This activity was also relatively higher among patients with IM. The association between urease activity and peptic ulcer was described by several studies. It seems that ammonia produced by the urease can induce apoptosis, whose action promotes tissue damage and ulcer formation (Igarashi et al., 2001). Although there is no report about higher level of urease activity in patients with peptic ulcer, the increased activity was previously established in strains from cancer patients (Ito et al., 1995). In a study by Xu et al. (1990) it was shown that urease inhibitor can cause a 75% drop in vacuolating gastric cells that had been induced by defined concentration of urease. While our results showed higher activity of this enzyme in the strains collected from ulcerative tissue, it remains to clarify its effect on gastric acid secretion and ulcer formation in these patients. Urease dependent NO production in the gastric tissue and its involvement in mucosal damage may explain its immunological role in the pathogenesis of H. pylori mediated gastritis and carcinogenesis (Gobert et al., 2002).

Based on the analyzed strains, the characterized relationships between H. pylori virulence genotypes, cagA, cagA/iceA1, or vacA s1m2 allelic forms, and IM or SAG proposed role of these virulence genes in forming histopathological changes that advance gastric malignancy. While an increased risk of peptic ulcer, compared with gastritis, was seen in the infected patients with the *dup*A positive strains, no statistically significant relationship was found for the studied virulence factors in these patients. Analysis of the association between urease activity of the H. pylori strains and ureB nucleotide polymorphisms showed that this subunit is conserved among most of the strains. The sole amino acid change (Ala>Thr) in these strains didn't show any possible influence on enzymatic activity in these strains. The putative role of *H. pylori* urease in the progression of ulcer formation was postulated in this study, since greater urease activity was seen among the strains that were isolated from patients with PUD compared with those from NUD patients. The noted activity seems to be affected by the CagA cytoplasmic translocation, so the highest activity was determined in the cagA negative strains. Because urease comprises 10% of total H. pylori cell proteins, it is important to realize the association between the higher inflammatory response and pathological changes of gastric tissue and risk of *H. pylori*-associated gastric cancer because of its activity in the stomach.

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Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL PAPER

Interaction of Gram-Positive and Gram-Negative Bacteria with Ceramic Nanomaterials Obtained by Combustion Synthesis – Adsorption and Cytotoxicity Studies

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Abstract

This paper presents the interactions of Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas putida*) bacteria with ceramic materials obtained by combustion synthesis. These studies were conducted based on an analysis of the adsorption of bacteria onto aggregates of ceramic materials in an aqueous suspension. The materials used in the studies were of a nanostructured nature and consisted mainly of carbides: silicon carbide (SiC) in the form of nanofibers (NFs) and nanorods (NRs), titanium carbide, and graphite, which can also be formed by combustion synthesis. Micrometric SiC was used as a reference material. Gram-positive bacteria adsorbed more strongly to these materials. It seems that both the point of zero charge value and the texture of the ceramic material affected the bacterial adsorption process. Additionally, the viability of bacteria adsorbed onto aggregates of the materials decreased. Generally, *P. putida* cells were more sensitive to the nanomaterials than *S. aureus* cells. The maximum loss of viability was noted in the case of bacteria adsorbed onto NRSiC and NFSiC aggregates.

Key words: Pseudomonas putida, Staphylococcus aureus, adsorption, ceramic nanomaterials, loss of viability

Introduction

The growing interest in nanostructured materials involves their potential practical use. In this regard, nanostructured materials built from chemically inert and thermally stable carbides, such as silicon carbide (SiC) or titanium carbide (TiC), are particularly important. Recent studies show that nanostructures of silicon and titanium carbides can be obtained via self-propagating combustion synthesis (Huczko et al., 2005; Cudziło et al., 2007). In this process, not only carbide, but also carbon materials such as nanostructured graphite forms, can be produced. Possible applications of such materials include their use in various kinds of filters that would retain microorganisms and, due to their cytotoxic properties, inhibit the growth of microorganisms and biofilms on their surface. Therefore, such filters can be free from the disadvantages of filters based on activated carbon. The available research shows that nanostructured materials could be used in wastewater treatment (Farre et al., 2009; Reddy et al., 2010; Joseph et al., 2012). Additionally, it has demonstrated the possibility of the adsorption of bacteria onto surfaces of various kinds of ceramic and nanostructured materials. Nanostructured materials may also exhibit strong antibacterial properties, but studies on the interaction between nanostructured carbides and bacteria are still limited. A significant number of studies have been devoted to examining the interactions between bacteria and nanostructured carbon materials, such as single-walled and multi-walled carbon nanotubes, graphene, and fullerenes (Lyon et al., 2006; Kang et al., 2007; 2008a; 2008b; Akhavan and Ghaderi, 2010), as well as modified carbonaceous materials containing metals such as zinc (Yamamoto et al., 2001). Some of these studies are also related to the adsorption of bacteria onto the surface of such materials. This is an important aspect of the research that involves the potential applications of these methods for microbiological water treatment (Rivera-Utrilla et al., 2001; Savage and Diallo, 2005; Li et al., 2008; Qu et al., 2013; Hossain et al., 2014). Similar investigations have focused not only on

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Experimental

Materials and Methods

µmSiC powder was purchased from Sigma-Aldrich

are associated with biofouling, microbial corrosion, weathering, and mechanisms of biofilm formation (Yee et al., 2000; Rong et al., 2008). In this context, studies have shown the possibility of adsorbing Gram-positive and Gram-negative bacteria to minerals such as quartz, corundum, and iron-containing minerals. The adsorption of bacteria to clay minerals was also studied (Jiang et al., 2007), although it was difficult to separate the bacteria and mineral particles. Similar difficulties may arise when examining the adsorption of bacteria onto aggregates of nanostructures in aqueous suspensions, but the appropriate use of reagents that increase the density of the aqueous environment allows the separation and measurement of unadsorbed bacteria (Jiang et al., 2007). Some of the carbides seem to be completely inert in their interactions with living cells, but in the nanostructured form, they can interact with cells like other nanomaterials. Based on the literature, two basic mechanisms of such interactions can be described: mechanical cell damage and oxidative stress caused by the presence of highly reactive chemical species (e.g., free radicals) on the surface of nanostructures (Cadet et al., 1999; Fenoglio et al., 2006; Barillet et al., 2010). The investigations conducted by Szala and Borkowski (2014) showed a significant toxicity of nanofibers and nanorods of SiC (NFSiC and NRSiC, respectively) toward Pseudomonas putida bacteria. In these experiments, mechanical damage to cells, a reduction in dehydrogenase activity, and a decrease in CO₂ production were found in the bacterial cultures as a result of the antibacterial activity of nanostructured SiC.

nanostructured materials, but also on the biogeochemi-

cal interactions between minerals and bacteria, which

The aim of the present studies was to investigate the interaction between bacteria and ceramic materials obtained by self-propagating high temperature synthesis (SHS). Similar results concerning P. putida adsorption onto nanofibers and nanorods of SiC were presented previously (Borkowski et al., 2015). In this work, we included a part of repeated investigations in order to compare with the results of bacteria adsorption onto the other ceramic materials and to conduct the viability experiment. The materials used were nanostructured carbides, such as nanofibers SiC (NFSiC), nanorods SiC (NRSiC) and TiC, but also included graphite and a mixture of graphite and TiC (TiC/C), which can be synthesised via the SHS route. The studies mainly involved the adsorption process and the viability of Gram-positive and Gram-negative bacteria on the surface of aggregations of the aforementioned materials in aqueous suspensions. These studies were conducted in relation to standard micrometric SiC (µmSiC), which was used as reference material to verify the hypothesis that the adsorption process and the loss of cell viability depend on the textures of the materials.

(St. Louis, MO, USA) and used without purification. Carbon monofluoride (CF), poly(tetrafluoroethene) (PTFE), hexachloroethane (C₂Cl₆), CaSi₂, and TiSi were purchased from commercial sources (Sigma-Aldrich and Alfa Aesar, Ward Hill, MA, USA). Ceramic nanomaterials were produced by combustion synthesis (SHS) in a stainless steel autoclave according to our previously published methods. NFSiC were synthesized by the SHS method using a PTFE/CaSi, system (Huczko et al., 2005). NRSiC were produced during the combustion of the CF/AlSi system (Szala and Borkowski, 2014). Nanometric TiC was synthesized using a CF/ TiSi system, and a mixture of titanium carbide with graphite (TiC/C) was obtained in using a TiSi/C₂Cl₄ system (Szala, 2010). Graphite nanoparticles were synthesized during the combustion of a CF/Al mixture (Cudziło et al., 2007). The point of zero charge (PZC) of the investigated materials was analyzed according previously described potentiometric titration methods (van der Wal et al., 1997; Bourikas et al., 2003; Borkowski et al., 2015). Scanning electron microscopy (SEM) images of the investigated materials are presented in Fig. 1. An example of mass titration curves is shown in Fig. 2. The PZC values of the investigated materials are presented in Table I.

Microorganisms and media. Strains of P. putida and Staphylococcus aureus (ATCC6538) were obtained from our own collection of pure strains of microorganisms (Geomicrobiology Laboratory, Faculty of Geology, University of Warsaw). Bacteria belonging to the genus Pseudomonas are the most commonly investigated Gram-negative microorganisms in relation to adsorption onto different materials. Similarly, S. aureus together with Bacillus subtilis are considered typical Gram-positive bacteria. (Yamamoto et al., 2001; Ams et al., 2004; Jiang et al., 2007; Rong et al., 2008). Taxonomic affiliation was confirmed by sequence analysis of the 16S rRNA gene. The bacteria were cultivated in both liquid and solid nutrient media (pH 7.5) comprising the following (g L⁻¹): glucose, 10; peptone, 5; yeast extract, 2; NaCl, 4; and agar (in the case of solid medium), 20. The media were autoclaved at 121°C for 15 min.

Protein measurements. To analyze the number of adsorbed bacteria without using the cultivation method, the correlation between protein content and bacterial counts was plotted for *P. putida* and *S. aureus* separately. The protein measurement was conducted according to Borkowski *et al.* (2015).

Adsorption tests. Adsorption tests were designed based on previous work (Jiang *et al.*, 2007) with impor-

tant modifications published by Borkowski et al. (2015). Adsorption tests were conducted in phosphate buffer (1/15 M) at three pH values: 3.0, 6.8, and 9.0. The pH was adjusted with small aliquots of NaOH (approximately $20 \,\mu$ l, $6 \,M$) or H₂PO₄ (approximately $20 \,\mu$ l, 80%). Adsorption was measured as follows. Twenty milligrams of investigated nanomaterials or 50 mg of µmSiC were mixed with 2 ml of buffer containing 109 P. putida or S. aureus cells ml⁻¹. Next, the suspensions were shaken (120 rpm) for 3 h at 25°C. After mixing, 1 ml of the suspension was placed into an Eppendorf tube and 0.3 ml of sucrose (60%) was added. Then, the mixture was centrifuged for 2 min at 4000 rpm (2600 g) to remove the solid materials. A total of 1 ml of supernatant containing unadsorbed bacteria was used to determine the protein concentration as described above. The number of adsorbed bacteria was calculated from the difference between the number of bacteria in suspension before and after adsorption. The adsorption tests were conducted in triplicate. The parameters of the Langmuir and Freundlich isotherms were calculated for the adsorption at pH 6.7. The adsorption was measured in the same way as described above in buffer containing $0-10 \times 10^8$ P. putida or S. aureus cells ml⁻¹. The Langmuir isotherm is described by the following equation:

$$n = \frac{A_m \cdot K_L \cdot C_{eq}}{1 + K_L \cdot C_{eq}} \tag{1}$$

where *n* is the amount of adsorbed bacteria (×10¹⁰ cells g¹), $A_{\rm m}$ is the maximal number of adsorbed bacteria (×10¹⁰ cells g⁻¹), $K_{\rm L}$ is the Langmuir constant, and $C_{\rm eq}$ is the equilibrium bacterial concentration (×10⁸ cells g¹).

The Freundlich isotherm is described by the equation:

$$n = K_{\rm F} \cdot C_{\rm eq}^{\ b} \tag{2}$$

where $K_{\rm F}$ is the constant of the isotherm, *b* is a parameter that has value in the range <0; 1>, and *n* and $C_{\rm eq}$ are as described for the Langmuir isotherm.

To fit the experimental data to the Langmuir model, a statistical spreadsheet (Statistica 10, StatSoft. Inc. Tulsa, OK, USA) was applied using the method of least squares for nonlinear models, while the Freundlich model was fitted to the experimental data using the linearized isotherm:

$$\log n = b \cdot \log C_{eq} + \log K_{F}$$
(3)

Measurement of the affinity of bacteria for the investigated materials. The adsorption of bacteria onto the surface of the tested materials does not always allow one to use the Langmuir isotherm parameters to evaluate the affinity of bacteria for aggregates of nanostructures in aqueous suspensions. Therefore, it was decided to first measure the affinity by approximating the first three data points with a second-degree polynomial function to give:

$$n = A \cdot C_{eq}^{2} + B \cdot C_{eq} + C \tag{4}$$

where *n* is the amount of adsorbed bacteria (×10¹⁰ cells g^{-1}), C_{eq} is the equilibrium bacterial concentration (×10⁸ cells g^{-1}), and A, B and C are the parameters of the polynomial.

Next, the differentiation of the obtained function was taken at the point $C_{eq} = 0$, and this value was considered to be a constant for bacterial adsorption, which may be regarded as a measure of affinity:

$$\frac{dn}{dC_{\rm eq}(0)} = B = K_{\rm ads} \tag{5}$$

where *n* is the amount of adsorbed bacteria (× 10¹⁰ cells g⁻¹), C_{eq} is the equilibrium bacterial concentration (× 10⁸ cells g⁻¹), B is both the parameter of the polynomial and the value of the derivative at the point of C_{eq} = 0, and K_{ads} is a constant of bacterial adsorption. The slope of the polynomial at the point C_{eq} describ-

The slope of the polynomial at the point C_{eq} describing the initial isotherms is shown in diagrams (Fig. 4) as a tangent (straight line) to the initial section of the isotherm. In the studies by Borkowski *et al.* (2015), the K_L was used as a measure of the bacterial affinity; however, in our study, such an approach did not always make sense.

Viability test. The viability test was performed according to Szala and Borkowski (2014). Briefly, to analyze the loss of viability, solutions of propidium iodide (PI) (2 mg/0.1 l, pH=7.4) and acridine orange (AO) (5 mg/0.1 l, pH = 7.4) were prepared in phosphate buffer. In a 100 ml sterile glass bottle (Simax), 10 ml of sterile saline solution (0.9% NaCl) and 40 mg of the investigated nanomaterials were added. Subsequently, 1 ml of *P. putida* or *S. aureus* inoculum (approximately 10⁸ colony-forming units (cfu)/ml in 0.9% NaCl) was added to the mixture and mixed for 120 min at 25°C (200 rpm). Then, the suspension was mixed with sucrose (60%) in order to separate of unadsorbed bacteria. After centrifugation, the residuum was stained. Next, ten representative fluorescence images of cells adsorbed onto the surface of aggregates of the nanostructures were acquired using an epifluorescence microscope with a B-filter. The results of the microscopic analysis were expressed as the ratio of the number of cells stained with PI (red-orange) divided by the number of cells stained with PI plus cells stained with AO (green).

Statistical analysis. The obtained data (viability test) were analyzed for significant mean differences using one-way analyses of variance (ANOVA) at p < 0.05. Post hoc tests for pair-wise differences and the identification of homogeneous subgroups were conducted using Tukey's HSD procedure. Homogenous subgroups are indicated by diagrams marked by the same lower case letters. The ANOVA was computed with Statistica 10 software (StatSoft. Inc. Tulsa, OK, USA).

Results and Discussion

SEM images of commercial SiC (Fig. 1A) showed that the powder contained irregular polygonal grains with sharp edges, with dimensions of about 100–200 μ m. The graphite that was used as a reference (Fig. 1B) had irregular particle sizes of about 1 μ m. The TiC obtained with the SHS method (Fig. 1C) formed rod-like particles with dimensions of about 50 × 150 nm. The TiC/C composite (Fig. 1D) was built from spheroidal agglomerates with diameters of about 5–100 μ m, but the spheroids were built from nanometric particles. Silicon carbide nanorods (Fig. 1E) obtained by the combustion method have dimensions 500×1000 nm. NFSiC

(Fig. 1F) were 10–100 nm in diameter and were over many micrometers in length. The PZC of the materials is presented in Table I. Examples of mass titration curves are presented in Fig. 2. The materials had different PZC values. The PZC values for the SiC materials ranged from 2.7 to 3.5, and in neutral pH, the surface charge of the investigated SiC materials was negative. For TiC and graphite, the PZCs were 7.2 and 9.4, respectively; thus, at neutral pH, their surface charge is close to zero or positive, respectively.

The isoelectric point of the bacteria used in the presented studies was 2.8 and 3.4 for *P. putida* and *S. aureus*, respectively (Table I). This indicates that the surface charge of *P. putida* was negative at each pH,



Fig. 1. Ceramic materials used in the studies.
A – µmSiC used as reference material, B – graphite, C – titanium carbide (TiC), D – titanium carbide with graphite (TiC/C), E – nanofibers of silicon carbide (NFSiC), F – nanorods of silicon carbide (NRSiC).

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| | μmSiC | graphite | TiC | TiC/C | NRSiC | NFSiC | P. putida | S. aureus |
|-----|---------|----------|---------|---------|---------|---------|-----------|-----------|
| PZC | 2.7 [1] | 7.5 [2] | 9.4 [2] | 7.2 [2] | 3.5 [1] | 2.8 [1] | 2.8 [1] | 3.4 [2] |

Table I The point of zero charge of materials used in the studies

¹ Borkowski et al., 2015; ² This study

while the surface charge of *S. aureus* was positive at pH 3 and negative at pH 6.8 and 9. Generally, based on the literature, it can be stated that the PZC values for most bacteria are relatively low, ranging from about 2 to 3.5. The charge of the bacterial cell wall originates from the dissociation of acidic groups, such as phosphate, carboxyl, and amino groups (van der Wal *et al.*, 1997).

Adsorption of bacteria. Bacterial adsorption onto the investigated materials, as well as the shape of the isotherms, seemed to depend on the texture of the materials and the species of bacteria. Generally, the adsorption of *P. putida* was less than than that of *S. aureus*, and adsorption depended on the pH of the solution (Fig. 3). Significant adsorption was noted at pH 3 and 6.8; at pH 9, the adsorption was substantially lower. Only in the case of TiC was this relationship reversed. The adsorption of bacteria onto graphite, TiC, and TiC/C were significantly higher at pH 6.8. The strongest adsorption of *P. putida* (70%) and *S. aureus* (90%) was found for NRSiC at pH 3 and graphite at pH 6.8, respectively.

The isotherms confirmed the data presented above. Generally, the adsorption isotherms of *P. putida* reflected its lower adsorption compared with *S. aureus* (Fig. 4). The shapes of isotherms were similar to the Langmuir and Freundlich functions, but in the case of TiC, the



Fig. 2. Example of mass titration curves. The measurement was realized according to Bourikas *et al.*, (2003). Straight lines represent the point of intersection with blank titration curve.

shape was different, indicating multilayer sorption or aggregation of the cells with TiC. It is important to note that the shape of the isotherms was very similar for both bacteria and that only the TiC seemed to interact differently with the bacteria. In the presented figures, straight lines were plotted tangentially to the initial fragment of the isotherms at $C_{eq} = 0$. These lines represent the adsorption constant (K_{ads}) and the affinity of the bacteria to the investigated materials. The measured affinities (as derivatives of an approximated polynomial function at $C_{eq} = 0$) are presented in Table II. These values indicate a high affinity of *P. putida* for C, TiC/C, and NRSiC, a moderate affinity for NFSiC, and a low affinity for µmSiC and TiC. In the case of S. aureus, similar results were found, but the measured affinities were much higher, which resulted in a significantly higher adsorption of these bacteria onto the investigated materials. Some authors interpreted the Langmuir constant (K_{t}) as the degree of affinity between the bacteria and the tested materials (Jiang et al., 2007). Therefore, a higher K, value indicates a higher affinity of bacteria for the



Fig. 3. Adsorption of bacteria *P. putida* (upper) and *S. aureus* (lower) onto the investigated materials at different pH. Error bars indicate standard deviations.

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Fig. 4. Isotherms of *P. putida* and *S. aureus* adsorption onto investigated materials at pH 6.8. Symbols represent experimental data, solid curve line – Langmuir model, dashed curve line – Freundlich model, solid straight line is a tangent to polynomial function in C_{eq} = 0. Standard deviation has been marked.

tested materials. Borkowski *et al.* (2015) showed that the K_L values for the adsorption of bacteria *P. putida* onto SiC nanostructures were inversely proportional to pH. This indicates, therefore, a decrease in the affinity of bacteria for the investigated material with increasing pH. In the presented studies, such an approach seems to be insufficient due to the different shapes of the obtained isotherms, especially in the case TiC. Table II allows one to compare the obtained values of K_L and K_{ads} . Generally, but not always, a higher value of K_L corresponds to a higher K_{ads} . Additionally, sometimes one can come to an incorrect conclusion when comparing the K_L values for the adsorption of both bacteria. For instance, based on K_L , the affinity of *P. putida* for µmSiC was greater than that of *S. aureus*. Similar results were observed for NRSiC, but the K_{ads} showed an inverse

| | Р. р | utida | | S. a | ureus | |
|----------|--|-------|------------------|--|-------------|------------------|
| | $A_{\rm m} \\ [\times 10^{10} {\rm cells} {\rm g}^{-1}]$ | K | K _{ads} | $A_{\rm m} \\ [\times 10^{10} {\rm cells} {\rm g}^{-1}]$ | $K_{\rm L}$ | K _{ads} |
| μmSiC | 0.22 | 0.52 | 0.17 | 1.25 | 0.35 | 0.79 |
| graphite | 2.57 | 0.40 | 1.12 | 5.12 | 2.16 | 8.65 |
| TiC | _ | - | 0.08 | _ | - | 5.99 |
| TiC/C | 3.25 | 0.63 | 2.14 | 7.17 | 1.33 | 10.1 |
| NRSiC | 2.55 | 0.53 | 1.08 | 5.41 | 0.39 | 2.50 |
| NFSiC | 7.32 | 0.07 | 0.41 | 7.13 | 0.68 | 3.61 |

| Table II |
|--|
| Parameters of Langmuir isotherms of bacteria adsorption at pH 6.8 and the constant |
| of bacteria adsorption as a value of the derivative of polynomial at a point of $C_{eq} = 0$ |

 A_{m} – the maximal number of adsorbed bacteria (× 10¹⁰ cells g⁻¹),

 $K_{\rm L}$ – the Langmuir constant,

 K_{ads} - constant of bacteria adsorption – affinity of bacteria to investigated materials.

relationship. A similar problem can arise when analyzing the maximal amount of adsorbed bacteria. It seems that some results can be overestimated because some curves did not reach the equilibrium stage. However, this problem could not be solved by increasing the concentration of bacteria in the experiments due to the possibility of bacterial flocculation (Borkowski et al., 2015). The results of the experiments presented above were confirmed by epifluorescence microscopy (Fig. 5A-E). The selected pictures present bacteria attached to the investigated materials. These images were obtained for preparations of bacteria and materials in aqueous suspensions; hence, the bacteria and the materials did not form aggregates due to the drying of the suspension on the microscopic slides. It can be stated that the bacteria, both Gram-positive and Gram-negative, can adsorb onto aggregates of graphite, TiC, or SiC nanostructures. Bacteria were also observed to adsorb onto micrometric SiC, but the adsorption values were significantly lower. Based on the results, it seems that the adsorption of bacteria strongly depends on the texture of the materials, and partially on pH. In the case of NRSiC, NFSiC, and µmSiC, the adsorption of bacteria was inversely proportional to pH generally. Adsorption could be only partially related to the isoelectric points of the bacteria and SiC, as was presented by Borkowski et al. (2015) for *P. putida*. Some authors emphasize the importance of the isoelectric points of bacteria and mineral materials in the aggregation process. In the paper by Ams et al. (2004), the adsorption of Gram-positive B. subtilis and Gram-negative Pseudomonas mendocina onto the surface of Fe-oxyhydroxide-coated and uncoated quartz grains as a function of pH was studied. Adsorption appeared to be controlled by the surface charges of the bacteria and the mineral surface. The possibility of bacterial adsorption onto the surface of nanostructures has been presented, e.g., in the work of Kang et al. (2007) and Singh et al. (2011). Similarly, the ability to create a biofilm and the adsorption of bacterial cells onto the surface of mineral materials, including carbon materials and modified clay minerals, were also demonstrated (Yee et al., 2000; Rivera-Utrilla et al., 2001; Yamamoto et al., 2001; Jiang et al., 2007; Rong et al., 2008). These studies also indicated the important roles of pH and the ionic strength of the solution as significant factors affecting the adsorption of bacterial cells onto the surface of minerals. In the case of the experiment with SiC and bacteria presented in this paper, the PZC value does not explain the observed phenomena sufficiently. On the contrary, the PZC values seem to affect adsorption to TiC, TiC/C, and graphite. In these cases, the adsorption of both P. putida and S. aureus was strongest at neutral pH. For instance, the isoelectric point of graphite was about 7.5, and taking into account the PZC of the bacteria, the adsorption should be strongest at a neutral or slightly acidic pH. At pH 9, the charges of both the bacteria and graphite are negative. At pH 3, the surface charge is positive, and only at a more neutral pH is the electrostatic effect the strongest. A similar relationship was found in case of adsorption to TiC/C and TiC. The PZC of TiC was about 9.4, and even at pH 9, adsorption should be significant. The obtained results showed that at pH 9, TiC formed aggregates with bacteria and behaved as the strongest adsorbent among the investigated materials.

The presented results showed the stronger adsorption of *S. aureus* compared with *P. putida*. Both the A_m and K_{ads} showed that *S. aureus* can aggregate with materials more effectively than Gram-negative bacteria, but the shape of the obtained isotherms was quite similar in both cases. Ams *et al.* (2004) showed that the differences in the adsorption of *B. subtilis* and *P. mendocina* onto some mineral phases can be explained by differences in their electrostatic properties and cell wall structures. Generally, in the presented studies, the isotherms were approximated by the Langmuir and

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Fig. 5. Bacteria adsorbed onto the investigated materials.

A – S. aureus on graphite; B – S. aureus on TiC/C; C and D – S. aureus on NFSiC and NRSiC respectively; E – P. putida on NRSiC. Bacteria are orange, mineral phases are green. Bars indicate 100 μm. Pictures E – 600x. F – Probably the bacteria P. putida and TiC particles associated into aggregations after the adsorption experiment. SEM, bar indicates 1 μm.

Freundlich equations, except for the isotherms obtained with TiC. In this case, the Langmuir isotherm did not make any physical sense, and the shape of the isotherm indicated a multilayer adsorption. It is interesting that these phenomena were observed for both of the investigated bacteria. It seems that TiC particles did not form aggregates in aqueous suspension, and the bacteria did not adsorb to the surfaces of TiC. It is possible that cells and TiC particles form aggregates together, and, as a heavier structure, they are easy to separate from free cells in the applied procedure. The SEM images (Fig. 5F) revealed that the bacteria formed structures surrounded by TiC particles, but it cannot be stated that the observed structures are truly aggregates that consist of cells and TiC.

Viability test. The viability test was presented in Figure 6. Based on the measurements, generally it can be stated that P. putida cells were more sensitive to the investigated nanomaterials than S. aureus cells. The maximum loss of viability was noted in the case of bacteria adsorbed onto NRSiC and NFSiC aggregates. The values reached 90% and 80%, respectively. The other materials did not exhibit such strong antimicrobial activity in relation to the control and reference material (µmSiC). Completely different values were found for S. aureus. The maximum loss of viability for bacterial cells adsorbed onto NRSiC and TiC/C reached about 30% and 15%, respectively. In other cases, the measured values did not differ statistically from the control. Many recent studies focused on the antimicrobial activity of carbon nanostructures, such as single- and multiwalled nanotubes (Kang et al., 2007; Kang et al., 2008a; 2008b; Akhavan et al., 2011; Su et al., 2013) or other carbon materials, such as graphite, graphene, and fullerene (Lyon et al., 2006; Akhavan and Ghaderi, 2010; Liu et al., 2011). The mechanism proposed to explain the antibacterial properties of these materials is primarily based on oxidative stress and the physical interactions with the cell membrane (Liu et al., 2011). Physical interactions with the cell membrane leading to the loss of integrity have been proposed as a key antibacterial mechanism of carbon nanotubes. It is possible that the same mechanism could play a crucial role in the effects of NFSiC and NRSiC on Gram-negative bacteria. It is interesting that Gram-positive bacteria, generally, were much more resistant, and the tested materials did not affect cell viability to the same extent as was observed for P. putida. It seems that Gram-positive bacteria, due to the presence of thick layer of murein in their cell walls, were not strongly affected by the tested materials. Gram-negative bacteria also contain murein, but this layer is much thinner and the cells are enveloped by a lipid outer membrane.

Conclusions

In the presented experiments, we demonstrated that ceramic materials obtained by self-propagating combustion synthesis can efficiently adsorb *P. putida* and *S. aureus*. The hypothesis that adsorption depends on the texture of materials was confirmed by comparing the adsorption of bacteria to NFSiC and NRSiC aggregates to µmSiC aggregates.

It can be concluded that Gram-positive bacteria adsorbed more strongly to these materials. It seems that both the PZC value and the texture of the ceramic





material affected bacterial adsorption. Additionally, on the one hand, Gram-positive bacteria showed a greater adsorption onto the surface of the tested materials. On the other hand, these bacteria had a significantly higher survival rate at the ceramic material surface, probably due to the presence of a thick layer of murein. However, in both cases of the tested bacteria, the greatest toxicity was exhibited by nanorods of SiC.

Based on the obtained results of adsorption and the analysis of the affinity of the bacteria, the investigated materials can be ordered from the largest to smallest sorption capacity, as can the affinity of the bacteria for the tested materials. For *P. putida*, the following order of the sorption capacity of the materials was observed: NFSiC>TiC>TiC/C>graphite>NRSiC>µmSiC. In regard to bacterial affinity, the tested materials can be ordered as follows: TiC/C>graphite>NRSiC>NFSiC>MSiC>NFSiC>µmSiC>TiC. Similarly, for *S. aureus*, the sorption capacity of the materials was: TiC>TiC/C>NFSiC>NRSiC>graphite>µmSiC. Regarding bacterial affinity, the materials can be ordered as follows: TiC/C>NFSiC>NRSiC>graphite>µmSiC. Regarding bacterial affinity, the materials can be ordered as follows: TiC/C>graphite>TiC>Sic>PiC>NRSiC

These rankings reveal an interesting relationship. On the one hand, the bacterial affinity to TiC is relatively weak. On the other hand, in dense cell suspensions, TiC behaves like a very good adsorbent of bacteria. This

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ORIGINAL PAPER

Biocontrol of Gray Mold Decay in Pear by *Bacillus amyloliquefaciens* Strain BA3 and its Effect on Postharvest Quality Parameters

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Abstract

The economic losses caused by postharvest fruits diseases have attracted global attention. Traditional chemical fungicide could not meet the need of humans. In recent years, microbial agent which has begun to take the place of chemical fungicide comes into people's vision. The aim of this paper was to investigate the potential of *Bacillus amyloliquefaciens* strain BA3 for its biocontrol capability on gray mold decay of pears and its effect on postharvest quality of pears. Compared with other treatments, the inhibition effect on gray mold of washed cell suspension of *B. amyloliquefaciens* was the best. Consequently it was utilized in subsequent experiments. Spore germination and germ tube length of *Botrytis cinerea* was 18.72% and 12.85 µm treated with BA3, while the control group was 62.88% and 30.44 µm. We confirmed that increase of the concentration of *B. amyloliquefaciens*, improved the efficacy of BA3 in controlling gray mold decay of pears. Colonization variation of BA3 in wounds of pears was recorded. To begin with, the populations of *B. amyloliquefaciens* increased rapidly and remained stable. On the fourth day, there was a declining trend , after that the population increased to 4×10^5 CFU/wound and remained stable. BA3 had no significant effect on mass loss, titratable acidity, firmness and total soluble solids of pears that were stored at 25°C for 7 days comparing with control group. However, the effect of *B. amyloliquefaciens* on ascorbic acid was significantly higher than that of the control group. Our study indicates that *B. amyloliquefaciens* has a potential acidity as postharvest biocontrol agent on pears.

Key words: Bacillus amyloliquefaciens, Botrytis cinerea, biocontrol agents, gray mold, quality parameters

Introduction

Postharvest diseases are the primary damages of fruits during cultivation, transportation and storage. Postharvest decay of fruits has caused significant levels of economic losses worldwide (Sugar and Basile, 2011; Luo *et al.*, 2015). It is reported that in developed countries 20–25% of harvested fruits decay because of postharvest diseases and 50% in developing countries (Sharma *et al.*, 2009; Lutz *et al.*, 2013).

The pear is one of the world's cultivated fruits, more than 70 countries and regions all over the world produce pears. In recent years, the cultivation area and the production of pears increased rapidly in China, which has been ranked first in the world (Yang *et al.*, 2015).

Gray mold decay spreads widely and is caused by *Botrytis cinerea* which becomes one of the most important postharvest pear diseases (Lutz *et al.*, 2013). Currently, postharvest diseases are controlled mainly by chemical means (Zhang *et al.*, 2008a). However, chemical control is unfriendly to the environment and even leads to hazardous effects on humans and the environment (Solanki *et al.*, 2013). Due to the increasing public concern about the potential detrimental effects of synthetic fungicides abuse, it is necessary to explore the best pollution-free means to control postharvest diseases (Sansone *et al.*, 2005; Liu *et al.*, 2010; Liu *et al.*, 2013). Microbial biocontrol agents show great potential for controlling postharvest decay of fruits as an alternative to chemical control (Fan and Tian, 2000).

Bacillus species can be isolated from a variety of substances and it has been proved that they can produce inhibiting substances acting against a wide range of pathogens (Arguelles-Arias *et al.*, 2009; Solanki *et al.*, 2012; 2013). The objective of this study was focused on the *Bacillus amyloliquefaciens* strain BA3 isolated from "douchi", controlling postharvest decay of pears by *B. amyloliquefaciens* and exploring: (1) biocontrol activity of *B. amyloliquefaciens* on conidial germination of

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B. cinerea, (3) population studies of *B. amyloliquefaciens* in wounds, (4) efficacy of *B. amyloliquefaciens* on control of gray mold decay of pears, (5) efficacy of *B. amyloliquefaciens* on quality attributes of postharvest pears.

Experimental

Materials and Methods

Pathogen inoculum. *B. cinerea* Pers, purchased from Guangdong Microbiology Culture Center (GIMCC), was cultivated on synthetic potato medium (extract of boiled potatoes, 200 ml; dextrose, 20 g; KH_2PO_4 , 3 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; vitamin B1, 8 mg; agar powder, 20 g and deionized water, 800 ml; pH, 6 at 25°C for 7 days. To prepare a spore suspension of *B. cinerea*, the plates were flushed with 10 ml sterile distilled water, and then the spore concentration was determined with a hemacytometer and adjusted to required concentration.

Antagonist. B. amyloliquefaciens strain BA3, was isolated from "douchi" (a kind of Chinese soy products) and identified by BioSune Company (Shanghai, China). GenBank number is KF192921. BA3 was incubated on NA (Nutrient Agar: peptone, 5 g; beef extract, 30 g; NaCl, 5 g; agar powder, 15 g; 1000 ml distilled water; pH, 7.0-7.2) at 28°C. B. amyloliquefaciens strain BA3 was cultivated in 250 ml Erlenmeyer flasks with 50 ml of NB (NA without agar powder) which had been inoculated with a loop of the culture on a rotary shaker at $200 \times g$ for 24 h at 28°C. Then four treatments of liquid cultures were used: (A) liquid cultures (LC): mentioned above; (B) autoclaved cultures (AC): autoclaving the liquid cultures for 20 min at 121°C; (C) Culture filtrates (CL): filtering centrifuged liquid supernatant of the antagonist through a 0.2 µm polycarbonate membrane filter; (D) washed cell suspension (WCS): the cells were harvested by centrifugation at $8000 \times g$ for 10 min, washed twice and then re-suspended with distilled water. The concentration of cells in the suspension was counted with a hemacytometer and adjusted to 1×10^8 CFU/ml with sterile distilled water and (E) sterile distilled water acted as the control (CK).

Fruits. Pear fruits (*Pyrus pyrifolia*) cultivar "huangguan" were harvested at commercial maturity, selected on uniformity size without physical injuries or infections. Steep the fruit for 2 min in 0.1% sodium hypochlorite to sterilize and air dry before being wounded.

In vitro antagonism. To estimate the interactions between the antagonist and the pathogen *in vitro*, we coated 200 μ l spore suspension of *B. cinerea* on synthetic potato medium plates with a glass rod evenly. Then cut a 3-mm-diameter disk from the plates and added 100 μ l A-E suspensions of treatments into each well. One hour later, the plates were incubated at 28°C

(Liu *et al.*, 2010). The inhibition zone diameters were recorded after 7 days. Each test consisted of three replicate trials of 3 plates. The test was repeated three times.

Efficacy of BA3 on conidial germination of B. cinerea. The efficacy of BA3 on spore germination and germ tube elongation of B. cinerea was assessed in potato dextrose broth (PDB) (Feng et al., 2011). One hundred µl spore suspension of *B. cinerea* $(1 \times 10^6$ spores/ml) was added to glass tubes containing 4.8 ml of PDB. One hundred μ l quantity of 1×10^8 CFU/ml of B. amyloliquefaciens suspension or sterile distilled water (as control) was added into each tube, respectively. After 12 hours' incubation at 28°C on a rotary shaker $(200 \times g)$, a total of 100 spores per replicate were observed microscopically with a light microscope and at least 5 microscope fields were observed. Conidia were considered germinated when the germ tube length was equal to or longer than the conidia length (Lutz et al., 2013). All treatments consisted of three replicates, and experiments were repeated three times.

Population studies of B. amyloliquefaciens strain BA3 on fruit wounds. A uniform 5 mm deep and 3 mm wide wound was made at the equator of each fruit (put on its side) using the tip of a sterile inoculating needle. We injected 20 μ l cell suspension of BA3 (1 \times 10⁸ CFU/ ml) into the wound of each pear. Then the treated pears were cultivated at 25°C (90% relative humidity). Population of BA3 was recorded after being incubated for 0 (2 h after treatment), 1, 2, 3, 4, 5, 6 and 7 days, respectively. Wounded tissue was removed with an sterile 7 mm (internal diameter) cork borer and ground with an autoclaved mortar and pestle in 50 ml of sterile 0.85% sodium chloride solution. Serial tenfold dilution was made and 0.1 ml of each dilution was spread in NA. The plates were incubated at 28°C for 2 days and the colonies were counted. Population densities of B. amyloliquefaciens BA3 were expressed as log₁₀ CFU per wound. There were three single fruit replicates per treatment, and the experiments were repeated three times (Yu et al., 2012).

Efficacy of BA3 in controlling of gray mold decay of pears. A uniform 5 mm deep and 3 mm wide wound was made at the equator of each fruit (put on its side) using the tip of a sterile inoculating needle. The cell suspension of BA3 was adjusted to gradient concentration consisting of 1×10^6 , 1×10^7 , 1×10^8 CFU/ml with sterile distilled water by hemacytometer, respectively. We added thirty microliters washed cell suspension of 1×10^6 , 1×10^7 , 1×10^8 and 0 (as control) CFU/ml into each wound, respectively. After two hours, we injected fifteen microliters of spore suspension of *B. cinerea* $(1 \times 10^5$ spores/ml) to each wound. Treated fruits were stored at 25°C for 7 days or 0°C for 30 days after airdrying. Infection rates and lesion diameters on fruits were measured. There were three replicate trials of 10 fruits per treatment with complete randomization in each test. The test was repeated twice.

Quality parameters of postharvest pears treated with BA3. To appraise the effect of *B. amyloliquefaciens* on quality attributes of postharvest pear, the pear fruits were soaked in washed cell suspensions $(1 \times 10^8 \text{ CFU/ml})$ or sterile distilled water as a control respectively for 30 second, and then air-dried. The treated fruits were stored in artificial climate chamber with polyethylenelined plastic boxes to retain high relative humidity at 25°C for 7 days. After storage, quality attributes were measured. Quality attributes of postharvest treated pears were made on three replicates of five fruits each, the test was repeated twice.

Mass loss. The weight of pear was measured by a JA31002 balance $(\pm 0.01 \text{ g})$ (Shanghai Jingping Balance Instruments, China) before treatment (A) and after storage (B), respectively, and the mass loss was calculated as (A–B)/A (Zhang *et al.*, 2008b).

Ascorbic acid. The 2, 6-dichloroindophenol titrimetric method (AOAC) was used to determine the ascorbic acid content of pressed fruit juice. Results were expressed as milligrams of ascorbic acid/100 g sample (AOAC, 1995).

Titratable acidity. Titratable acidity was determined by the method described by Özden and Bayindirli (2002). Titratable acidity was calculated as the percent of malic acid.

Fruit firmness. Firmness values of each pear were measured at three points. Insert a fruit ripeness tester (Wagner Instruments) by 90° at the equator of each fruit after the removal of 1 mm thick slice of peel. The firmness of each pear was measured three times on different sides.

Total soluble solids (TSS). We measured total soluble solids (TSS) with a hand-held refractometer, and recorded the refractive index of the same juice. The results were expressed as percentages (g per 100 g fruit weight) (Luo *et al.*, 2015).

Statistical analysis. Statistical analyses were performed with SPSS version 19.0. The data were analyzed by analysis of the variance (ANOVA). Statistical significance was assessed at P-value < 0.01 and Duncan's Multiple Range Test was used to separate means.

Results and Discussion

Microorganism is a kind of useful biocontrol agent that can inhibit several pathogens fungi which cause postharvest diseases of fruits (Chen *et al.*, 2009; Li *et al.*, 2011; Askarne *et al.*, 2012; Solanki *et al.*, 2012; Yu *et al.*, 2012; Hu *et al.*, 2015). But to our knowledge, there is little information concerning about the effect of *B. amyloliquefaciens* on controlling postharvest gray



Fig. 1. Efficacy of different treatments in controlling of growth of *B. cinerea* on plates.

LC, liquid cultures; AC, autoclaved cultures; CL, Culture filtrates; WCS, 1×10^8 CFU/ml washed cell suspension; CK, sterile distilled water. Inhibition zone diameters were measured after 7 days at 28°C. Means are averaged values of three trials ±standard error. Bars represent standard errors. Values followed by different lowercase letters are significantly different at the 0.01 probability level according to analysis by Duncan's multiple range tests.

mold decay of pears and the influence of *B. amylolique-faciens* on quality parameters of pears. Thus, it is in need of exploring the efficacy of *B. amyloliquefaciens* in control of gray mold decay and its effect on postharvest quality attributes of pears.

Antagonism *in vitro*. On synthetic potato medium plates, the inhibition zone diameter of washed cell suspension of BA3 was 7.6 cm (Fig. 1) and was significantly larger than that of control and other treatments. Liquid cultures and autoclaved cultures also significantly inhibited the development of *B. cinerea*. Inhibition zone diameters, respectively, were 4.8 cm and 2.9 cm. But those were significantly smaller than that of washed cell suspension. Culture filtrates and sterile distilled water did not have any inhibitory effect on *B. cinerea* so the inhibition zone diameters were 0 cm.

The inhibition zone diameters of washed cell suspension of BA3 were obviously larger than control and all the other treatments and it indicated the cell suspension was the best agent among what we have used against *B. cinerea* (Hu *et al.*, 2015). So the cell suspension was utilized in the next experiments.

Efficacy of BA3 on conidial germination of *B. cinerea*. The spore germination of control was 62.88% after incubated at 28°C for 20 hours, and the germination of treatment was 18.72%. At the same time, the germ tube length of *B. cinerea* of control was 30.44 μ m, which of treatment was 12.85 μ m (Table I).

B. amyloliquefaciens significantly controlled spore germination and germ tube length of *B. cinerea*. Lutz *et al.* (2013) indicated that there are differences between different strains' inhibiting effect and different pathogens. The germination inhibition percentage of BA3 is higher than most yeast strains in Lutz *et al.* (2013) and Spadaro *et al.* (2013). Several biocontrol mechanisms have been suggested as being effective against postharvest fruit diseases (Jamalizadeh *et al.*, 2011),

Table I Efficacy of *B. amyloliquefaciens* strain BA3 on conidial germination of *B. cinerea*

| Treatments | Spore germination (%) | Germ tube length (µm) |
|----------------------|--------------------------|--------------------------|
| Control | 62.88±3.78a | 30.44±1.79a |
| B. amyloliquefaciens | 18.72±1.26b | $12.85 \pm 0.75b$ |

Germination rate and germ tube length were measured after 20 h incubation at 28°C in PDB. Means are averaged values of three trials \pm standard error. Values followed by different lowercase letters are significantly different at the 0.01 probability level according to analysis by Duncan's multiple range tests. The same as below.

competition for nutrients is the possible mechanism that BA3 inhibit the spore germination.

Population studies of BA3 on fruit wounds. At the start of the experiment (time 0), the population of *B. amyloliquefaciens* was 6×10^4 CFU/wound, and then it increased rapidly to 4.7×10^6 CFU/wound (Fig. 2).



Fig. 2. Population dynamics of *B. amyloliquefaciens* on fruit wounds incubated at 25°C. Data were pooled from three experiments and each point representing

the mean colony counts from three replicate fruits.

In the following three days (1st day to 3rd day), the populations remained stability basically. But on the fourth day, there was a declining trend of. When on the fifth day, *B. amyloliquefaciens* population touched bottom of 2.4×10^4 CFU/wound, then increased to 4×10^5 CFU/ wound and kept stable.

To select an antagonist suitable for postharvest application, it is necessary to look for what can be adapted for the environment of wounds and make use of nutrient sources, then grow it and proliferate it well (Manso and Nunes, 2011; Yu et al., 2013). The population of *B. amyloliquefaciens* strain BA3 increased rapidly and remained stability on the wounds of pears in the first three days which demonstrated that BA3 has the potential as an antagonist. But the population declined to 2.4×10^4 CFU/wound on the fourth day. The pathogens might attach to the wounds, begin to grow, and then compete for nutrition and space against *B. amyloliquefaciens*. Afterwards the antagonist became the dominant bacteria and the population of BA3 kept stable. These suggested that the competition for nutrient sources and space may be one of the mechanisms of action to inhibit the pathogen. This result is different from Zhang et al. (2008a) and Hu et al. (2015). It is probable that *B. amyloliquefaciens* needs a transitional period to control the pathogens.

Efficacy of BA3 in control of gray mold decay of pears. After incubation at 25°C for 7 days, the infection rate of control fruit was 100%. And at the concentration of *B. amyloliquefaciens* of 1×10^8 CFU/ml, it decreased to 53%, which was significantly lower than the control (Table II). The infection rate of the concentration of *B. amylolique faciens* at 1×10^8 CFU/ml was not significantly lower than 1×10^7 CFU/ml, but data indicated that the lesion diameters at 1×10^8 CFU/ ml were significantly smaller than at 1×10^7 CFU/ml. Moreover, lesion diameter of the control was 4.80 cm, which of *B. amyloliquefaciens* at the concentration of 1×10^8 CFU/ml was significantly smaller than the control, being 0.91 cm. After cultivation for 30 days at 0°C, the infection rate of the control was 86.3%, while at concentration 1×10^8 CFU/ml it was significantly lower than the control, being 29.8%. Being different from cultivated at 25°C, infection rate at 1×108 CFU/ml was significantly lower than that at 1×10^7 CFU/ml. However, the lesion diameters of the concentration at 1×10^6 CFU/ml and 1×10^7 CFU/ml did not show significant differences. Both infection rate and lesion

| Concentrations of | 25 | °C | 0°C | | |
|----------------------------------|-----------------------|-------------------------|-----------------------|-------------------------|--|
| B. amyloliquefaciens (CFU/ml) | Infection rate (%) | Lesion diameter (cm) | Infection rate (%) | Lesion diameter (cm) | |
| 0 (CK) | $100 \pm 0a$ | $4.80 \pm 0.06a$ | 86.3±1.26a | $4.18 \pm 0.19a$ | |
| 1×10^{6} | 91.5±3.38b | 3.82±0.11b | 61.2±1.68b | $2.73 \pm 0.08b$ | |
| 1×10^{7} | $68.2 \pm 4.44c$ | $2.78\pm0.27c$ | $47.8 \pm 1.40c$ | $2.37 \pm 0.09b$ | |
| 1×10^{8} | 53.0±5.38c | 0.91±0.14d | 29.8±1.30d | 0.75±0.11c | |

 Table II

 Efficacy of *B. amyloliquefaciens* strain BA3 in control of gray mold decay of pears

Infection rate and lesion diameter were recorded after storage at 25°C for 7 days or 0°C for 30 days.

Biological control of gray mold decay

| Treatments | Mass loss (%) | Ascorbic acid (mg/100g) | Titratable acidity (% malic acid) | Fruit firmness (N) | Total soluble solids (%) |
|------------|------------------|----------------------------|--------------------------------------|-----------------------|-----------------------------|
| Control | 1.74±0.49a | 0.651±0.056a | $0.067 \pm 0.0053a$ | 11.96±0.40a | 11.11±0.423a |
| Antagonist | $1.25 \pm 0.04a$ | $0.881\pm0.083b$ | $0.078 \pm 0.0070a$ | $13.24 \pm 0.40a$ | 11.22±0.324a |

 Table III

 Efficacy of *B. amyloliquefaciens* strain BA3 on postharvest quality parameters of pears

diameter of each concentration at 0° C were smaller than at 25° C.

The results reported in Table II show that, the concentration of BA3 significantly influenced the development of *B. cinerea* in pear. Hu *et al.* (2015) and Li *et al.* (2011) demonstrated that the higher the concentration of *B. amyloliquefaciens*, the lower the infection rate and the smaller the lesion diameter. Similarly, there is a direct correlation between the concentration of *B. amyloliquefaciens* strain BA3 and biocontrol effectiveness. When the concentration of *B. amyloliquefaciens* was 1×10^8 CFU/ml, the infection rate was reduced almost by one half, and the lesion diameters were contained within 1 cm.

Since most fruits are stored at low temperatures to extend shelf-life, being able to inhibit decay at low temperatures condition is the criterion for selecting an antagonist (Manso and Nunes, 2011). Stored at 0°C, the effect of BA3 in controlling of gray mold decay is better than at 25°C, suggesting that *B. amyloliquefaciens* had great potential in inhibiting gray mold decay of pears and could be utilized as a biological control agent at low temperatures.

According to studies conducted by Lutz *et al.* (2013), limiting spore germination of pathogens may be one of the major mechanisms of action of *B. amyloliquefaciens*. However, the interactions among host, pathogen, antagonist and microorganisms are complicated (Liu *et al.*, 2010), and the action mechanisms of antagonists against pathogens are not single (Arguelles-Arias *et al.*, 2009). This requires further research and more accurate descriptions.

Quality parameters of postharvest pears treated with BA3. According to Table III, the pears treated with *B. amyloliquefaciens* showed no significant differences with regard to mass loss, titratable acidity, firmness and total soluble solids compared with the control. But the ascorbic acid of pears treated with *B. amyloliquefaciens* was significantly higher than that of control fruits.

Not impairing the quality attributes of fruits is one of the conditions of an ideal biocontrol agent (Liu *et al.*, 2010). Moreover, the ascorbic acid of pears treated with *B. amyloliquefaciens* was significantly enhanced. This is different from other literature data (Özden and Bayindirli, 2002; Zhang *et al.*, 2008a; Luo *et al.*, 2015) which discussed the effect of antagonists on quality parameters of postharvest fruits. All of this further illustrates that *B. amyloliquefaciens* strain BA3 has a commercial potential.

In our experiments, *B. amyloliquefaciens* was isolated from the "douchi" and therefore is not harmful to human health. All of the results from the study indicate that *B. amyloliquefaciens* strain BA3 has the potential to be used as a biological control agent.

Our experiments did not fully assess the effect of *B. amyloliquefaciens* in control of gray mold decay of fruits, considering storage conditions or the mixture of other materials that improve the biocontrol efficacy so as to explore better means of biocontrol. We should also focus our further studies on the commercial application of *B. amyloliquefaciens*.

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ORIGINAL PAPER

Enzymes Involved in Naproxen Degradation by Planococcus sp. S5

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Abstract

Naproxen is a one of the most popular non-steroidal anti-inflammatory drugs (NSAIDs) entering the environment as a result of high consumption. For this reason, there is an emerging need to recognize mechanisms of its degradation and enzymes engaged in this process. *Planococcus* sp. S5 is a gram positive strain able to degrade naproxen in monosubstrate culture (27%). However, naproxen is not a sufficient growth substrate for this strain. In the presence of benzoate, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid or vanillic acid as growth substrates, the degradation of 21.5%, 71.71%, 14.75% and 8.16% of naproxen was observed respectively. It was shown that the activity of monooxygenase, hydroxyquinol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase and protocatechuate 4,5-dioxyegnase in strain S5 was induced after growth of the strain with naproxen and 4-hydroxybenzoate. Moreover, in the presence of naproxen activity of gentisate 1,2-dioxygenase, enzyme engaged in 4-hydroxybenzoate metabolism, was completely inhibited. The obtained results suggest that monooxygenase and hydroxyquinol 1,2-dioxygenase are the main enzymes in naproxen degradation by *Planococcus* sp. S5.

Key words: Planococcus, biodegradation, naproxen, enzymes induction, aromatic plant compounds

Introduction

In recent years, non-steroidal anti-inflammatory drugs (NSAIDs) have been widely detected in the environment. These biologically active compounds and their continuous inflow into the environment may lead to their accumulation in the environment and chronic exposure of organisms. As a result, this may cause potential negative effects on living organisms. While the transformation mechanisms of non-steroidal antiinflammatory drugs in the human body and in other animals have been extensively studied, the degradation of these drugs by bacteria has been seldom investigated and remains largely unknown (Quintana et al., 2005; Marco-Urrea et al., 2010; Wojcieszyńska et al., 2014). It is suggested that the transformation of naproxen by Trametes versicolor or activated sludge may occur by 2-(6-hydroxynaphthalen-2-yl)propanoic acid and 1-(6-methoxynaphthalen-2-yl)ethanone formation (Quintana et al., 2005; Marco-Urrea et al., 2010; Lahti and Oikari, 2011). Marco-Urrea et al. (2010) suggested participation of laccase and cytochrome P-450 system in biotransformation of this drug in fungi. Among bacteria, only Stenotrophomonas maltophilia KB2 is known to be able to cometabolically degrade naproxen (Wojcieszyńska et al., 2014). After growth of strain KB2 on naproxen activity of phenol monooxygenase, naphthalene dioxygenase, hydroxyquinol 1,2-dioxygenase and gentisate 1,2-dioxygenase was observed. It suggests that degradation of this compound consists in its hydroxylation and aromatic ring cleavage (Wojcieszyńska et al., 2014). In monosubstrate culture naproxen is degraded by strain KB2 with low efficiency. The addition of a carbon source, such as glucose, acetate or powdered milk, increases the level of naproxen degradation (Quintana et al., 2005; Lahti and Oikari, 2011; Wojcieszyńska et al., 2014). However, the non-aromatic substrates do not induce enzymes of aromatic ring degradation/transformation pathways. It is observed that the introduction of aromatic compounds (e.g. phenol) as an additional carbon source increase the degradation of toxic, persistent aromatic compounds (Kulkarni and Chaudhari, 2006; Greń et al., 2010; Wojcieszyńska et al., 2014). However, the introduction of phenol or its derivatives into the environment may constitute an additional ballast and disturb ecological balance. Therefore, a good alternative seems to be the use of naturally occurring aromatic compounds, such as protocatechuic

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acid produced by *Prunus domestica*, *Melissa officinalis* or *Vitis vinifera* or vanillic acid identified in *Juglans regia* or *Chenopodium murales* (Ghareib *et al.*, 2010; Kakkar and Bais, 2014). The objective of our study is to examine the ability of *Planococcus* sp. S5 to degrade naproxen in either mono- or disubstrate cultures with plant aromatic compounds as growth substrates. *Planococcus* sp. S5 was chosen for this study since it degrades salicylate (Łabużek *et al.*, 2003), which is not only the intermediate of naphthalene degradation and mimetic of naproxen, but also belongs to non-steroidal antiinflammatory drugs. Additionally, the aim of this study is to identify enzymes engaged in naproxen degradation by *Planococcus* sp. S5 in cometabolic conditions.

Experimental

Materials and Methods

Media and culture conditions. Planococcus sp. S5 was routinely cultivated in BBL nutrient broth at 30°C and 130 rpm for 24 hours. Then 6 mg/l naproxen was added to the culture. After 48 hours, cells were harvested by centrifugation $(5.000 \times \text{g at } 4^{\circ}\text{C} \text{ for } 15 \text{ min})$, washed with fresh sterile medium and used as inoculum. Degradation of naproxen in a monosubstrate, as well as cometabolic systems, was performed in 500 ml Erlenmeyer flasks containing 250 ml of the mineral salts medium (Greń et al., 2010) inoculated with cells to a final optical density of about 1.5 and 0.5 at $\lambda = 600$ nm (OD600) for the monosubstrate and cometabolic systems, respectively. For degradation experiments two control cultures were prepared. The uninoculated control (I) consisted of 250 ml of sterile mineral salts medium, while the heatkilled control (II) consisted of 250 ml of autoclaved culture prepared under conditions identical to those of the experimental cultures. Naproxen was added to each flask to obtain a final concentration of 6 mg/l, and all cultures were incubated with shaking at 30°C for 28 days.

For studies on the cometabolic transformation of naproxen, as well as the induction of enzymes, 3 mM aromatic compound of plant origin: benzoate (BA), 4-hydroxybenzoic acid (4-HB), 3,4-dihydroxybenzoic acid (3,4-DHB) or vanillic acid (VA) was added. Cultures in 250 ml of sterile mineral salt medium supplemented with appropriate growth substrate and 6 mg/l naproxen were inoculated with cells to a final optical density of about 0.5 at λ =600 nm (OD600), and incubated at 30°C with shaking at 130 rpm. If the complete degradation of the suitable growth substrate was observed, a successive dose of plant aromatic compound was introduced and the culture was left for incubation until it reached OD600=1.0. All cultures were grown in triplicate.

Analytical methods. To study the degradation of naproxen, 1 ml samples were taken periodically (every 7 days) from the culture medium and centrifuged ($6.000 \times g$, 15 min). The concentration of aromatic substrates in the culture supernatant was determined by HPLC (Merck HITACHI) equipped with a LiChromospher[®] RP-18 column (4×250 mm), liChroCART[®] 250-4 Nucleosil 5 C18 and a DAD detector (Merck HITACHI). The mobile phase was acetonitrile and 1% acetic acid (50:50 v/v) at a flow rate of 1 ml/min. The detection wavelength was set at 260 nm. Naproxen and plant aromatic compounds in the supernatant were identified and quantified by comparing the HPLC retention times and UV-visible spectra with those of the external standards.

Preparation of cell extracts. After 28 days in culture, cells of *Planococcus* sp. S5 were harvested by centrifugation $(4.500 \times \text{g} \text{ for 15 min at } 4^{\circ}\text{C})$ and the pellet was washed with 50 mM phosphate buffer, pH 7.0, and resuspended in the same buffer. Cell-free extracts were prepared by sonication of the whole cell suspension (6 times for 15 s) and centrifugation at 9.000 × g for 30 min at 4°C. Clear supernatant was used as a crude cell extract for enzyme assays.

Enzyme assays. Monooxygenase activity was determined spectrophotometrically by measuring NADH oxidation (ϵ^{340} = 6.220/M cm) (Divari *et al.*, 2003). In order to determine the activity of dioxygenase-catalysed dihydroxylation, the formation of cis, cis-dihydrodiol was measured at 262 nm (ϵ^{262} = 8.230/M cm) (Cidaria et al., 1994). The activity of catechol 1,2-dioxygenase was measured spectrophotometrically by the formation of cis,cis-muconic acid at 260 nm $(\epsilon^{260} = 16.800/M \text{ cm})$. In order to determine catechol 2,3-dioxygenase activity, the formation of 2-hydroxymuconic semialdehyde was measured at 375 nm $(\varepsilon^{375} = 36.000/M \text{ cm})$ (Wojcieszyńska *et al.*, 2011). The activity of protocatechuate 3,4-dioxygenase was assayed by measuring oxygen consumption (Hou et al., 1976). The activity of protocatechuate 4,5-dioxygenase was measured spectrophotometrically by the formation of 2-hydroxy-4-carboxymuconic semialdehyde at 410 nm $(\epsilon^{410} = 9.700/M \text{ cm})$ (Wojcieszyńska *et al.*, 2011). In order to determine gentisate 1,2-dioxygenase activity, the formation of maleylpyruvate was measured at 330 nm $(\epsilon^{330} = 10.800/M \text{ cm})$ (Feng *et al.*, 1999). The activity of hydroxyquinol 1,2-dioxygenase was measured spectrophotometrically by the formation of maleylacetate at 243 nm (ϵ^{243} = 44.520/M cm) (Wei *et al.*, 2010).

One unit of enzyme activity was defined as the amount of enzyme required to generate $1 \mu mol$ of product per minute. Protein concentration in the crude extract was determined by the Bradford method using bovine serum albumin as a standard (Wojcieszyńska *et al.*, 2011). All experiments were performed in three

replicates. The values of enzyme activities were analyzed by one-way ANOVA (p < 0.05) using STATIS-TICA 10.0 PL software package.

Results and Discussion

Naproxen degradation in mono- and disubstrate cultures. Naproxen belongs to the polar acidic drugs that are often used by human population as a non-steroidal anti-inflammatory drug without prescription (Grenni *et al.*, 2014). For this reason, a great amount of naproxen or its metabolites is excreted and enters sewage treatment plants, where they are barely reduced and, consequently, released into the environment. Although these compounds are microcontaminants, detected in the range $0.01-2.6 \mu g/l$, they can exert toxic effects on non-target organisms (Rodriguez-Rodriguez *et al.*, 2010; Grenni *et al.*, 2013; Qurie *et al.*, 2014). That is why preliminary studies on microorganisms able to degrade naproxen, as well as enzymes involved in its degradation, are so important.

The presented paper is the first report on the degradation of naproxen by gram-positive bacterium – *Planococcus* sp. S5. As we demonstrated previously, strain S5 is able to grow on salicylate, benzoate or phenol and express either catechol 1,2-dioxygenase or catechol 2,3-dioxygenase depending on the inductor (Łabużek *et al.*, 2003; Hupert-Kocurek *et al.*, 2012). Since naproxen, the derivative of naphthalene, may be metabolized by acetylic acid or benzoic acid as intermediates (Annweiler *et al.*, 2000), the degradation potential of strain S5 suggests that this strain is a good candidate for naproxen biotransformation.

The chemical oxidation of naproxen in abiotic control, as well as adsorption of this drug on bacterial cells was not observed (data not shown). In monosubstrate culture, approximately 27.5% of naproxen was removed after 28 days. However, this compound was an insufficient carbon source for strain S5 and decrease in bacterial growth was observed (Fig. 1). This gave rise to the need for introducing an additional source of carbon into the culture. Due to the fact that aromatic plant compounds show a similar structure to naproxen, the use of such compounds as growth substrates may cause the induction of enzymes engaged in the metabolism of aromatic ring. Moreover, aromatic plant compounds are substrates naturally present in the environment. The results of studies on the transformation of naproxen in the presence of benzoate, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid or vanillic acid have shown that 4-hydroxybenzoic acid is a good growth substrate for cometabolic degradation of this drug. The increased efficiency of naproxen degradation was observed only in the presence of 4-hydroxybenzoic acid as an additional



Fig. 1. Degradation of 6 mg/l naproxen by *Planococcus* sp. S5 and changes of microbial biomass monitored as optical density at 600 nm

carbon source (Fig. 2). This confirms the results of our studies on nitrophenol degradation by *Stenotrophomonas maltophilia* KB2. Addition of 4-hydroxybenzoic acid into the culture allowed the transformation of about 30% of mononitrophenols (Greń *et al.*, 2010).

Induction of degradation enzyme. Knowledge on enzymes engaged in naproxen degradation by microorganisms is very limited. Therefore, the aim of our study was to determine if/how the presence of naproxen affects the induction of key enzymes in aromatic degradation pathways. Planococcus sp. S5 is known to synthesizes two types of catechol dioxygenases: catechol 1,2-dioxygenase (in the presence of salicylate, benzoate or low concentration of phenol) and catechol 2,3-dioxygenase which synthesis is induced by salicylate or phenol (Łabużek et al., 2003; Hupert-Kocurek et al., 2012). Due to the low biomass obtained in the culture of strain S5 with naproxen as the only carbon source, as well as very low rate of naproxen degradation in the cultures with benzoate, 3,4-dihydroxybenzoic acid or vanillic acid as a growth substrate, degradation enzymes were isolated from cells grown in the presence of 4-hydroxybenzoate or 4-hydroxybenzoate and naproxen. After growth of the strain with 4-hydroxybenzoate activity of monooxygenase, hydroxyquinol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase and gentisate 1,2-dioxygenase was observed (Table I, Fig. 3A). Monooxygenase is known to be engaged in hydroxylation of the aromatic ring of 4-hydroxybenzoate to 3,4-dihydroxybenzoic acid or 1,2,4-benzenotriol (Sze and Dagley, 1984; Eppink et al., 1997; Wang et al., 2002). The aromatic ring of 3,4-dihydroxybenzoate formed as a result of monooxygenase activity may be then cleaved by protocatechuate 3,4-dioxygenase, whereas degradation of 1,2,4-benzenetriol is catalyzed by hydroxyquinol 1,2-dioxygenase (Sze and Dagley, 1984; Park et al., 2006). Additionally, hydroxylation of 4-hydroxybenzoate may lead to gentisic acid formation

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Fig. 2. Degradation of 6 mg/l naproxen by *Planococcus* sp. S5 in cometabolic systems and changes of microbial biomass monitored as optical density at 600 nm (a – with 3 mM benzoate; b – with 3 mM 4-hydroxybenzoate; c – with 3 mM 3,4-dihydroxybenzoate; d – with 3 mM vanillic acid)

that is connected with the intramolecular migration (NIH Shift) of carboxylic group (Fairley *et al.*, 2002; Deveryshetty *et al.*, 2007). After growth of *Planococcus*

Table I Specific activity of enzymes in the presence of 4-hydroxybenzoate or naproxen and 4-hydroxybenzoate

| | Specific enzyme activity (U/mg protein) | | |
|---------------------------------|--|----------------------|--|
| Enzyme | 4-HB | naproxen +4-HB | |
| monooxygenase | 25.74 ± 1.90 | 25.66 ± 7.40 | |
| naphthalene dioxygenase | 0.0 ± 0.0 | 0.0 ± 0.0 | |
| hydroxyquinol 1,2-dioxygenase | 46.56 ± 0.0 | 215.25 ± 90.74 | |
| catechol 1,2-dioxygenase | 0.0 ± 0.0 | 0.0 ± 0.0 | |
| catechol 2,3-dioxygenase | 0.0 ± 0.0 | 0.0 ± 0.0 | |
| protocatechuate 3,4-dioxygenase | $58.55\pm0.0^{*}$ | $30.47 \pm 2.12^{*}$ | |
| protocatechuate 4,5-dioxygenase | $0.0\pm0.0^{*}$ | $3.68 \pm 1.74^{*}$ | |
| gentisate 1,2-dioxygenase | $104.16 \pm 29.46^{*}$ | $0.0\pm0.0^{*}$ | |

 * The asterisk indicates the values of enzymes activities which differ significantly (p < 0.05) in dependence on the growth substrate

sp. S5 with 4-hydroxybenzoate and naproxen changes in the activity of enzymes was observed (Table I, Fig. 3B). Activity of hydroxyquinol 1,2-dioxygenase increased approximately fourfold, while protocatechuate 3,4-dioxygenase and gentisate 1,2-dioxygenase, enzymes engaged in 4-hydoxybenzoate cleavage, were inhibited (Table I). As it is shown in Table I, in the presence of naproxen, protocatechuate 3,4-dioxygenase showed about 52 % of its initial activity while gentisate 1,2-dioxygenase was completely inhibited. The increase in hydroxyquinol 1,2-dioxygenase activity could be connected with the engagement of this enzyme in naproxen degradation (Wojcieszyńska et al., 2014). Decrease of protocatechuate 3,4-dioxygenase activity was observed by Luo et al. (2008) in the presence of naphthalene, which is the structural mimetic of naproxen. However, in Planococcus sp. S5 culture with naproxen and 4-hydroxybenzoic acid activity of protocatechuate 4,5-dioxygenase was observed (Table I). Activity of this enzyme was also observed by Yun et al. (2004) during their studies on 4-hydroxybenzoic acid degradation. We assume that induction of protocatechuate 4,5-dioxygenase

Α

HO

HO

HO

В

NAD(P)*

H₂Ò

НÓ



СООН

3-carboxy--cis,cis-muconic acid TCA

HOOC

in the presence of naproxen and 4-hydroxybenzoate could be the response of bacterial strain to stress connected with inhibition of the main enzyme engaged in 4-hydroxybenzoate degradation (Fig. 3).

In conclusion, *Planococcus* sp. S5 has the ability of efficient degradation of naproxen in the presence of 4-hydroxybenzoate as a carbon source. In this condition, activity of monooxygenase, hydroxyquinol 1,2-dioxygenase, and two different protocatechuate dioxygenases is observed. The presence of various metabolic pathways and induction of different oxygenases involved in the degradation of aromatic compounds enable the use of *Planococcus* sp. S5 in the degradation of various aromatic pollutants including non-steroidal anti-inflammatory drugs.

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ORIGINAL PAPER

Characterization of Bacteria Isolation of Bacteria from Pinyon Rhizosphere, Producing Biosurfactants from Agro-Industrial Waste

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Abstract

Two hundred and fifty bacterial strains were isolated from pinyon rhizosphere and screened for biosurfactants production. Among them, six bacterial strains were selected for their potential to produce biosurfactants using two low cost wastes, crude glycerol and lactoserum, as raw material. Both wastes were useful for producing biosurfactants because of their high content in fat and carbohydrates. The six strains were identified by 16S rDNA with an identity percentage higher than 95%, three strains belonged to *Enterobacter* sp., *Pseudomonas aeruginosa, Bacillus pumilus* and *Rhizobium* sp. All strains assayed were able to grow and showed halos around the colonies as evidence of biosurfactants production on Cetyl Trimethyl Ammonium Bromide agar with crude glycerol and lactoserum as substrate. In a mineral salt liquid medium enriched with both wastes, the biosurfactants were produced and collected from free cell medium after 72 h incubation. The biosurfactants produced the surface tension from 69 to 30 mN/m with an emulsification index of diesel at approximately 60%. The results suggest that biosurfactants produced by rhizosphere bacteria from pinyon have promising environmental applications.

K e y w o r d s: agro-industrial wastes, biosurfactants, emulsification index, surface tension

Introduction

Biosurfactants (BS) are exopolymers mainly produced by bacteria, yeasts and fungi (Desai and Banat, 1997). BS are amphiphilic biomolecules, with hydrophobic and hydrophilic portions. The hydrophobic part of the molecule is based on saturated or unsaturated fatty acids. The hydrophilic portion can be either cationic and anionic amino acids or mono-, di-, and polysaccharides (Banat et al., 2000). Among the various species of BS, rhamnolipids have been studied extensively; they are among the most effective BS synthesized by Pseudomonas aeruginosa (Prieto et al., 2008). Some other BS widely known are surfactin produced by Bacillus subtilis, sophorolipids by Candida bombicola (Rosenber and Ron, 1999) and emulsan by Acinetobacter calcoaceticus (Karanth et al., 1999). Some Burkholderia sp. have demonstrated to be very useful in producing a considerable diversity of glycolipids (Pérez et al., 2010).

BS reduce surface and interfacial tension at gasliquid-solid interfaces and have emulsification and foaming properties (Dubey *et al.*, 2012; Freitas *et al.*, 2013; Vecino *et al.*, 2013). BS have advantages over the highly used synthetic surfactants, such as lower toxicity or higher stability over extreme temperature, pressure, pH values and salinity conditions (Gudiña *et al.*, 2013). Because of these advantages BS have extensive applications in oil recovery (Youssef *et al.*, 2007), bioremediation of persistent organic pollutants, and also in industrial fields as food production and pharmaceutical (Banat *et al.*, 2010; Ferhat *et al.*, 2011; Fonseca *et al.*, 2011).

Exploration of the microbial diversity of water and soil environments, along with the isolation and cultivation of biosurfactant-producing microorganisms (Makkar and Cameotra, 2002), are highly desired in order to enhance the biotechnological production of BS (Yañez-Ocampo and Wong-Villareal, 2013). The search for biosurfactant-producing microorganisms in the pinyon plant (*Jatropha curcas* L.) seems to have tremendous potential. This is primarily due to the natural ability of this plant to survive and grow under extreme environmental conditions, such as dryness and marginal soil (Abou-Kheira and Atta, 2009), and besides

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the lack of knowledge about the interaction between microbes and pinyon roots.

Additionally, agro-industrial organic wastes has recently been used as substrate to cultivate BS due to its low cost and high availability (Cassidy and Hudak, 2001; Kitamoto et al., 2002; Abbasi et al., 2012; 2013). It is well known that wastes from palm oil, canola oil, soybean, glycerol and buttermilk, may be used to achieve an increase in production of about ten times in the mass of BS produced higher than that obtained when the production media contain glucose as the carbon source (Makkar and Cameotra, 2002). For instance, Ron and Rosenberg (2001) and Abbasi et al. (2012) demonstrated that BS can be produced by P. aeruginosa through fruit wastes, waste vegetable oil and foodindustry wastes as the substrate. In this context, agroindustrial wastes generated in Chiapas are low cost raw material, renewable and abundant throughout the year, with a total generation of about 28-140 thousand tons per year. These wastes include crops and processing residues of corn, coffee, cocoa, mango, banana, oil palm and buttermilk (SAGARPA, 2007; Valdéz-Vázquez et al., 2010). Likewise, huge amounts of glycerol were generated from the biodiesel fuel production in Chiapas during a 5 years operation period and, because this crude glycerol is expensive to purify for use in other industries, it remains without any disposal method and becoming a significant environmental problem, but potentially valuable as substrate to produce BS.

Hence, the isolation of bacteria strains from pinyon rhizosphere capable of biosurfactant production by using four agro-industrial wastes as substrate is of interest in commercial production. In the present study, the presence of potential biosurfactant producer bacteria was determined by the formation of a complex with CTAB with methylene blue as an indicator. Moreover, growth kinetics and BS production were described for those strains with potential biosurfactant producer properties. The surface tension and the emulsification index have also been measured. Finally, sequencing identification of 16S rRNA has also been used for bacterial identification.

Experimental

Materials and Methods

Agro-industrial waste material. Four different agro-industrial wastes were used as substrates to produce BS. Lactoserum, coffee husk and palm oil cake residues were obtained from the Ocosingo and Palenque regions of Chiapas in Mexico. Crude glycerol was obtained from biodiesel production process via transesterification of waste vegetable oil, also in Chiapas. **Physicochemical characterization of agro-industrial wastes.** Nitrogen content was measured by Kjeldahl method and protein content was estimated using an appropriate Nitrogen Factor, carbohydrates were measured by a gravimetric method and fat content was measured gravimetrically after Soxhlet extraction using hexane as a solvent according to the procedures outlined in Standard Methods (APHA, 2001). The results were expressed as weight percent.

Isolation of bacterial strains from pinyon rhizosphere. Pinyon plants (Jatropha curcas L.) were collected from the municipality of Mazatán, Tapachula, Huixtla, Huehuetán, Mapastepec, Pijijiapan and Tonalá in the state of Chiapas, Mexico. From each sample, approximately 1 g of the plant root was first mixed with 9 ml of MgSO₄·7H₂O 10 mM in tubes, and shaken vigorously to obtain a solution containing microorganisms from pinyon rhizosphere. Two hudred µl of sample solution was then streaked on semisolid medium Baz (g/l): azelaic acid 2.0, K₂HPO₄ 0.4, KH₂PO₄ 0.4, MgSO₄·7H₂O 0.2, CaCl₂ 0.02, Na₂MoO₄·H₂O 0.002, FeCl₃ 0.01, bromothymol blue 0.075, agar 2.3, pH 5.7 and incubated at 28°C for a week. Samples that showed bacterial growth were streaked twice again. Selected samples were inoculated in solid medium BAc: 0.2% azelaic acid, 0.02% L-citrulline, 0.04% K₂HPO₄, 0.04% KH₂PO₄, 0.02% MgSO₄·7H₂O (Estrada de los Santos et al., 2001) enriched with cyclohexamide (100 mg/l). After 7 h of incubation at 28°C, colonies with different morphology were selected and further purified in BAc medium. To confirm the purity, isolates were transferred to PY medium (g/l): peptone 5, meat extract 3, agar 15. Purified colonies were prepared in 70% glycerol and stored at -70°C, to preserve them for further characterization.

Preliminary experiments to evaluate BS formation. Strains were transferred to PY agar (as activation medium), and incubated for 24 h at 30°C. Subsequently, strains were inoculated again on CTAB agar (g/l): KH₂PO₄ 0.7, Na₂H₂PO₄·7H₂O 1.7, (NH₄)₂SO₄ 2.32, MgSO₄ · 7H₂O 0.4, hexadecyltrimethylammonium bromide (CTAB) 0.2, agar base 15, agro-industrial waste 20, methylene blue (MB) 0.03; with each agro-industrial waste, and incubated for 48 h at 30°C. P. aeruginosa ATCC 27853 was used as a positive control. Bacterial colonies surrounded by a translucent halo were identified as a potential biosurfactant producer. The former, based on the property that BS can be determined by the formation of a complex with CTAB with methylene blue as indicator. In this method, methylene blue is reduced to methylene white by an excess of reducing sugar that generates translucent halos. The detection of the translucent halos formed due to complexation between anionic rhamnolipids and cationic MB/CTAB confirmed the presence of potential biosurfactant producer bacteria.

Growth kinetics in liquid culture medium. Growth kinetics and BS production were described for those strains with potential biosurfactant producer bacteria. A pre-inoculum in PY medium was incubated at 30°C for 24 h. For biomass collection, samples were centrifuged at $10.000 \times g$ during 20 min at 4°C. Growth kinetic was conducted in Erlenmeyer flask (125 ml) containing 80 ml aliquots of mineral salt media. For each strain, the mineral media was supplemented with 20 g/l carbon source, from its respective agro-industrial residue, and the flasks were inoculated with a 1% (v/v) inoculum. Kinetics were monitored for 72 h. At regular intervals, samples were submitted to analysis of biomass by dry weight and to BS production by surface tension.

Surface tension measurement. BS production was quantified through the determination of surface tension. Culture samples were centrifuged at $15.000 \times g$ for 20 min at 4°C to remove the cells and the supernatant was submitted to surface tension measurements. Surface tension was carried out by the Whilhelmy plate method using an Easy Dine KRÜSS K20 Tensiometer. This method consists in determining the force exerted on the liquid surface by a platinum plate until it penetrates a certain depth from the liquid surface and breaks the interfacial surface tension. For the measurements, distilled water was used as a control with a surface tension of 66–67 mN/m.

Emulsification Index (EI₂₄%). The emulsification index of culture samples was determined by adding 2 ml of cell-free supernatant to 2 ml of diesel in a test tube, mixing with a vortex for 1 min and leaving to stand for 24 h at environmental temperature. Emulsions formed by the isolates were compared to those formed by Sodium Dodecyl Sulfate (SDS) 5% (w/v) as control. The emulsification index was calculated by dividing the height of the emulsion layer (H_E) by the total height of the mixture (H_T) and multiplying by 100, as following: EI_{24} (%) = (H_E/H_T)*100. Finally, EI_{24} was carried out at the beginning of the test and 24 h later, to confirm its stability through time (Abbasi *et al.*, 2011).

All experimental measurements were performed in triplicate, data were averaged and was calculated standard deviation using Minitab[®] 17 software package.

Sequencing identification of 16S rRNA. 16S ribosomal RNA gene was amplified by using rD1 and fD1 oligonucleotides according to conditions described by Saitou and Nei (1987) and Weisburg *et al.* (1991). The amplification products were purified in gel using the purification kit GeneJET (Thermo Scientific). The purified products were sent for sequencing construction to the Sequencing Unit at the Biotechnology Institute of the National Autonomous University of Mexico (UNAM). Sequence of the 16S rRNA isolates were compared with 16S rRNA genes from the Gen-Bank database.

Results

Physicochemical characterization of agro-industrial residues. Agro-industrial residues were analysed to determine its composition. Protein, carbohydrate and fat content were quantified from lactoserum, coffee husk, palm oil cake and crude glycerol residues (Table I). Crude glycerol showed the highest carbohydrate content among the four residues.

Strains selection from preliminary detection of biosurfactant assays. Two hundred fifty bacterial strains from pinyon rhizosphere were isolated and further inoculated on CTAB agar using lactoserum, coffee husk, palm oil cake and crude glycerol residues. Blue methylene was used as indicator of BS production. Translucent halos were detected in six strains from lactoserum and crude glycerol residues, demonstrating the presence of BS. Strains were named based on the agro-industrial residue that allowed the evident halo presence. Strains showing halo in lactoserum were named LS1, LS8 and LS147; strains showing a halo in crude glycerol were named CG12, CG18 and CG101 (Table II).

Growth kinetics in liquid culture medium with CG and LS. Growth kinetics were carried out in liquid culture medium with lactoserum and crude glycerol using LS1, LS8, LS147 strains and CG12, CG18, CG101 strains respectively. As shown in Fig. 1, the exponential growth of biomass from CG18 strains was observed and the maximum biomass (2.1 g/l) was reached at 48 h of cultivation; while the maximum growth of biomass using lactoserum was reached at 48 h of cultivation with LS8 strain (1.34 g/l). All strains showed typical bacterial growth with both agro-industrial wastes.

Surface tension and emulsification index. BS production was quantified by surface tension measurements and emulsification index using diesel as hydrophobic

 Table I

 Physicochemical analysis of agro-industrial wastes

| Physicochemical | | | | |
|-----------------|------------------|-------------------|-----------------|------------------|
| parameter (%) | Lactoserum | Crude glycerol | Coffee waste | Oil palm cake |
| Proteins | 0.54 ± 0.06 | 0.93 ± 0.013 | 0.94 ± 0.06 | 2.59 ± 0.13 |
| Carbohydrates | 7.03 ± 0.024 | 31.76 ± 0.15 | 21.21 ± 0.00 | 12.84 ± 0.00 |
| Fats and oils | 4.23 ± 0.36 | 53.69 ± 0.136 | 2.7 ± 0.64 | 6.6±0.09 |

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| Agar CTAB and | Bacterial strains isolated from pinyon rhizosphere | | | | | | |
|-----------------------|--|-----|-------|------|------|-------|----|
| agro-industrial waste | LS1 | LS8 | LS147 | CG12 | CG18 | CG101 | PA |
| Lactoserum | + | + | + | _ | _ | _ | - |
| Crude glycerol | NC | NC | NC | + | + | + | + |
| Coffee husk | - | - | - | NC | NC | NC | NC |
| Oil palm cake | - | - | - | NC | NC | NC | NC |

Table II Test result of BS presence on agar CTAB and agro-industrial waste

PA = Pseudomonas a eruginosa ATCC 27853. NC = no growth. + = positive response and halo presence. - = negative response, growth without halo

phase (EI_{24} %). Cell-free supernatant from lactoserum and crude glycerol culture samples were analysed. Figures 2A and 2B showed the response surfaces determined at 72 h of the growth kinetic assays. The results obtained showed that BS produced by CG12, CG18 and CG101 strains reduced the surface tension to 28–30 mN/m, meanwhile LS1, LS8 and LS147 strains reduced the surface tension to 38–43 mN/m. On the other hand, emulsification activity was quantified by determining EI_{24} %. The biosurfactants produced by lactoserum strains showed emulsification index values of 44 to 69% whereas BS produced by crude glycerine strains showed EI_{24} values of 5 and 26%.

Sequencing of 16S rRNA gene. Sequencing of 16S rDNA showed that strain LS1 was closely related to *Enterobacter* sp. CIFRI D-TSB-9-ZMA, strain LS8 to *Enterobacter* sp., strain LS147 to *P. aeruginosa* SWD, strain CG12 to *B. pumilus* OCOB5, strain CG18 to *Rhizobium* sp. BGC8 and finally, strain CG101 was related to *Enterobacter* sp. NCCP-291 (table III).



Fig. 1. Growth of CG and LS strains during cultivation at 30°C in a culture medium with mineral salts, crude glycerol (up) and lactoserum (down) as carbon source



Fig. 2. Surface tension and emulsification index of BS from CG and LS strains, after 72 h incubation. ST = surface tension (up). $EI_{24} = emulsificaton index (down)$. PA ATCC 27853 = *P. aeruginosa* reference strain. SDS = Sodium Dodecyl Sulfate. DW = Distilled water

Discussion

It is estimated that the raw material accounts for 30–40% of the total production cost in most biotechnological processes (Liu *et al.*, 2011; Pereira *et al.*, 2013). In order to reduce cost for biosurfactants production at industrial scale, it is desirable to use low cost raw materials (Jain *et al.*, 2013a; 2013b). In this paper, crude glycerol and lactoserum wastes, due to their content in fat and carbohydrates, were used as carbon source for biosurfactant production by bacteria. According to Gudiña *et al.* (2015), the high nutritional content

Table III Molecular characterization by 16S rDNA gene and the percentage of identity of the bacterial strains isolated from pinyon rhizosphere

| Strain code | Name of specie | % Identity |
|-------------|------------------------------------|------------|
| LS1 | Enterobacter sp. CIFRI D-TSB-9-ZMA | 95 |
| LS8 | Enterobacter sp. | 95 |
| LS147 | P. aeruginosa SWD | 96 |
| CG12 | B. pumilus OCOB5 | 97 |
| CG18 | Rhizobium sp. BGC8 | 98 |
| CG101 | Enterobacter sp. NCCP-291 | 93 |

of both substrates, along with their low price, make them useful to be used as culture medium or nutrient supplements for microorganisms in diverse industrial fermentation processes.

In contrast, the low content in fat and carbohydrate in coffee husk and palm oil cake wastes, do not suggest that they can be used as raw material for BS production (Makkar and Cameotra, 2002). There are few reports on utilizing these wastes to BS production by microorganisms (Jain *et al.*, 2013a; 2013b; Rocha e Silva *et al.*, 2014).

In the CTAB agar test for tensoactive agents, growth and presence of halo around bacterial colonies of LS1, LS8, LS147, CG12, CG18 and CG101 strains was observed. However, on CTAB agar with coffee husk and palm oil cake wastes, LS strains grew but there was no halo. Same behaviour was observed with CG strains on lactoserum, there was no evidence of a halo. This suggests that thecarbon source from both wastes, employed for synthesis of biomass.

CTAB agar, is a selective culture medium that confirms glycolipids presence. This medium has been used for anionic biosurfactants detection like rhamnolipids produced by *Pseudomonas* sp. (Siegmund and Wagner, 1991; Soberón-Chavez *et al.*, 2005; Smyth *et al.*, 2010). In the CTAB agar assay, the strain *P. aeruginosa* ATCC 27853 was employed as positive control, so probably the BS produced by CG and LS strains are glycolipidic nature.

The LS strains were not capable to grow on crude glycerol. Probably impurities from crude glycerol produced an inhibitory growth effect. According to da Silva *et al.* (2009), Fonseca *et al.* (2009; 2011), glycerol is a biodegradable molecule by aerobic or anaerobic pathway, however because its alkalinity and impurities, it is necessary to neutralize and filter it, in order that microorganisms can use it as carbon source. Liu *et al.* (2011) and Rywinska *et al.* (2013) have reported alkali 12–16%, methyl esther 15–18%, methanol 8–12% and water 2–3% as glycerin impurities.

During the kinetics performed in liquid medium with crude glycerol and lactoserum, the strains CG and LS had a typical microbial behaviour growth. In stationary growth phase (50 h), the quantity of biomass, in dry weight, for CG12, CG18 and CG101 strains was 1.51, 1.54, 1.06 g/l respectively, whereas LS1, LS8 and LS147 strains produced 1.31, 1.34 y 0.84 g/l respectively. These results indicate that the bacterial strains isolated from pinyon rhizosphere are capable to grow on crude glycerol and lactoserum, as the only carbon source.

In parallel with kinetic growth, the surface tension (ST) from cell-free supernatants was quantified in order to evidence BS production. The CG strains reduced ST between 28–30 mN/m and LS strains between 38–42 mN/m. Gudiña *et al.* (2015) and Al-Bahry *et al.* (2013) also used ST to show BS production in a liquid medium with molasses as carbon source, in which *B. subtilis* and *P. aeruginosa* were grown. The ST reported by both authors was 23 and 30 mN/m respectively.

The emulsification indexes of cell-free supernatants showed that BS produced by the bacterial strains isolated from pinyon rhizosphere, have emulsifier properties stable by 24 hours (strains CG 5–26% and strains LS 44 a 69%). The ability to form stable emulsions is an important feature to be considered for the application of biosurfactants, mainly for environmental applications such as bioremediation and enhanced oil recovery (Gudiña *et al.*, 2015).

BS produced by CG and LS strains cultured with crude glycerol and lactoserum had a similar behaviour in ST and EI_{24} %, to positive controls: BS from *P. aeru-ginosa* ATCC 27853 (25 mN/m, 38 EI_{24} %) also synthetic surfactant SDS 5% (29 mN/m, 56 EI_{24} %). Distilled water was used as negative control (69 mN/m, 1.2 EI_{24} %). Thus, a cell-free broth-containing biosurfactant can be directly used without purification steps, which would further reduce the biosurfactant production cost (Déziel *et al.*, 1999). Biosurfactants produced by CG and LS strains, can be applied in enhanced oil recovery. Similar results were obtained by Nalini and Parthasarathi (2014).

In this study, two hundred and fifty strains were isolated from the pinyon rhizosphere of plants collected in Chiapas. Six of them demonstrated potential biosurfactant production. It was evident that crude glycerol and lactoserum are wastes that can be used as raw material for biosurfactant production. These BS can reduce the surface tension and emulsify diesel. The strains CG12 and CG18 identified as B. pumilus OCOB5 and Rhizobium sp. BGC8 are not yet reported as biosurfactants producers. The results are of great interest for biotechnology applications in bioremediation of heavy metals, hydrocarbons and pesticides (Sastoque-Cala et al., 2010). They suggested that strains that showed BS are also capable to solubilize phosphates, grow in insoluble carbon sources such as benzene and phenol and, at the same time, desorb heavy metals in soil.

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ORIGINAL PAPER

Levels of Organic Compounds, Number of Microorganisms and Cadmium Accumulation in *Festuca ovina* Hydroponic Culture

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Abstract

Understanding the microbiological, biochemical and physiological aspects of phytoremediation of soil and water environments polluted to different degrees with heavy metals has very important theoretical and practical implications. In this study, a comparison was made between total cadmium concentration in root and shoot tissues as well as concentrations of particular fractions of Cd immobilized by roots of *Festuca ovina* (Sheep's fescue) hydroponically cultivated in nutrient solutions supplemented with 1 μ g Cd ml⁻¹ and those cultivated at 10 μ g Cd ml⁻¹. After three weeks of *E ovina* cultivation, the number of bacterial CFU and the amounts of organic chelators, siderophores, proteins and reducing sugars in the growth medium and on the root surface were higher at 10 than at 1 μ g Cd ml⁻¹. The grass also reacted to the high Cd concentration by a decrease in plant growth and dehydrogenase activity in root tissues. The concentration of Cd determined in fractions bound with different strength in roots was significantly dependent on Cd concentration in the growth medium. When the plants were grown at 1 μ g Cd ml⁻¹, 9% of the immobilized cadmium was loosely bound to the root surface, 20% was exchangeable adsorbed, and 28% was bound by chelation; at 10 μ g Cd ml⁻¹, the respective values were 12%, 25%, and 20%. About 43% of the immobilized cadmium remained in roots after sequential extraction, and bioaccumulation factors in shoots had the same values independently of Cd concentration. At both Cd concentrations, the cadmium translocation index for *E ovina* was low (< 1), which is why this grass can be recommended for phytostabilization of the metal under study.

Key words: hydroponic culture under different Cd concentrations, metal chelators, phytoremediation, sequential extraction of Cd

Introduction

Cadmium is not essential for plant, animal and human metabolism (Van der Perk, 2006). The only known biological function of Cd has been described in marine diatoms under zinc limitation. In those conditions, a low concentration of Cd acted as a cofactor in Cd-carbonic anhydrase (Xu and Morel, 2013). Cadmium tends to be less strongly adsorbed than other divalent metals and, therefore, it is more labile in soil and more available to plants (An et al., 2011; Kacálková et al., 2014). Areas located in industrial regions can contain high concentrations of heavy metals (HM). For example, in southern Poland, there are regions with HM-polluted sites (e.g. Bolesław near Olkusz, Upper Silesia) where the concentrations of Zn, Pb and Cd exceed 40,000 mg kg⁻¹, 5,000 mg kg⁻¹ and 300 mg kg⁻¹, respectively (Majewska et al., 2011). In such cases, it is very important to prevent the migration of these metals to surrounding uncontaminated soils. Apart from physico-chemical stabilization (compost application, liming etc.), phytostabilization can be taken into account as a method of remediation of HM-contaminated soils (Alvarenga et al., 2008). This method is an aesthetically pleasing strategy that uses the ability of metal-tolerant plants to grow in and cover contaminated ground. Phytostabilization consists in sequestration of metals within roots and the rhizosphere without translocating them into the above-ground plant tissues (Mendez and Maier, 2008; Ganesan, 2012; Zhang et al., 2012). Plants are colonized by endophytic and rhizospheric microorganisms which increase the resistance of their hosts to environmental stresses (Jha et al., 2013). These microorganisms can play an important role in phytoremediation of soils polluted with heavy metals (Soleimani et al., 2010) and many other, especially organic, contaminants (Gerhardt et al., 2009).

Organic components of root exudates are a good source of nutrients and energy for microorganisms, hence, a microbial community living in the rhizosphere can be 10–100 times larger than the same type of community found in bulk soil (Huang and Germina, 2002).

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Microbial immobilization of a HM from rhizosphere soil solution can involve binding of the metal by cell envelopes, its intracellular accumulation, formation of insoluble complexes with extracellular biopolymers or precipitation with inorganic anions, such as sulfides and phosphates (Kurek and Majewska, 2012). On the other hand, microorganisms can accelerate the decomposition of organic matter (e.g. dead roots) and, consequently, mobilize HM in the rhizosphere. Dissolved organic ligands (e.g. carboxylates and phenolics) exuded by plant roots and those produced by microorganisms are also responsible for HM mobilization. Soluble ligands which have a higher affinity for HM than roots and other solids, and those ligands which acidify the root zone are important factors in increasing HM desorption (Haoliang et al., 2007; Kim et al., 2010; Kurek and Majewska, 2012). These processes can increase HM concentrations in soil solution and decrease the capacity of phytostabilization.

The aim of the present study was to compare speciation of Cd (water-soluble, exchangeable absorbed and chelate-bound fractions) immobilized by roots of *Festuca ovina* grown at 1 or 10 μ g Cd ml⁻¹ of the growth medium. Also, the numbers of microorganisms inhabiting the growth medium and the root surface, and the concentrations of organic compounds (*i.e.* phenols, proteins, citric acid, reducing sugars, total Fe(III)chelators and siderophores) released by roots and/or root-associated microorganisms after three weeks of plant cultivation in both media were compared.

Experimental

Materials and Methods

Plant growth conditions. Half a gram of non-sterilized grass seeds (about 625) were transferred to sterile plastic sieves with a surface area of 170 cm². The sieves were placed in sterile plastic containers (2,000 ml) holding 600 ml of autoclave-sterilized (non-aerated) Hoagland medium (Atlas, 1995; Sipos *et al.*, 2013) supplemented with 1 or 10 μ g Cd ml⁻¹ (as CdCl₂ · 2.5H₂O). Cadmium-free Hoagland medium was used as a control. All glassware and plastic containers were soaked in 7.5M HNO₃ for at least 2 h, rinsed thoroughly with deionized water and sterilized before use.

The plants were grown in a growth chamber (Biogenet, FD147 Inox) at 24/18°C (day/night), with a 12/12 h (day/night) photoperiod, at a photosynthetic photon flux density of 250 µmol m⁻² s⁻¹ for 3 weeks. The volume of the growth medium was kept constant by replenishing the volume evaporated during plant growth with sterile Hoagland medium without added Cd (Majewska and Kurek, 2011). After 3 weeks, 30 randomly-selected plants were removed from the medium and separated into roots and shoots. The roots were washed with sterile deionized water. Shoot height and root length as well as fresh and dry weight of the plants were determined. The dry weight of the plants was estimated after heating the plant material at 105°C until a constant weight was obtained.

Estimation of cadmium concentration in growth medium and in plants. The growth medium was collected and centrifuged $(9,000 \times g \text{ for } 15 \text{ minutes})$, and its pH was measured using a Beckman pH-meter. The concentration of cadmium ions was determined using a cadmium ion selective electrode from SENTEK with a detection limit of 10⁻⁷ M. Next, 20 ml of the medium was evaporated to dryness and digested with concentrated HNO₂. In order to determine the concentration of the metal in shoots and roots, 30 mg of dry plant material was digested with concentrated HNO₂. Next, the acid was evaporated to dryness, and then the residue was dissolved in 10 ml of 1M HNO, (Majewska et al., 2006). Cadmium concentrations were determined by mixing 1 ml of mineralizate, 2 ml of 3 M NaOH, 2 ml of an aqueous solution of Triton X-100 (5%) containing 0.3 mM dithizone, and 5 ml deionized water. The absorbance of the samples was determined at $\lambda = 549$ nm using a Varian UV-visible spectrophotometer (Fiedler et al., 2004; Majewska and Kurek, 2011). The bioaccumulation factor (BAF) was calculated as Cd concentration in roots or shoots (mg kg⁻¹) divided by the initial concentration of Cd in the medium $(mg l^{-1})$, and the translocation index (TRL) was described as mg Cd kg⁻¹ of dry weight of shoots divided by mg Cd kg⁻¹ of dry weight of roots (Mendez and Maier, 2008; Cheraghi et al., 2011).

Determination of dehydrogenase activity in root tissues. Metabolic activity in root tissues was assessed by the tetrazolium chloride (TTC) reduction method according to the procedure described by Comas et al. (2000). In the tissues, TTC was reduced to formazan (a bright-red pigment) by dehydrogenase enzymes. Roots (50 mg of fresh weight) were submerged in 3 ml of 0.6% TTC in 0.05 M Na₂HPO₄-KH₂PO₄ buffer (pH 7.4) containing 0.05% Triton X-100. The samples were incubated at 28°C for 24 h, rinsed twice with distilled water, and extracted with 10 ml of 95% ethanol for 10 minutes in a water bath at 85°C. The total volume of the extract was made up to 25 ml, and the absorbance was measured at $\lambda = 490$ nm (Varian, UV-visible spectrophotometer). Control roots were boiled for 15 minutes in distilled water to denature the enzyme. Dehydrogenase activity in roots was expressed as µg of formazan formed by 1 mg of root dry weight.

Sequential extraction of Cd immobilized in roots. After three weeks of plant growth, speciation of the metal immobilized by roots was analyzed using a threestep sequential extraction procedure. This method makes it possible to fractionate metals into fractions operationally defined as the water-soluble fraction (extracted with deionized water), the exchangeable absorbed fraction (extracted with 0.1 M NaNO₂) and the chelate-bound fraction (extracted with 0.02 M EDTA). In addition, a residual fraction of cadmium, not removed during sequential extraction, was determined (Majewska and Kurek, 2011). Roots (500 mg fresh weight) were suspended in 100 ml of each extractant and shaken (250 rotations per minute) at room temperature for 30 minutes. The extracts were evaporated to dryness, and Cd concentration was determined in the residues as described above. After sequential extraction, the roots were collected by filtration, dried and digested with HNO₂, and the amount of Cd in root tissues was determined. Total cadmium concentrations as well as Cd ions were determined in all of the tested fractions as described above.

Determination of microorganisms CFU number. After three weeks of plant growth, the numbers of microorganisms in the growth medium, those released from the root surface during sequential extraction and those inhabiting interior root tissues were estimated by the plate count method. Fungi were cultivated on Martin medium (Martin, 1950) and bacteria on nutrient agar (Atlas, 1995) for 7 days at 28°C. The number of microorganisms was expressed as log₁₀ colony forming units (CFU) per milligram of dry weight root mass.

Determination of organic compound concentrations. Dissolved organic compounds, such as reducing sugars, proteins, phenols, citric acid, total Fe(III)chelators and siderophores were quantitated in water extracts and 0.1 M NaNO₂ extracts from roots, and in the growth medium. Before determination of organic compounds, the plant growth medium was centrifuged $(9,000 \times g \text{ for } 15 \text{ minutes})$. The amounts of reducing sugars were measured according to the method of Nelson and Somogyi (Alef and Nannipieri, 1995). Bradford reagent was used to determine protein concentrations, with albumin as a standard (Bradford, 1976). Total soluble phenolic compounds were measured by reaction with the Folin-Ciocalteau reagent, and calibration was performed using ferulic acid as a standard (de Ascencao and Dubery, 2003). Citric acid was determined using a commercial kit (R-BIOPHARM Enzymatic Bioanalysis, Cat. No 10 139 076 035). Reactions with FeCl₂ were carried out to estimate the total amount of organic Fe(III)-chelators. In this method, desferrioxamine B was used to construct the calibration curve (Atkin et al., 1970). Catechol and hydroxamate siderophores were determined by the Arnow method with 3,4-dihydroxybenzoic acid as a standard (Arnow, 1937) and the Csaky method with hydroxylamine hydrochloride (Csaky, 1948), respectively.

Data analysis. Three experimental settings were used for each treatment (0, 1 and $10 \,\mu\text{g}$ Cd ml⁻¹ of growth medium), and the experiment was performed three times. One-way analysis of variance was carried out to compare the mean values. When significant *p*-values were obtained (*p* < 0.05), differences between the individual means were compared using Student's t-test (Brandt, 1999). All data analyses were performed using Microsoft*Excel 2010.

Results

Plant growth and dehydrogenase activity under different Cd concentrations. Symptoms of cadmium toxicity to grass seedlings were developed 3 weeks after the treatments, especially in the presence of 10 µg Cd ml⁻¹ in the growth medium. Yellow lesions on shoots and dark-brown root tips were found. Cd concentration in the growth medium did not affect the number of plants surviving at the end of the experiment (Table I). Plant growth, expressed as shoot height and shoot fresh weight, was significantly inhibited when the plants were exposed to 1 µg Cd ml⁻¹. Cd had a stronger effect on shoots than on roots (Table I). No significant reduction in fresh and dry weights and length of roots in the presence of 1 µg Cd ml⁻¹ was observed. Inhibition of plant growth by 10 µg Cd ml⁻¹ of the medium was much more severe than that caused by 1 µg Cd ml⁻¹. The activity of dehydrogenase enzymes in root tissues declined with the increase in Cd concentration in the growth medium (Table I). The highest decrease in the activity of these enzymes was observed in roots of plants cultivated in the presence of $10 \,\mu g \,\text{Cd ml}^{-1}$.

Cd concentrations in shoots and roots. The concentration of cadmium immobilized by the plant biomass increased with the increase in Cd concentration in the growth medium, and was always higher in roots than in shoots. Cd concentration in roots of plants grown at 1 µg Cd ml⁻¹ was approximately three times lower than in roots of plants grown in the medium containing 10 µg Cd ml⁻¹ whereas for shoots this difference was almost 10-fold (Table I). The value of the bioaccumulation factor (BAF) for roots of plants cultivated in the medium containing 10 µg Cd ml⁻¹ was significantly lower than for those grown at 1 µg Cd ml⁻¹. On the other hand, there was no significant difference between the values of BAF for shoots from the two treatments (Table I). Cadmium concentration in the growth medium significantly affected the values of TRL. For plants cultivated in the growth medium at 10 µg Cd ml-1, TRL was 3.5 times higher than for plants cultivated at 1 μg Cd ml $^{-1}$ (Table I).

Concentrations of Cd in the growth medium and in the individual fractions of the root extraction. At

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Table I

Effect of Cd on *F. ovina* growth parameters, dehydrogenase activity and Cd accumulation in its roots and shoots. Mean values with marked the same letter within the same row do not differ significantly (p > 0.05). Values are expressed as mean ± S.D. (n = 9)

| Parameters | | Cadmium concentration in the growth medium ($\mu g m l^{-1}$) | | | |
|--|------------------------|---|----------------------------|---------------------|--|
| | | 0 | 1 | 10 | |
| Number of plants | | 509±27 a | 536±41 a | 505±28 a | |
| Shoot height (mm) | | 95±9 a | 63 ± 19 b | 49±14 b | |
| Root length (mm) | | 91±30 a | 65±31 a | 16±7 b | |
| FW (mg) Biomass of 100 plants | Shoots | 1046±106 a (100%) | 700 ± 76 b (67%) | 297 ± 48 c (28%) | |
| | Roots | 501±68 a (100%) | 602±81 a (120%) | 53±14b (11%) | |
| DW (mg) Biomass of 100 plants | Shoots | 148±16 a (100%) | 128±9a (86%) | 79±1 c (53%) | |
| | Roots | 57±11 a (100%) | 58±20 a (102%) | 24±6b (42%) | |
| Activity of root dehydrogenase (µg formaza | n mg ⁻¹ DW) | 2.78±0.74 a | 1.58±0.96 a | 0.52 ± 0.16 b | |
| Cd concentration (µg Cd mg ⁻¹ DW) | Shoots | 0 | $0.07\pm0.01~b$ | 0.76±0.17 a | |
| | Roots | 0 | $1.74 \pm 0.18 \mathrm{b}$ | 5.39±0.30 a | |
| BAF | Shoots | - | 71 ± 12 a | 76±17 a | |
| | Roots | - | 1742 ± 188 a | 539±35 b | |
| TRL | | _ | $0.04 \pm 0.01 \mathrm{b}$ | 0.14 ± 0.04 a | |

- not determined, FW - fresh weight, DW - dry weight, BAF - bioaccumulation factor, TRL - translocation index

Table II

Distribution of Cd between plant biomass (Cd bioaccumulation), sediment (Cd precipitation) and growth medium (soluble complexes and ionic form of Cd) after 3-week cultivation of *F. ovina* in the hydroponic system. Values are expressed as mean \pm S.D. (n=9)

| Parameters | | Cadmium concentration in growth medium $(\mu g \ ml^{-1})$ | | |
|--|------------------|--|----------------|--|
| | | 1 | 10 | |
| Initial amount of Cd in the hydropo | onic system (µg) | 527±52 (100%) | 5917±42 (100%) | |
| Amount of Cd accumulated by plants | | 357±41 (64%) | 841±67 (16%) | |
| Amount of Cd in sediment (insoluble forms of Cd) | | 35±2 (6%) | 661±112 (13%) | |
| Amount of Cd in growth medium | Ionic form | 17±3 (3%) | 3550±148 (60%) | |
| | Non-ionic form | 147±3 (27%) | 687±48 (11%) | |

1 μ g Cd ml⁻¹, plants accumulated 64% of Cd present in the growth medium, 6% was converted to insoluble forms, which were separated by centrifugation, and 30% of Cd remained in the growth medium. The metal in the medium was either in the ionic form or bound to soluble compounds (Table II). When the concentration of Cd was increased to 10 μ g Cd ml⁻¹, 16% of the metal was accumulated in the plant biomass, 13% was stored as insoluble forms and 71% was still found in the medium, primarily in the ionic form (Table II).

A three-step sequential extraction of the Cd immobilized by grass roots was applied to evaluate the participation of the dissolution (fraction 1, Cd extracted with deionized water), ionic exchange (fraction 2, Cd extracted with 0.1 M NaNO₃) and chelation (fraction 3, Cd extracted with 0.02 M EDTA) processes, as well as intracellular accumulation (fraction 4, residual Cd) in cadmium immobilization by roots of sheep's fescue plants. When Cd concentration in the medium was 1 µg ml⁻¹, 9% was extracted with deionized water, 20% was extracted with 0.1 M NaNO₃ and 28% with 0.02 M EDTA (Fig. 1B). The increase in metal concentration in the medium from 1 to 10 µg Cd ml⁻¹ resulted in an elevated concentration of Cd in all the fractions tested (Fig. 1A). The percentage distribution of Cd between the fractions was also changed: it increased in the watersoluble (12%) and ionic exchange (25%) fractions and decreased in the fraction extracted with 0.02 M EDTA



Fig. 1. Sequential extraction of Cd immobilized by roots of *F. ovina* after 3-week cultivation in Hoagland's growth medium supplemented with 1 or $10 \,\mu$ g Cd ml⁻¹.

A – concentration of Cd in the individual fractions (μ g Cd per 1 ml of extract); B – percentage distribution of Cd between the fractions tested. Bars marked with the same letter are not significantly different (p >0.05). Standard deviations are shown as deviation bars (n=9)

(20%) (Fig. 1B). At both Cd concentrations in the growth medium, the roots of the grass accumulated an average of 43% of the immobilized metal in their tissues.

Cadmium extracted from roots was released as metal bound to soluble organic compounds and as free ions. When the metal was extracted with 0.02 M EDTA, the whole pool of cadmium removed was bound to EDTA in both treatments. At 1 μ g Cd ml⁻¹, 12.3% of the metal extracted from roots with 0.1 M NaNO₃ was in the ionic form, whereas at 10 μ g Cd ml⁻¹, as much as 48.5% of Cd was present in the extracts in this form. No cadmium ions were found in water extracts from roots of plants grown at 1 μ g Cd ml⁻¹, but at 10 μ g Cd ml⁻¹, about 7.1% Cd was in the ionic form (data not presented).

Concentrations of organic compounds. Sheep's fescue grass reacted to the presence of cadmium in the growth medium with an increased release of organic

compounds from roots into the growth medium. After plant cultivation, the growth medium supplemented with 10 µg Cd ml-1, was enriched 3-fold with total chelators and citric acid, 4-fold with catechol siderophores and 5-fold with hydroxamate siderophores, proteins and reducing sugars compared to the control medium without added metal. At 1 µg Cd ml⁻¹, the growth medium was significantly enriched with catechol siderophores, proteins and reducing sugars only (Table III). Also, the amounts of total chelators, siderophores, proteins and reducing sugars extracted with deionized water and 0.1 M NaNO, from root surfaces were increased, but the amount of citric acid dropped with the increase in Cd concentration in the growth medium (Fig. 2). The solution of NaNO, mobilized more organic compounds from roots than did deionized water, except for total chelators and citric acid, which were efficiently released

| Organic compounds in the | growth medium after 5-week cultivation of <i>F. ovinu</i> |
|---|--|
| in Hoagland medium. The am | nounts of the individual compounds are expressed as |
| μg mg ⁻¹ dry weight of roots. Me | an values marked with the same letter within the same |
| row do not differ significantly | $(p > 0.05)$. Values are expressed as mean \pm S.D. $(n = 9)$ |

Table III

| Organic compounds | Cd in the growth medium (μg Cd ml ⁻¹) | | | | |
|--------------------------|---|-------------------|-------------------|--|--|
| Organic compounds | 0 | 1 | 10 | | |
| Total chelators | 3.43±0.41 b | 4.24±0.31 b | 11.54±1.74 a | | |
| Hydroxamate siderophores | 2.14±1.53 b | 2.65±0.46 b | 10.01 ± 4.06 a | | |
| Reducing sugars | 2.31±0.56 b | 7.13±1.67 a | 11.84±4.08 a | | |
| Phenols | 0.25±0.01 a | 0.98±0.16 a | 0 | | |
| Citric acids | 0.90±0.36 b | 1.00±0.18 b | 2.62 ± 0.47 a | | |
| Proteins | 0.72±0.53 b | 3.48±0.62 a | 3.81±2.32 a | | |
| Catechol siderophores | 1.50±0.48 c | 2.88 ± 0.18 b | 5.58±0.40 a | | |



Fig. 2. Two-step sequential extraction of organic compounds (OC) from roots of *F. ovina* after 3-week cultivation in Hoagland's growth medium supplemented with 1 or 10 μ g Cd ml⁻¹. The amounts of the individual compounds are expressed as μ g OC mg⁻¹ dry weight of roots. Bars marked with the same letter are

not significantly different (p > 0.05). Standard deviations are shown as deviation bars (n = 9)

to the water fraction. There were no differences between the treatments in the amounts of phenols extracted from roots.

Microorganisms colonizing grass roots and the growth medium. After three-week cultivation of plants, a microbiological analysis of the plant growth media and the extracts obtained during sequential extraction of plant roots was performed. Regardless of Cd concentration in the growth medium, bacteria were the only microorganisms isolated. The number of bacteria in the growth medium without Cd added was significantly lower than in the medium supplemented with 1 or 10 μ g Cd ml⁻¹ (Table IV). After sequential extraction of plant roots cultivated in Cd-contaminated media, the number of bacteria in the water and 0.1 M NaNO₃extracts was significantly higher than in extracts from control roots (without added Cd). The concentration of Cd was without effect on the number of bacteria

| Table | IV |
|-------|----|
|-------|----|

The number of bacteria in the growth medium and four sequential extraction fractions obtained from *F. ovina* roots (\log_{10} CFU mg⁻¹ DW of roots). Mean values marked with the same letter within a column do not differ significantly (*p* > 0.05). Values are expressed as mean \pm S.D. (*n* = 9)

| Cd concentration | Bacteria | Bacteria released from roots during sequential extraction | | | | |
|---|-----------------------|---|--|-----------------------------------|------------------------------------|--|
| in Hoagland medium (μg Cd ml ⁻¹) | in Hoagland medium | Fraction 1 extracted with H ₂ O | Fraction 2 extracted with NaNO ₃ | Fraction 3 extracted with EDTA | Fraction 4 inhabiting root tissues | |
| 0 | 5.60±0.12 b | 5.95 ± 0.27 b | $4.92\pm0.06~b$ | 5.13±0.32 a | 5.62±0.05 a | |
| 1 | 6.02±0.01 a | 6.72±0.21 a | 5.89±0.06 a | 4.96±0.11 a | 5.23±0.16 a | |
| 10 | 6.69±0.37 a | 6.96±0.28 a | 5.96±0.03 a | 5.28±0.10 a | 3.09±0.21 b | |

released during extraction of roots with 0.02 M EDTA. The number of bacteria isolated from root tissues after sequential extraction was significantly decreased when Cd concentration in the plant growth medium was increased (Table IV). The water fraction was the richest in bacteria. At the concentration of 10 μ g Cd ml⁻¹, the number of bacteria on the root surface (extracted with water) was 10,000 higher than the number of bacteria isolated from root tissues. Release of microbial and plant metabolites into the growth medium during cultivation of the plants resulted in a decrease in pH values from 6.6 ± 0.1 to 5.8 ± 0.3 regardless of the concentration of Cd added.

Discussion

The root surface is a site of very important processes involved in plant growth and of interactions between organic compounds and microorganisms. These interactions affect biogeochemical cycling, enhancing plant growth, and increase plant tolerance to biotic and abiotic stress (Kamaludeen and Ramasamy, 2008; Philippot et al., 2013). In the present study, the grass F. ovina (var. Bornito - a commercially available cultivar) was used because of its resistance to drought and nutrient deficiency and because it does not require special soil conditions (as noted on packet). This choice was also influenced by observations of Grodzińska and Szarek-Łukaszewska (2009), who had found that F. ovina was one of the dominant plant species on postindustrial waste heaps containing zinc, lead, cadmium, thallium and other metals.

Cadmium tends to be more labile in soil and more available to plants than other divalent metals (Kacálková *et al.*, 2014). An *et al.* (2011) compared the accumulation of Cd, Pb, Cu, Cr and Fe in different plant species (tomato, maize, greengrocery, cabbage and Japanese clover herb). Among the metals tested, cadmium was easily absorbed and had the highest enrichment coefficient values for most plant tissues. In the present study, the values of the Cd bioaccumulation factor (BAF) calculated for roots of *F. ovina* growing at 1 or

10 µg Cd ml⁻¹ of hydroponic solution were very high (1742 and 539, respectively) because metal retention by soil solid components was eliminated. Cadmium concentration was always higher in roots of F. ovina than in its shoots and increased with the elevated Cd concentration in the growth medium. A comparison of cadmium accumulation in roots and shoots of other grasses, such as Lolium perenne (Lou et al., 2013), Elymus elongatus (Sipos et al., 2013) and Secale cereale (Majewska and Kurek, 2011), also shows that roots tend to contain more of this metal than shoots. Mendez and Maier (2008), Cheraghi et al. (2011) and Zhang et al. (2012) have recommended plants (monocotyledons and dicotyledons) with a high BAF (>1) and a low translocation index (TRL < 1) as the best candidates for phytostabilization. The values of TRL for F. ovina growing at 1 µg ml⁻¹ as well as at 10 µg ml⁻¹ were much lower than 1 (0.04 and 0.14, respectively). The same situation was observed in *F. ovina* growing at 5, 10, 50 and 100 µg Cd g⁻¹ dry weight of soil (data unpublished).

The exposure of *F. ovina* to 10 µg Cd ml⁻¹ resulted in a significant decrease in shoot height, root length, fresh and dry mass of the plants, and vitality of plant roots, while at 1 µg ml⁻¹ these parameters were not significantly changed (Table I). This indicates that low concentrations of Cd may have no impact on plants. In the present study, the vitality of plant roots was determined by measuring dehydrogenase activity expressed as TTC reduction. According to Comas et al. (2000), there is a relationship between respiration and dehydrogenase activity, and respiration is a standard test of metabolic activity of cells. A low TTC reduction can be indicative of a generally low metabolic activity or necrosis of root tissues. TTC reduction can be evaluated in the stele but not in the cortex of old roots. Heavy metal toxicity can accelerate the senescence and death of roots and can cause a decline in root function. In these cases, roots can only adsorb metals on their surface without transporting them into root tissue cells and translocating them into shoots.

Another reaction of the grass's roots to Cd treatment was an increased release of organic compounds to the growth medium and their storage on the root sur-

face. Numerous authors have observed that Cd-stress induces changes in plant metabolism, with plants synthesizing concentrations of organic compounds that are significantly higher than those synthesized by plants growing in unpolluted environments (dell'Amico et al., 2005; Madhaiyan et al., 2007; Adeniji et al., 2010; Kim et al., 2010). Li et al. (2014) have reported that exudation of high amounts of dissolved organic carbon by roots of Triticum aestivum is an important protective mechanism under Cd stress. In our study, after cultivation of *F. ovina*, the growth medium contaminated with 10 µg Cd ml⁻¹ was significantly enriched with various organic chelators, siderophores, citric acid, reducing sugars and different proteins (Table III). By contrast, the amounts of phenols in the growth medium decreased to zero at this Cd dose. This drop could have been caused by accumulation of soluble phenolics in root cells and their use in the phenylpropanoid (phenyloalanine) pathway connected with induction of plant resistance to different biotic and abiotic stresses (Atkinson and Urwin, 2012; Khan et al., 2015; Zhang et al., 2015). One of the main results of the induction of this pathway is lignin biosynthesis, which leads to straightening of the plant cell wall by its lignification (Schützendüdel et al., 2001; Elobeid et al., 2012). Acceleration of cell wall lignification under Cd-stress can, on the one hand, negatively affect root elongation, but, on the other, increase Cd immobilization and detoxification, and ultimately enhance tolerance of the plants to this metal.

The exposure of sheep's fescue to $10 \ \mu g \ Cd \ ml^{-1}$ also significantly increased the amounts of organic compounds, other than citric acid, attached to the root surface (Fig. 2). A similar increase has been observed for *S. cereale* cultivated in the same conditions (Majewska and Kurek, 2011). The thick layer of organic material developing on the root surface acted as the first barrier against Cd entering into root tissues while organic compounds secreted into the growth medium played an important role in decreasing the concentration of free metal ions. After *F. ovina* cultivation, only part of the Cd introduced into the hydroponic solution remained in the medium in the ionic form (Table II).

Non-sterilized grass seeds were used in the experiments. The microbial community of these seeds inhabited the growth medium and the root surface of cultivated plants (Table IV). Proliferation of microorganisms was stimulated in the presence of organic compounds, which were efficiently released by roots of plants growing at $10 \,\mu\text{g}$ Cd ml⁻¹. The numbers of bacteria in the growth medium were significantly higher at $10 \,\mu\text{g}$ Cd ml⁻¹ than in the control medium without cadmium. Also, the numbers of bacteria attached to the root surface and removed during extraction were the highest at $10 \,\mu\text{g}$ Cd ml⁻¹. Fungi were found neither in the growth medium nor on the root surface in either

of the Cd treatments. The numbers of bacteria isolated from F. ovina roots after extraction declined with the increase in Cd concentration in the growth medium, and at 10 µg Cd ml-1 their titer was two orders of magnitude lower than at $1 \mu g$ Cd ml⁻¹ (Table IV). In the natural environment, plant roots are always associated with microorganisms, which utilize organic compounds released to the root zone and produce their own metabolites. Endophytes (strains inhabiting the internal tissues of plants), such as Serratia nematodiphila, Enterobacter aerogenes, Enterobacter sp. or Acinetobacter sp. (Chen et al., 2010), and rhizosphere bacteria, such as those belonging to the genera Agrobacterium, Arthrobacter, Mycobacterium, Pseudomonas (dell'Amico et al., 2005), Burkholderia and Methylobacterium (Madhaiyan et al., 2007), could protect plants from the toxic effect of metals (biosorption and precipitation), produce plant growth-promoting compounds (indole acetic acid, siderophores and ACC-deaminase) and improve Fe and P nutrition. In contaminated habitats, some sensitive microbes would be eliminated while others would develop resistance to heavy metals and proliferate. Kao et al. (2006), for example, have demonstrated that microorganisms adapted to high Zn, Cu and Pb concentrations in soil, and that their respiration, expressed as CO₂ production, strongly increased within 7 days after metal supplementation.

Cadmium applied at the concentrations of 1 and $10\,\mu g\ ml^{\mbox{--}1}$ of the growth medium was immobilized by *F. ovina* roots in different amounts (1.7 and 5.4 mg g^{-1} , respectively). Roots are composed of many different compounds which are responsible for immobilization of metals. Chen et al. (2013) have suggested that pectin and cellulose, but not hemicellulose, present in the cell walls of Salix jiangsuensis J172 were responsible for Cd adsorption. Next to cellulose, the most abundant natural raw material is lignin. Lignin is an aromatic polymer which contains several functional groups, e.g. methoxyl, aliphatic and phenolic hydroxyl, and carboxyl groups, that can act as very efficient binding agents in the immobilization of heavy metal ions (Šćiban et al., 2011). The results of the present study revealed that 43% of Cd immobilized by the roots of plants grown at both cadmium concentrations was retained in their tissues and not released during sequential extraction (Fig. 1). Yin et al. (2015), in a study of Boehmeria nivea, have recorded the highest concentration of Cd in the cytoderm fraction (precipitated Cd or Cd bound in cell walls), which was found to be the most stable fraction of the metal in plant tissues. This metal could be mobilized only after cell lysis and microbial mineralization of dead tissues. The remaining part of Cd immobilized by F. ovina roots was loosely bound (deionized water extractable metal), exchangeably bound (extracted with 0.1 M NaNO₃) and bound by chelation (Cd extracted

with 0.02 M EDTA). Cadmium mobilized by deionized water and 0.1 M NaNO₃ solution with an ionic strength equal to the strength of soil solution is considered to be the most labile. Cadmium extracted with EDTA is strongly bound and difficult to remove by soil solution (Kurek and Majewska, 2004). The increase in Cd concentration in the medium from 1 to $10 \,\mu g \,ml^{-1}$ resulted in elevated amounts of Cd extracted from roots with deionized water and 0.1 M NaNO₃, and decreased amounts of Cd extracted with 0.02 M EDTA (Fig. 1B), which means that the stability of Cd immobilized by roots was weakened.

Cadmium extracted from F. ovina roots was released as free ions and as metal bound to soluble organic compounds. Metal-organic complexes present in the root zone have different degrees of toxicity, bioavailability and degradation. It has been proven that the rate of mineralization of metal-organic complexes such as Cd-acetate and Cd-citrate was significantly lower than for complexes with Mg or Zn (Renella et al., 2004). In such cases, metal-organic complexes can be incorporated into the soil organic matter during humification and humus accumulation. In the natural environment, microbiological decomposition of plant residues is a source of substrates for the synthesis of macromolecular polymers that are combined into aggregates of humic substances (Semenov et al., 2013). If grass shoots with a low Cd concentration (~ $0.76 \,\mu g \,m g^{-1} \,DW$) return to the soil as plant residues, they could facilitate humification and efficiently increase metal stabilization in re-vegetated soils. Further studies are required, however, to fully understand the mechanisms of HM phytostabilization in contaminated soils.

Conclusion

In order to exclude Cd retention by soil solid components, hydroponic cultures were chosen for the experiments. The results of the present study show that Cd-stress decreased *F. ovina* biomass, shoot height, root length and root activity but increased the quantity of root exudates, especially at $10 \,\mu g \,ml^{-1}$ of the growth medium. At this Cd concentration, root exudates containing organic chelators, siderophores, proteins and reducing sugars significantly stimulated proliferation of bacteria introduced into the hydroponic solution with non-sterilized seeds. Fungi were not detected.

F. ovina immobilized Cd in roots (BAF=539), with a very low translocation of this metal to shoots (TRL=0.14). Therefore, this grass could be potentially used for phytostabilization of metals in contaminated soils. Cadmium immobilized by roots was released during sequential extraction as free ions or in a form

bound with organic compounds. These observations suggest that the dissolution, ion exchange and chelation processes occurring in the close vicinity of roots can release Cd immobilized in those roots. The root zone is a specific habitat created by root exudates and microorganisms, and mechanisms controlling Cd phytostabilization are difficult to determine precisely due to the multiple factors acting in this zone.

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ORIGINAL PAPER

Prevalence of Parasitic Contamination in Salad Vegetables Collected from Supermarkets and Street Vendors in Amman and Baqa'a – Jordan

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Abstract

One of the main ways in transmitting parasites to humans is through consuming contaminated raw vegetables. The aim of this study was to evaluate the prevalence of parasitological contamination (helminthes eggs, *Giardia* and *Entamoeba histolytica* cysts) of salad vegetables sold at supermarkets and street vendors in Amman and Baqa'a – Jordan. A total of 133 samples of salad vegetables were collected and examined for the prevalence of parasites. It was found that 29% of the samples were contaminated with different parasites. Of the 30 lettuce, 33 tomato, 42 parsley and 28 cucumber samples examined the prevalence of *Ascaris* spp. eggs was 43%, 15%, 21% and 4%; *Toxocara* spp. eggs was 30%, 0%, 0% and 4%; *Giardia* spp. cysts was 23%, 6%, 0% and 0%; *Taenia/Echinococcus* eggs was 20%, 0%, 5% and 0%; *Fasciola hepatica* eggs was 13%, 3%, 2% and 0%; and *E. histolytica* cysts was 10%, 6%, 0% and 0%, respectively. There was no significant difference in the prevalence of parasite in salad vegetables either between supermarkets and street vendors, or between Amman and Baqa'a, *Ascaris* spp. was found to be the highest prevalent parasite in salad vegetables from supermarkets and street vendors and from Amman and Baqa'a. Our results pointed out that, the parasitic contamination of salad vegetables found in our study might be caused by irrigating crops with faecal contaminated water. We concluded that salad vegetables sold in Amman and Baqa'a may cause a health risk to consumers.

Key words: Amman, Baqa'a refugee camp, contamination, parasite, salad vegetable

Introduction

Foodborne disease causes diarrhoea, diarrhoea was reported by the world health organization to be one of the five most common disease causes of death in the world (1.6 million deaths per year) (Baldursson and Karanis, 2011). Foodborne disease could be caused by viruses, bacteria and parasites, while transmission can occur by contaminated food such as contaminated vegetables. Vegetables can become contaminated during growth, harvesting, processing and distribution (Slifko *et al.*, 2000; Dawson, 2005). The sources of vegetable parasite contamination during growth are sewage sludge, untreated waste water, contaminated slurry, farm livestock, and indigenous wildlife (Slifko *et al.*, 2000; Lanata, 2003; Dawson, 2005).

Parasites (protozoa or helminthes) could be transmitted to humans by ingesting one of the parasite environmental transmission stages (protozoa: cysts and oocytes, helminthes: eggs, and larval) (Slifko *et al.*, 2000; WHO, 2003). Consuming improperly washed vegetables such as salad vegetables represent one of the major forms in transmitting parasites into humans, in which raw vegetables play a role as a vehicle in the parasite transportation. The prevalence of parasites in vegetables was reported in many developed and developing countries (da Silva *et al.*, 1995; Robertson and Gjerde, 2000; 2001; Al-Binali *et al.*, 2006; Daryani *et al.*, 2008; Uga *et al.*, 2009; Abougrain *et al.*, 2010; Al-Megrm, 2010; Iournals, 2014).

Jordan is a small Middle Eastern country with limited natural resources. Jordan water resources is one of the lowest in the world, the renewable fresh water resources is approximately 135 m³ per capita for all uses and 90% of the country receive annual precipitation less than 200 mm (Raddad, 2005; Al-Jaloudy, 2006). Jordan population was estimated at 6.3 million in 2012 as reported by the Jordanian department of statistics (DSJ, 2014). Vegetables are one of the major components of the daily diet in Jordan, from which vegetable salad (cucumber, tomato, lettuce and parsley) being one of the daily dishes. The main parasites detected in

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vegetables in the neighbouring countries were Ascaris spp., Taenia spp., Fasciola spp., Toxocara spp., Echinococcus spp., Giardia spp. and Entamoeba histolytica (Sharif, 2002; Al-Shawa and Mwafy, 2007; Hadi, 2011; Hassan et al., 2012; Adanir and Tasci, 2013; Ali and Ameen, 2013). To our knowledge, no published study to date has evaluated the parasitological contamination of salad vegetables sold at Jordanian markets. Therefore, the aim of this study was to evaluate the prevalence of parasitological contamination (helminthes eggs, Giardia and E. histolytica cysts) of salad vegetables at supermarkets and street vendors in Amman and Baqa'a – Jordan.

Experimental

Materials and Methods

The study area. Samples were collected from different areas in Amman and Baga'a. Amman is the capital city of Jordan, with a population of almost 2.5 million inhabitants (39% of Jordan population) (DSJ, 2014). Amman is situated in the mountain heights plateau of Jordan with an elevation ranging from 400 to 1000 m above sea level (Al Rawashdeh and Saleh, 2006), Amman has a mediterranean climate; moderate and dry in summer (average temperature 25°C) cold and wet in winter (average temperature 9°C) (JOME-TEO, 2015) with an annual rainfall of almost 500 mm (Dahamsheh and Aksoy, 2007).

Baqa'a is the largest Palestinian refugee camp in Jordan with more than 100.000 registered refugees. Baqa'a is situated almost 20 km north of Amman (UNRWA, 2015) with a similar climate to Amman. Baqa'a refugee camp has a higher poverty and a poorer environmental hygiene compared to Amman (UNRWA, 2015).

Sampling. A total of 133 samples of salad vegetables were collected from supermarkets and street vendors in Amman and Baqa'a as shown in Table I. Salad vegetables were collected randomly between March 2014 and August 2014.

| Table I | |
|--|--|
| Number of samples collected from each area | |

| Total | Number of samples collected from Amman | | Number of samples collected from Baqa'a | | Total |
|----------|--|-------------------|---|-------------------|-------|
| | Super- market | Street vendors | Super- market | Street vendors | |
| Lettuce | 8 | 8 | 7 | 7 | 30 |
| Tomato | 7 | 8 | 10 | 8 | 33 |
| Parsley | 10 | 9 | 11 | 12 | 42 |
| Cucumber | 7 | 7 | 7 | 7 | 28 |

Determination of intestinal parasites. Each sample was weighed (250 g) and placed into sterile plastic bags, samples were then washed with one litter of sterile normal saline solution (0.85% NaCl) by shacking for 20 minutes. The washing saline was then left to sediment overnight. The supernatant was discarded and the remaining washing saline (50 ml) was centrifuged at 2000 RCF for 15 minutes. Supernatant was then discarded and the remaining pellet with 5 ml of the saline was collected (Erdoğrul and Sener, 2005). Three simple and three iodine stained smears were then prepared from each sample, smears were used to detect helminthes eggs, Giardia and E. histolytica cysts using light microscope (Downes and Ito, 2001).

Statistical analysis. Fisher's exact test was used to assess the differences between proportions, significance was defined as P < 0.05. The analyses were made using GraphPad Prism 5 software (San Diego, CA).

Results

A total of 133 samples of salad vegetables (30 lettuces, 33 tomatoes, 42 parsley and 28 cucumbers; Table I) were examined for the presence of parasites (helminthes eggs, Giardia and E. histolytica cysts). It was found that 29% (39 out of 133) of the salad vegetable samples were contaminated with different parasites (helminthes eggs, Giardia and E. histolytica cysts). The highest percentage of contamination was detected in lettuce samples (63%), while tomato and parsley samples showed lower contamination percentage (27% and 24%, respectively). The least percentage of contamination was detected in cucumber (13%). Lettuce samples were contaminated significantly more often than tomato, parsley and cucumber with parasites (P = 0.006, Odds ratio = 4.6; P = 0.001, Odds ratio = 5.5)and P < 0.0001, Odds ratio = 46.6, respectively), further details are shown in Table II.

Twenty seven percent (18 out of 67) of salad vegetable samples sold at supermarkets and 32% (21 out of 66) of salad vegetable samples sold at street vendors were contaminated with different parasites (Table II), no significant difference was found between the prevalence of parasites in salad vegetables sold at supermarkets and street vendors (P>0.05, Table II). Ascaris spp. was found to be the highest prevalent parasite in salad vegetables sold at supermarkets and street vendors (18% and 24%, respectively; Table III).

The prevalence of Ascaris spp. eggs found in the 30 lettuce, 33 tomato, 42 parsley and 28 cucumber samples examined was 43%, 15%, 21% and 4%, respectively. Ascaris spp. eggs was found to be significantly higher in total lettuce samples than in total tomato and cucumber samples (P = 0.024, Odds ratio = 4.2 and

| Parasite contamination | | nation% Amman | an Parasite contamination% Baqa'a | | Total Parasite | |
|------------------------|-------------------------|---------------------------|-----------------------------------|---------------------------|-----------------------------------|--|
| | Supermarket % (No.)ª | Street vendors % (No.) | Supermarket % (No.) | Street vendors % (No.) | contamination | |
| Lettuce | 62.5% (5 out of 8) | 75% (6 out of 8) | 42.9% (3 out of 7) | 71.4 (5 out of 7) | 63.3% (19 out of 30) ^b | |
| Tomato | 14.3% (1 out of 7) | 25% (2 out of 8) | 40% (4 out of 10) | 25% (2 out of 8) | 27.3% (9 out of 33) ^c | |
| Parsley | 10% (1 out of 10) | 44.4% (4 out of 9) | 27.3% (3 out of 11) | 16.7% (2 out of 12) | 23.8% (10 out of 42) ^d | |
| Cucumber | 14.3% (1 out of 7) | 0% (0 out of 7) | 0% (0 out of 7) | 0% (0 out of 7) | 3.6% (1 out of 28) | |
| Total salad vegetables | 25% (8 out of 32) | 37.5% (12 out of 32) | 28.6% (10 out of 35) | 26.5% (9 out of 34) | 29.3 (39 out of 133) | |

| Table II |
|--|
| Prevalence of parasites in salad vegetables according to area, retail and vegetable type |

Note: 26.9% (18 out of 67) of salad vegetable samples sold at supermarkets were contaminated with parasites and 31.8% (21 out of 66) of salad vegetable samples sold at street vendors were contaminated with different parasites (no significant difference between the two ways of retail, P > 0.05). ^a No.: number of contaminated samples out of the total sample number.

^b Lettuce samples were contaminated significantly more often than tomato, parsley and cucumber samples with parasites (P=0.006, Odds ratio=4.6; P=0.001, Odd ratio=5.5 and P<0.0001, Odds ratio=46.6, respectively).

 $^{\circ}$ Tomato samples were contaminated significantly more often than cucumber samples with parasites (P = 0.016, Odds ratio = 10.1).

^d Parsley samples were contaminated significantly more often than cucumber samples with parasites (P=0.041, Odds ratio=8.4).

| Vegetables | Source | <i>Ascaris</i> spp. No. (%) | <i>Toxocara</i> spp. No. (%) | <i>Giardia</i> spp. No. (%) | Taenia/ Echinococcus No. (%) | <i>Fasciola</i> spp. No. (%) | Entamoeba histolytica No. (%) |
|----------------|-----------------------|--------------------------------|---------------------------------|--------------------------------|------------------------------------|---------------------------------|-------------------------------------|
| Lettuce | Supermarkets n = 15 | 5 (33.3)ª | 4 (26.7) | 2 (13.3) | 2 (13.3) | 1 (6.7) | 0 (0) |
| | Street vendors n = 15 | 8 (53.3) ^b | 5 (33.3) | 5 (33.3) | 4 (26.7) | 3 (20) | 3 (20) |
| | Total n = 30 | 13 (43.3)° | 9 (30)° | 7 (23.3) ^f | 6 (20.0) ^g | 4 (13.3) | 3 (10) |
| Tomato | Supermarkets n = 17 | 3 (17.6) | 0 (0) | 1 (5.9) | 0 (0) | 0 (0) | 2 (11.8) |
| | Street vendors n = 16 | 2 (12.5) | 0 (0) | 1 (6.3) | 0 (0) | 1 (6.3) | 0 (0) |
| | Total n = 33 | 5 (15.2) | 0 (0) | 2 (6.1) | 0 (0) | 1 (3.0) | 2 (6.1) |
| Parsley | Supermarkets n = 21 | 3 (14.3) | 0 (0) | 0 (0) | 1 (4.8) | 0 (0) | 0 (0) |
| | Street vendors n = 21 | 6 (28.6) | 0 (0) | 0 (0) | 1 (4.8) | 1 (4.8) | 0 (0) |
| | Total n=42 | 9 (21.4) ^d | 0 (0) | 0 (0) | 2 (4.8) | 1 (2.4) | 0 (0) |
| Cucumber | Supermarkets n = 14 | 1 (7.1) | 1 (7.1) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Street vendors n = 14 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Total n = 28 | 1 (3.6) | 1 (3.6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| All vegetables | Supermarkets n=67 | 12 (17.9) | 5 (7.5) | 3 (4.5) | 3 (4.5) | 1 (1.5) | 2 (3.0) |
| | Street vendors n = 66 | 16 (24.2) | 5 (7.6) | 6 (9.1) | 5 (7.6) | 5 (7.6) | 3 (4.5) |
| | Total n = 133 | 28 (21.1) | 10 (7.5) | 9 (6.8) | 8 (6.0) | 6 (4.5) | 5 (3.8) |

 Table III

 Prevalence of different parasites detected in salad vegetables from supermarkets and street vendors

^a The highest prevalent pathogenic intestinal parasite found in supermarkets was Ascaris spp.

^b The highest prevalent pathogenic intestinal parasite found in street vendors was Ascaris spp.

^cThe prevalence of Ascaris spp. eggs was significantly higher in total lettuce samples than in total tomato and cucumber samples

(P=0.024, Odds ratio=4.2 and P=0.0005, Odds ratio=20.7, respectively).

^d The prevalence of *Ascaris* spp. eggs was significantly higher in total parsley samples than in total cucumber samples (*P*=0.042, Odds ratio=7.4). ^e The prevalence of *Toxocara* spp. eggs was significantly higher in total lettuce samples than in total tomato, parsley and cucumber samples

(P=0.0006, Odds ratio=29.6; P=0.0002, Odds ratio=37.6 and P=0.013, Odds ratio=11.6, respectively).

¹ The prevalence of *Giardia* spp. cysts was significantly higher in total lettuce samples than in total parsley and cucumber samples

(P = 0.0014, Odds ratio = 27.1 and P = 0.011, Odds ratio = 18.2, respectively).

^g The prevalence of *Taenia/Echinococcus* eggs was significantly higher in total lettuce samples than in total tomato and cucumber samples

(P = 0.009, Odds ratio = 17.8 and P = 0.024, Odds ratio = 15.1, respectively).

P = 0.0005, Odds ratio = 20.7, respectively), it was also found to be significantly higher in parsley samples than in total cucumber samples (P = 0.042, Odds ratio = 7.4). Table III shows further details of the prevalence of parasites (helminthes eggs, *Giardia* and *E. histolytica* cysts) that have been detected in salad vegetable samples collected from supermarkets and street vendors.

Thirty one percent (20 out of 64) of salad vegetables samples sold in Amman and 28% (19 out of 69) of salad vegetables samples sold in Baqa'a were contaminated

| | Vegetables sold in Amman, No. (%), n=64 | Vegetables sold in Baqa'a, No. (%), n=69 |
|-----------------------|---|--|
| Non-infected | 44 (68.8) | 50 (72.5) |
| Infected | 20 (31.2) | 19 (27.5) |
| Ascaris spp. | 14(21.8) ^a | 13 (18.8) ^b |
| <i>Toxocara</i> spp. | 5 (7.8) | 5 (7.2) |
| <i>Giardia</i> spp. | 5 (7.8) | 4 (5.8) |
| Taenia/Echinococcus | 5 (7.8) | 2 (2.9) |
| <i>Fasciola</i> spp. | 2 (3.1) | 4 (5.8) |
| Entamoeba histolytica | 3 (4.7) | 2 (2.9) |

Table IV Prevalence of different parasites detected in salad vegetables from Amman and Baqa'a

^a The highest prevalent pathogenic intestinal parasite found in vegetables sold in Amman was *Ascaris* spp.

^b The highest prevalent pathogenic intestinal parasite found in vegetables sold in Baqa'a was *Ascaris* spp.

with different parasites (Table IV). No significant difference was found between the prevalence of parasites in salad vegetables sold at Amman and Baqa'a (P>0.05). *Ascaris* spp. was found to be the highest prevalent parasite in salad vegetables at Amman and Baqa'a areas (22% and 19%, respectively; Table III). Table IV shows further details of the prevalence of parasites that have been detected in salad vegetable samples collected from Amman and Baqa'a.

Discussion

Fresh vegetable could become contaminated by parasites while growing in the field through irrigation, soil or types of fertilizers used. Other sources of contamination include harvesting, distribution or the retail market (Beuchat, 2002).

When comparing the prevalence of parasites attached to salad vegetables in the neighbouring developing countries and the developed countries (Table V), it is clear that the prevalence is higher in neighbouring developing countries ranging from 16% to 58% (Al-Binali et al., 2006; Al-Shawa and Mwafy, 2007; Daryani et al., 2008; Abougrain et al., 2010; Al-Megrm, 2010; Fallah et al., 2012; Hassan et al., 2012; Ali and Ameen, 2013) when compared to the prevalence in more developed countries like Turkey (Adanir and Tasci, 2013) and Norway (Robertson and Gjerde, 2001) (6% for both countries). In our study, it was found that 29% of the 133 salad vegetables samples examined were contaminated with different parasites (helminthes eggs, Giardia and E. histolytica cysts). The similarity between the prevalence of parasites attached to salad vegetables found in our study and the neighbouring developing countries could reflect a common way of contamination. The previous studies (Al-Binali et al., 2006; Al-Shawa and Mwafy, 2007; Daryani et al., 2008; Abougrain et al., 2010; Al-Megrm, 2010; Hassan et al., 2012; Ali and Ameen, 2013) shared the finding that the main source of parasite contamination found in vegetables was from irrigating vegetables with faecal contaminated water.

High prevalence of transmitted helminthes and protozoa could be caused by poverty, poor environmental hygiene and poor sanitation (Montresor *et al.*, 1998; Jamaiah and Rohela, 2005). In our study we compared the prevalence of parasites in salad vegetables sold in Amman and in Baqa'a refugee camp (31% and 28%, respectively). Unexpectedly, no significant difference was found in the prevalence of parasites in salad vegetables between these two areas. The study also compared the prevalence of parasites attached to salad vegetables

Table V Prevalence of parasites in salad vegetables at different neighbouring developing countries and developed countries

| City | Prevalence of parasite in examined salad vegetables % (No.) ^a | Reference | |
|-----------------------|--|----------------------------------|--|
| Tripoli – Libya | 57.9% (73 out of 126) | (Abougrain <i>et al.</i> , 2010) | |
| Ardabil – Iran | 56.7% (80 out of 141) | (Daryani <i>et al.</i> , 2008) | |
| Sulaimani – Iraq | 49.8% (119 out of 239) | (Ali and Ameen, 2013) | |
| Gaza Governorates | 37.0% (80 out of 216) | (Al-Shawa and Mwafy, 2007) | |
| Shahrekord – Iran | 32.6% (99 out of 304) | (Fallah <i>et al.</i> , 2012) | |
| Abha – Saudi Arabia | 27.2% | (Al-Binali <i>et al.</i> , 2006) | |
| Alexandria – Egypt | 19.4% (19 out of 98) | (Hassan <i>et al.</i> , 2012) | |
| Riyadh – Saudi Arabia | 16.2%(76 out of 470) | (Al-Megrm, 2010) | |
| Burdur – Turkey | 6.3% (7 out of 111) | (Adanir and Tasci, 2013) | |
| Norway | 6.1% (29 out of 475) | (Robertson and Gjerde, 2001) | |

^a No.: number of contaminated samples out of the total sample number.

sold at supermarkets and street vendors (27% and 32%, respectively), yet again no significant difference in the prevalence of parasites in salad vegetables was found between these two modes of retail. These findings exclude poor environmental hygiene and poor sanitation of the marketing area or retail method as the main cause of salad vegetable contamination, and points to the vegetable field growing area as the main source of contamination.

In Tripoli – Libya Ascaris spp. eggs were reported to be the most predominant parasite attached to salad vegetables, the study reported that 68% of salad vegetables examined were contaminated with Ascaris spp. eggs (Abougrain et al., 2010). Ascaris spp. was also one of the most prevalent detected parasite attached to vegetables in the neighbouring developing countries ranging from 12% to 26% (Al-Shawa and Mwafy, 2007; Al-Megrm, 2010; Fallah et al., 2012; Ali and Ameen, 2013). In Jordan, a study by Al-Lahham et al. (1990) found that the highest intestinal parasite detected in the stool samples collected from 283 food handlers was Ascaris lumbricoides with a detection rate of 5%. In our study the prevalence of Ascaris spp. eggs attached to vegetables was 21% and were found to be the highest detected parasite in salad vegetables examined, our findings agrees with the previous studies. This study did not differentiate between A. lumbricoides and Ascaris suum eggs as these two species eggs have a highly similar morphological appearance (Blaszkowska et al., 2011).

Lettuce and parsley leafs have a more rough surface texture and surface area when compared to tomato and cucumber surfaces making it a more easier area for attaching parasites, lettuce and parsley vegetable are also in more direct contact with soil and irrigating water than in tomato and cucumber vegetables. In the present study the prevalence of Ascaris spp. eggs was found to be significantly higher in lettuce samples (43%) than in tomato (15%) and cucumber (4%) samples. Ascaris spp. eggs were also significantly higher in parsley samples (21%) than in cucumber samples (4%) and higher than in tomato (15%) samples. Ascaris spp. is usually found in sewage and untreated waste water and is used as an indicator organism for sewage and water treatment process (Gerba and Smith, 2005). These findings may indicate that the soil that crops grow on and water used in irrigating crops are faecal contaminated.

The prevalence of *Toxocara* spp. attached to vegetables in some neighbouring developing countries ranged from 3% to 18% (Hadi, 2011; Fallah *et al.*, 2012; Adanir and Tasci, 2013). In Jordan a study by Abo-Shehada (1989) found that 16% of soil samples collected from school playgrounds and public places were contaminated with *Toxacara* eggs. A serological survey conducted in north of Jordan by Abo-Shehada *et al.* (1992) showed a seroprevalence of *Toxocara canis* in 11% of tested individuals. In the present study *Toxocara* spp. eggs were detected in 10% of salad vegetables which agrees with the previous findings. According to our finding, consuming unwashed salad vegetables may cause *Toxocariasis*. This study did not differentiate between *Toxacara cati* and *T. canis* eggs as these two species eggs have a highly similar morphological appearance (Blaszkowska *et al.*, 2011).

A study conducted in Jordan by El-Shehabi *et al.* (1999) detected *T. canis* in the intestines of 1.2% of dogs infected with intestinal helminths. In our study, the prevalence of *Toxocara* spp. eggs attached to lettuce was found to be 30% and significantly higher than the prevalence found in tomato (0%), parsley (0%) and cucumber (4%) samples. As *Toxocara* spp. eggs are excreted by dogs and cats faeces (Deplazes *et al.*, 2011), our finding indicate that lettuce is cultivated in an opened lands that are inhibited with more roaming dogs and cats when compared to parsley, tomato and cucumber cultivation lands.

Giardia cysts are found on surface water such as lakes and rivers, and their concentration is positively associated with the water pollution by means of residential or agricultural faecal contamination (Rosen *et al.*, 2000; Karanis *et al.*, 2006). The prevalence of *Giardia* spp. cysts attached to vegetables in some neighbouring developing countries ranged from 7% to 10% (Abougrain *et al.*, 2010; Hassan *et al.*, 2012; Ali and Ameen, 2013). In our study we detected the eggs of *Giardia* spp. in 9% of salad vegetables which agrees with the previous studies, our finding suggest that using faecal contaminated water for irrigation could be the main source of contamination of salad vegetables with *Giardia* spp.

Giardia lamblia causes giardiasis in humans, it is reported that ingesting as low as 10 *Giardia* cysts by humans may cause disease (Arnone and Walling, 2007). A study by Shakkoury and Wandy (2005) reported that the prevalance of *G. lamblia* in stool samples collected from individuals visiting primary health care centres in Amman – Jordan was 30%. In our study, the prevalence of *Giardia* spp. cysts attached to lettuce was found to be 23% and significantly higher than the prevalence in parsley and cucumber samples (*Giardia* spp. was not detected in the parsley and cucumber samples). Our results indicate that the high prevalence of *Giardia* spp. in lettuce may pose a risk to consumer's health if unwashed prior to consumption.

The prevalence of *Taenia* spp. attached to vegetables in some neighbouring countries was 11% in Sulaimani – Iraq (Ali and Ameen, 2013) and 3% in Burdur – Turkey (Adanir and Tasci, 2013). In our study we did not differentiate between the eggs of *Taenia* spp. and the eggs of *Echinococcus* spp. as they are undistinguishable from each other. The prevalence of *Taenia/Echinococcus* attached to vegetables in the present study was 6%. 206

The prevalence of Taenia spp. attached to leafy vegetables in Riyadh - Saudi Arabia was 20% (Al-Megrm, 2010), and the prevalence of Taenia/Echinococcus attached to lettuce in Tripoli - Libya was 33% (Abougrain et al., 2010). In Jordan, a study by Al-Lahham et al. (1990) detected Taenia saginata in 0.4% of the stool samples collected from 283 food handlers. In our study, the prevalence of Taenia/Echinococcus attached to lettuce was found to be 20% and significantly higher than the prevalence in tomato and cucumber samples (Taenia/Echinococcus were not detected in the tomato and cucumber samples). The high prevalence of Taenia/ Echinococcus in lettuce may pose a risk to consumer's health if unwashed prior to consumption. In Jordan the annual incidence of hydatidosis (a human disease caused by Echinococcus granulosus) depending on the region was found to be 15-65 per 100.000 (Kamhawi and Hijawi, 1992).

A study by Ajlouni *et al.* in 1984 found that 14% of stray dogs in Jordan were infected with *E. granulosus* (Ajlouni *et al.*, 1984) and a study by Maraqa *et al.* (2005) reported that 20% of Jordan local sheep were infected with *E. granulosus*. As *Echinococcus* spp. eggs are excreted by dogs and sheep faeces (Deplazes *et al.*, 2011), our finding indicate that lettuce is cultivated in open lands that are inhabited with more roaming dogs or sheep when compared to tomato and cucumber cultivation lands.

The prevalence of *Fasciola* spp. attached to vegetables in some neighbouring developing countries was 21% in Sulaimani – Iraq (Ali and Ameen, 2013) and 15% in Riyadh – Saudi Arabia (Al-Megrm, 2010). A study by Maraqa *et al.* (2005) reported that 3.2% of the sheep imported to Jordan from Romania were infected with *Fasciola hepatica* and a study by Sharrif *et al.* (1998) found that the prevalance rate of *F. hepatica* in camels grown in Jordan was 4%. In our study we detected the eggs of *Fasciola* spp. in 5% of salad vegetables.

The prevalence of E. histolytica attached to vegetables in some neighbouring developing countries ranged from 7% to 38% (Al-Shawa and Mwafy, 2007; Hassan et al., 2012; Ali and Ameen, 2013). A study in Amman - Jordan by Al-Momani et al. (2006) detected E. histolytica in 20% of the positive parasite stool samples. Another study in Amman - Jordan by Chazal and Adi (2007) found that E. histolytica as the most prevalent parasite detected in stool samples, with an infection rate of 28%. In our study we detected the cysts of E. histolytica in 4% of salad vegetables and the highest prevalence was found in lettuce samples (10%). Our results indicate that, the high prevalence of E. histolytica in salad vegetables especially in lettuce may pose a risk to consumer's health, if unwashed prior to consumption. The presence of E. histolytica in salad vegetables indicates that salad vegetables are faecal contaminated. Our results point out that, the most probable source of the parasite contamination found in salad vegetables sold at Amman and Baqa'a is from using faecal contaminated water in irrigating crops. It seems that the scarceness of fresh water in Jordan has pushed farmers to use faecal contaminated water in irrigating their crops. So, action is needed to direct farmers to use treated wastewater in irrigating trees or vegetable crops that is not intended to be eaten row, educating farmers on the risk of using faecal contaminated water for irrigation on the public health and perform a proper treatment of sewage water. In order to confirm the source of parasite contamination found in salad vegetable, further studies is needed to examine the salad vegetables cultivation area and to examine the water sources used in irrigation.

Our study showed that salad vegetables sold in Amman and Baqa'a are contaminated with parasites (helminthes eggs, *Giardia* and *E. histolytica* cysts) that may cause health risk to consumers. So, programs in the essentiality of washing raw vegetables prior to consumption and personal hygiene must be intensified.

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SHORT COMMUNICATION

Identification and Localization of β -D-Glucosidase from Two Typical *Oenococcus oeni* Strains

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Abstract

 β -D-glucosidase (β G) gene from *Oenococcus oeni* SD-2a and 31MBR was cloned, sequenced and analyzed, also intracellular β G of the two strains was further localized. The results showed that β G gene of the two strains was in high homology (>99%) to reported β G gene, confirming both strains possess β G activity at the molecular level. Intracellular β G of SD-2a is a mainly soluble protein, existing mostly in the cytoplasm and to some extent in the periplasm. While for 31MBR, intracellular β G is mainly insoluble protein existing in the cytoplasmic membrane. This study provides basic information for further study of the metabolic mechanism of β G from *O. oeni* SD-2a and 31MBR.

Key words: *Oenococcus oeni*, β-D-glucosidase, βG cloned, gene localization

Wine fermentation is a complex process driven by microorganisms such as yeasts and lactic acid bacteria (LAB). Malolactic fermentation (MLF), taking place after alcoholic fermentation, is the bacterially driven decarboxylation of L-malic acid to L-lactic acid and carbon dioxide. This process improves the stability and quality of wines (Alexandre et al., 2004; Sumby et al., 2013). Oenococcus oeni is the most important LAB to conduct MLF in virtually all red wine and an increasing number of white wines (Bartowsky and Borneman, 2011; Olguin et al., 2011). Although commercial O. oeni starters are used to induce MLF in the practice of winemaking at present, the majority of wineries prefer MLF to occur spontaneously. Thus, now more attention is being focused on the isolation and study of native LAB starters possessing important oenological characteristics (Ruiz et al., 2008; 2010; Izquierdo et al., 2009; Capozzi et al., 2010; Mesas et al., 2011; Dong et al., 2014).

Several oenological characteristics, such as the presence of glycosidase activity, are considered as criteria for the selection of qualified MLF starters (Grimaldi *et al.*, 2005; Michlmayr *et al.*, 2012a). It has been well established that apart from free flavor compounds, a significant part of flavors remain in newly made wine as odorless non-volatile glycosides (Maicas and Mateo, 2005; Michlmayr *et al.*, 2010). The odorless glycosides containing aroma and flavor aglycones are not directly accessible to the olfactory mucosa and may affect wine quality greatly after hydrolysis (Mesas *et al.*, 2012; Michlmayr *et al.*, 2012b). β -D-glucosidase (β G) is one of the most important glycosidases to catalyze glycosylated precursors, releasing active aroma and flavor compounds during winemaking. β G activity in *O. oeni*, the main bacterial species conducting MLF, was confirmed more than 20 years ago (Guilloux-Benatier *et al.*, 1993). Over the past decades, numerous investigations have been conducted, providing evidence for the potential β G activity of *O. oeni* strains for flavor enhancement in wines (Spano *et al.*, 2005; Michlmayr *et al.*, 2010; Gagné *et al.*, 2011). It has been reported that the possession of glycosidic activities is widespread and strain dependent among *O. oeni* strains (Barbagallo *et al.*, 2004; Grimaldi *et al.*, 2005; Saguir *et al.*, 2009).

SD-2a and 31MBR are two important *O. oeni* strains widely used during winemaking in China. SD-2a is a patented strain screened from spontaneous MLF of wines in Yantai, Shandong Province, China, while 31MBR is a commercial strain prevalent in China with an excellent performance during MLF. The profile of β G activity for the two strains has been reported previously (Li *et al.*, 2012a; 2012b). It showed that both strains possess β G activity and the enzyme exists mainly in intracellular form. However, up to now, no information about its molecular basis is available. To better understand the nature of β G activity of the two strains, in the present study, β G gene of *O. oeni* SD-2a and 31MBR

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was cloned, sequenced and analyzed through bioinformatics, also the intracellular βG of the two strains was further localized.

O. oeni strains SD-2a and 31MBR, stored in our laboratory, were cultivated as described before (Li et al., 2012a). Bacterial growth was monitored by measuring the OD₆₀₀nm until the mid-log phase (about 40 h and 20 h for SD-2a and 31MBR respectively). Genomic DNA was extracted with Genomic DNA isolation Kit (TaKaRa, Shiga, Japan) as recommended by the manufacturer and verified on a 1% (w/v) agarose gel. Primers 5'TTGTCTAAGATTACTT CAATTATT TCA 3' and 5' TTAACTTTGATTGGCGA GTTTA3', deduced from the nucleotide sequences of BG gene previously identified in O. oeni PSU-1 (Makarova et al., 2006), were used. For the PCR experiments, 25 ng of genomic DNA isolated from O. oeni SD-2a or 31MBR was added to a 25 µl PCR mixture containing 0.5 U of ExTaq polymerase, 0.2 mM of dNTP mix, 1×PCR buffer (TaKaRa, Shiga, Japan), and 0.25 mM of each primer. The reaction was carried out at the following temperature profile: 94°C, 4 min; 94°C, 1 min; 58°C, 40 s; 72°C, 1.5 min - 35 cycles (using the icycler PCR Bio-Rad). The PCR reaction was terminated at 72°C for 10 min. PCR fragments were analysed on gel electrophoresis by applying 5 µl of sample to 1.0% agarose gel and a 2000 bp ladder (TaKaRa, Shiga, Japan) was used as the standard marker. The amplified fragments were purified with PCR Clean-up Kit (TaKaRa, Shiga, Japan), connected with vector PMD18-T (TaKaRa, Shiga, Japan), and transformed into Escherichia coli DH5a. Plasmid was extracted using Plasmid Extraction Kit (TaKaRa, Shiga, Japan) and sequenced by Takara Biotechnology Co., Ltd. Dalian, China.

The alignment of the deduced protein sequences against that of *O. oeni* PSU-1 was carried out with software Clustalx 1.81. Physical and chemical parameters of deduced amino acid sequences were computed with ProtParam (Gasteiger *et al.*, 2005). The amino acid scale of hydropathicity was defined following the ProtScale (Gasteiger *et al.*, 2005). Transmembrane helices were predicted with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMH MM-2.0/). The presence and location of signal peptide cleavage sites in amino acid sequence was predicted with SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Finally, the subcellular localization prediction was conducted with PSORTb v. 3.0.2 (Yu *et al.*, 2010).

 β G localization of SD-2a and 31MBR was conducted as described by de Cort *et al.* (1994) with some modifications. Bacterial growth was monitored by measuring the OD₆₀₀nm until the end of exponential growth phase (about 80 h and 40 h for SD-2a and 31MBR respectively). Then 10 ml cultures were centrifuged (5.000 g, 10 min, 4°C), washed with 0.5 M sodium malate buffer (pH 6.0), and recentrifuged to obtain whole cells.

The whole cells were resuspended in 10 ml 0.5 M sodium malate buffer (pH 6.0), then treated with lysozyme (Sigma) in a final concentration of 75 µg/ml and incubated for 2 h at 37°C until spheroplast formation was completed as confirmed by phase-contrast microscopic observation. Spheroplasts were harvested by slow-speed centrifugation (7.000 g, 20 min, 4°C), washed with 0.5 M sodium malate buffer (pH 6), and recentrifuged. The latter supernatant was mixed with the first supernatant as supernatant A (containing cell wall fragments and periplasmic-compounds). Spheroplasts were resuspended in a hypotonic solution (10 mM sodium malate buffer, pH 6), and lysed spheroplasts were centrifuged (24.000 g, 1.5 h, 4°C) to separate the particulate fraction (cell membrane) from the soluble fraction. Supernatant A was recentrifuged (24.000 g, 1.5 h, 4°C) to get the cell wall fragments and supernatant (containing periplasmic-compounds). The particulate fraction and cell wall fragments were resuspended in a minimum amount of 0.5 mM sodium malate buffer (pH 6) for enzyme assay along with other four parts prepared (spheroplasts, supernatant A, soluble fraction and periplasmic-compounds). βG activity assay was conducted following the method described previously (Li et al., 2012b)

PCR amplification resulted in single gene products of *ca* 2200 bp on the genomic DNA from both *O. oeni* SD-2a and 31MBR (Fig. 1). Purified PCR fragments were then transformed in *E. coli* DH5 α and subsequently sequenced. The alignment of protein sequence (Fig. 2) showed deduced amino acid sequences of gene



Fig. 1. PCR fragments with primers designed from β-D-glucosidase gene of *O. oeni* PSU-1 on genomic DNA of *O. oeni* SD-2a and 31MBR.

Lane 1: (M), molecular size marker (200 bp ladder). Lane 2: (31MBR), PCR fragment on genomic DNA of *O. oeni* 31MBR. Lane 3: (SD-2a), PCR fragment on genomic DNA of *O. oeni* SD-2a.

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Fig. 2. Alignment of β-D-glucosidase amino acid sequences from O. oeni SD-2a and 31MBR with that of O. oeni PSU-1. Identical residues are indicated by black background and similar residues by gray background.

cloned from the two strains exhibit high degree of homology (>99%) to the deduced protein sequence of β G gene identified in *O. oeni* PSU-1 (Makarova *et al.*, 2006), with five amino acid changes for SD-2a and six for 31MBR respectively, confirming βG gene was virtually cloned from the two strains. While for SD-2a and 31MBR, only three amino acids were observed to be different between them, indicating the βG gene is highly conserved. The coding region of βG gene cloned from the two strains was 2214 nucleotides long and initiates with the rare start codon TTG. While there is no possible start codon (ATG) upstream of this position, expression of the gene initiated with the next ATG codon in frame downstream resulted in inactive protein (Michlmayr et al., 2010). Besides, a ribosomal binding site (AAGGAG) was located upstream of the TTG codon. Analysis of the predicted protein sequence showed that βG of the two strains belongs to glycoside hydrolase family 3, which comprises enzymes with a broad specificity toward glycosylated plant metabolites. The β G with 701 amino acids, has a calculated molecular mass of 77.71 kDa and theoretical pI of 5.70. The protein with instability index 30.76 is classified as stable protein, and with a grand average of hydropathicity -0.395 is less hydrophilic. The Kyte-Doolittle hydropathy plot of the deduced protein sequence (data not shown) also showed the two similar protein is probably hydrophobic. Furthermore, no transmembrane domains or signal peptide cleavage sites was predicted within the two protein, indicating βG of the two strains is not secretory protein. The prediction of subcellular

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localization showed that βG of the two strains exists mainly in cytoplasm, some in cytoplasmic membrane and little in cell wall and extracellular.

In order to confirm the localization prediction, enzyme localization assay was also conducted for the two strains and the results are shown in Fig. 3. As for SD-2a (Fig. 3A), the highest β G activity occurred in the soluble fraction and some in the membrane, suggesting the enzyme exists mainly in the cytoplasm as a soluble protein. This is consistent with the localization prediction. The activity was also detected in the periplasm, as high as that in supernatant A, while low activity was observed in the cell wall. Furthermore, the spheroplast also exhibited high activity, but lower than the total activity of the soluble fraction and membrane, demonstrating the substrate may be transformed into the cells and then hydrolysed and the spheroplast may make the access difficult for substrate and intraspheroplast βG. As for 31MBR (Fig. 3B), some differences from that of SD-2a were observed. Otherwise than for SD-2a, the highest activity was observed in the membrane for 31MBR, much higher than that in the soluble fraction, indicating that the enzyme exists mainly in the cytoplasmic membrane as an insoluble protein and some in the cytoplasm as a soluble form. In addition, it should be noted that the spheroplast activity kept the same level with the soluble activity, indicating the intra-spheroplast soluble βG may be responsible for the spheroplast activity.

Recently, wine consumption, especially red wine, shows an increasing trend in China. However, wines



Fig. 3. β-D-glucosidase localization of *O. oeni* SD-2a (A) and 31MBR (B).

Supernatant A, containing cell wall fragments and periplasmic-compounds. Cell wall, sediment fraction from centrifugation of supernatant A. Periplasm, supernatant from centrifugation of supernatant A. Spheroplast, whole cells treated with lysozyme without cell wall. Membrane, particulate fraction from centrifugation of lysed spheroplast. Soluble, supernatant from centrifugation of lysed spheroplast.

made in China are negatively affected by low pH and bland flavor due to the climate and cultivars. Thus MLF is necessary during winemaking and the selection of starter cultures possessing important oenological characteristics is significant for the wine industry. SD-2a and 31MBR are two important *O. oeni* strains widely used during winemaking in China. The two strains possessing β G activity have been reported (Li *et al.*, 2012a; 2012b), whereas in this study, the β G of the two strains was further identified on a molecular level and characterized through bioinformatic analysis.

The β G gene cloned is probably widespread and highly conserved in *O. oeni* strains, since great identity of protein sequence (>99%) was observed in this study. Through the analysis of amino acid composition, β G of *O. oeni* SD-2a and 31MBR was probably hydrophobic, since many hydrophobic amino acids were included. However, the subcellular localization prediction showed the enzyme exists mainly in the cytoplasm as a soluble protein, which may be due to the secondary and tertiary structure of the protein that alters the hydrophobicity of β G. β G of the two strains was predicted as a non-secretory protein which mirrors the previous report that low β G activity was observed in the culture supernatant for both strains (Li *et al.*, 2012b), as well as the subcellular localization prediction that little of the enzyme was extracellular.

As for location of β G, intracellular enzyme has been reported for different O. oeni strains (Barbagallo et al., 2004; Michlmayr et al., 2010; Perez-Martin et al., 2012). The same results have also been observed for O. oeni SD-2a and 31MBR (Li et al., 2012b). However, up to now it's not quite clear where the metabolic process of these intracellular enzymes lies and no further localization has been reported. In this study, intracellular βG of SD-2a and 31MBR was further localized through bioinformatic analysis and localization assay. For SD-2a, the results by two methods are coincident. While for 31MBR, the result of localization assay is opposite to the prediction that βG activity was observed mainly in the cytoplasmic membrane instead of the cytoplasm. This could be explained by intra-specific phenotypic diversity. Intracellular β G of SD-2a is mainly a soluble protein, existing most in the cytoplasm and some in the periplasm. While for 31MBR, intracellular β G is mainly an insoluble protein existing in the cytoplasmic membrane, and some soluble enzyme existing in the cytoplasm and periplasm. Thus it may be concluded that the hydrolysis of glycosides may occur in the cytoplasm for SD-2a and on the cytoplasmic membrane for 31MBR.

Although SD-2a and 31MBR were verified to possess β G gene with potential to act on specific substrates, it remains imperative to understand how the gene is regulated under winemaking conditions, and to evaluate whether the expressed enzymes are active in wine. It is also interesting to compare the expression level of β G gene and phospho- β G gene of the two strains in glucose rich and depleted media as well to achieve a better understanding of their metabolic role, since the mechanism of whole cells of both strains possessing high βG activity has not been elucidated. In addition, it is also worth considering the possibility to use other hosts like Saccharomyces cerevisiae and Lactobacillus plantarum for over-expression of this gene. Related work is being done in our lab and this study is therefore the basis of all further research.

In conclusion, βG gene from *O. oeni* SD-2a and 31MBR was cloned, sequenced and analyzed. Deduced amino acid sequences showed high homology (> 99%) to the βG sequences reported, thus βG activity for the two strains was confirmed on a molecular level. Prediction of enzyme localization by bioinformatic analysis was not totally consistent with the result of localization assay, so to some extent bioinformatic analysis can only be considered as a reference. Intracellular βG of SD-2a was mainly a soluble protein existing in the cytoplasm

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SHORT COMMUNICATION

Characterization of Staphylococcal Cassette Chromosome *mec* (SCC*mec*) in Methicillin-Resistant *Staphylococcus epidermidis* Strains Isolated from Biomaterial-Associated Infections and their Antibiotic Resistance Patterns

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Abstract

This work aims to provide an insight into staphylococcal cassette chromosome *mec* elements and antibiotic resistance in clinical isolates of *Staphylococcus epidermidis*. The dominating type was SCC*mec* – IV. Fifteen isolates were assigned to SCC*mec* type III, two isolates to SCC*mec* type II. Most isolates were resistant to at least three of the non- β -lactam antibiotics tested. None of the strains exhibited resistance to new generation antibiotics, such as daptomycin and linezolid. Also, none of these strains showed resistance to tigecycline and only four strains were resistant to rifampin *i.e.* antibiotics which are very efficient in treating biofilm-associated infections.

Key words: Staphylococcus epidermidis, SCCmec type, antibiotic resistance

Among coagulase-negative staphylococci (CoNS), Staphylococcus epidermidis is the leading cause of hospital-acquired and biomaterial-associated infections. This bacterium can be responsible for endocarditis, peritonitis, bone and joint infections, septicaemia and bacteremia (Voung and Otto, 2002). The main virulence factor associated with S. epidermidis is the ability to form biofilm on implanted medical devices or damaged tissues. Strains belonging to this species have been particularly efficient at developing resistance to antimicrobial agents, which is due in part to the presence of the mobile genetic elements caring resistance genes (Schoenfelder et al., 2010). The mecA gene, which encodes PBP2a, a transpeptidase with a low affinity for beta-lactam antibiotics, is carried on a mobile genetic element called the staphylococcal chromosome mec (SCCmec). In addition, resistance gene for macrolides, tetracyclines and aminoglycosides can accrue on the SCCmec cassette. This element is bound by terminal inverted repeat sequences (IR) and integrated at the 3' end of the orfX gene, which is located near the origin of replication in the chromosome. Eleven types (I to XI) of SCCmec have been assigned for staphylococci based on the composition of the ccr gene complex and the class of the mec gene complex (Ito et al., 2001; 2004; IWG, 2009; Shore et al., 2013; Turlej et al., 2011). The mec gene complex is composed of mecA gene, intact or truncated sets of regulatory genes (mecRI and mecI), hypervariable region (HVR) and associated insertion sequence (IWG, 2009). The ccr gene complex encodes the reccombinase that plays an important role in integration and excision of SCCmec from the chromosome. Three district *ccr* genes, *ccr*A, *ccr*B and *ccr*C have been described in staphylococci strains. In addition to the ccr and mec gene complex, SCCmec cassette contains various mobile genetic elements (MGE), e.g. insertion sequences, transposons and plasmids, which are located in the joining regions (IWG, 2009). The occurrence of a very similar SCCmec cassette in different species provided evidence that genetic transfer of this element occurs between species in nature (Barbier et al., 2010; Hanssen and Ericson Sollid, 2006; Smyth et al., 2010). Moreover, Bloemendaal et al. (2010) demonstrated in vitro the transfer of SCCmec IV from S. epidermidis to the most virulent staphylococcal species, Staphylococcus aureus.

The aim of this study was to investigate the distribution of SCC*mec* types among *S. epidermidis* strains

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recovered from biomaterial-associated infections and analyse the antibiotic resistance patterns of these strains.

Sixty five CoNS collected from hospitalized patients were analyzed. Forty-six strains were isolated from the catheter-related bloodstream infection of hospitalized patients, which were regarded as causative agents of blood stream-infections (the same strain isolated from blood culture from the catheter and at the same time from blood culture by venous puncture). Nine strains were isolated from infection of the prosthesis, ten from peritoneal fluid. Isolates were identified by using the Vitek 2 system (bioMérieux, France).

The bacterial genomic DNA was isolated from clinical isolates using the Genomic DNA Plus kit (A&A Biotechnology, Poland). The SCC*mec* types were identified using multiplex PCR (Zhang *et al.*, 2005). The amplification products were electrophoresed in 1.5% agarose gel. The gels were stained with ethidium bromide, visualized on a UV light transilluminator, and documented with V.99 Bio-Print system (Vilber Lourmat, Torcy, France).

Resistance to β -lactams were determined by the cefoxitin (30 µg) screen test. Susceptibility to the following antibiotical agents: fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin), aminoglycosides (gentamicin, tobramycin), glycopeptides (teicoplanin, vancomycin), macrolides and lincosamides (clindamycin, erythromycin), tetracyclines (tetracycline, tigecycline) and others (linezolid, rifampin, trimethoprim-sulfamethoxazole) was performed using Vitek 2 system (bioMérieux, France) according to EUCAST recommendations (http://www.eucast.org/clinical_breakpoints). Minimum inhibitory concentrations (MICs) of daptomycin were determined by microdilution method in Mueller-Hinton broth, supplemented to yield final concentration of 50 mg/l calcium (EURO-CAST, 2007). Results were read after incubation at 37°C for 18-24 h. Susceptibility to daptomycin was defined as MIC value of $\leq 1 \text{ mg/l}$.

Tests were performed using software Excel (2010, Microsoft). The Pearson test was used to analyze correlation between SCCmec types and the resistance to different antibiotics. A P-value of < 0,05 was considered significant.

We have previously documented the occurrence of biofilm-associated genes in the majority of clinical *S. epidemidis* as well as their ability to form biofilm structures *in vitro* (Szczuka and Kaznowski, 2014). This work aims to provide an insight into staphylococcal cassette chromosome *mec* elements and antibiotic resistance. Among these strains, 82% were multiresistant. Resistance to erythromycin, clindamycin, and tetracycline was found in 45 (69%), 43 (66%) and 35 (54%) of the isolates, respectively. Twenty four (37%) were resistant to ciprofloxacin, twenty one (32%) to gentamicin and nineteen (29%) to trimethoprim-sulfamethoxazole. None of the strains exhibited resistance to glycopeptides. However, only four strains were resistant to rifampin and all were susceptible to tigecycline, antibiotics which are very effective in the treatment of biofilm-associated infections. All clinical strains were susceptible to linezolid, even though a few isolates of linezolid-resistant *S. epidermidis* were reported elsewhere (Hong *et al.*, 2007; Treviño *et al.*, 2009; Bonilla *et al.*, 2010; Gu *et al.*, 2013). In addition, all strains were susceptible to the new agent daptomycin, which demonstrated excellent *in vitro* activity against bacteria embedded in biofilms (Stewart *et al.*, 2009).

It is believed that the increasing resistance of S. epidermidis to methicillin and other beta-lactam antibiotics is due to the presence of the SCCmec, which can be easily transferred between staphylococci strains, especially in biofilm structures (Garza-Gonzalez et al., 2010a; 2010b). Sixty two S. epidermidis isolates were classified into three SCCmec types. Forty five (69%) S. epidermidis isolates harbour SCCmec type IV, which is believed to be the most mobile version of this element. Results of this study are in agreement with previously reported data, which indicated that type SCCmec type IV was the most prevalent in S. epidermidis strains among adults treated in a French hospital (Barbier et al., 2010; Garza-Gonzalez et al., 2010a; 2010b). Also SCCmec type IV dominated among S. epidermidis isolated from outpatients living in Algeria, Mali, Moldavia and Cambodia (Ruppé et al., 2009). In contrast, Li et al. (2009) found that only two out of 38 S. epidermidis strains recovered from patients treated in China carried SCCmec type IV, whereas SCCmec type III was the most prevalent. Our results indicate that 15 S. epidermidis strains (23%) carried SCCmec type III. Only two isolates harboured SCCmec type II. None of the isolates carried type I. Our results demonstrated that the strains harbouring SCCmec cassette type III were in a significantly higher proportion resistant to non beta-lactam drugs, except rifampin as compared to isolates with SCCmec type IV (Table I). It could be explained by the presence of several resistance genes in the

Table I Association between SCC*mec* types and resistance patterns to selected antimicrobial agents

| Antimicrobial | Number of resis | | |
|----------------------------------|-----------------------------------|----------------------------------|---------|
| agents | SCC <i>mec</i> type III (n=15) | SCC <i>mec</i> type IV (n=45) | p-value |
| ciprofloxacin | 11 (73) | 11 (24) | < 0.001 |
| clindamycin | 13 (86) | 27 (60) | 0.031 |
| gentamicin | 9 (60) | 7 (15) | < 0.001 |
| tetracycline | 14 (93) | 16 (35) | < 0.001 |
| trimethoprim/ ulfamethoxazole | 9 (60) | 10 (22) | < 0.001 |

SCC*mec* cassettes type III. The distribution of SCC*mec* types among *S. epidermidis* are comparable to the results of the studies conducted by Wisplinghoff *et al.* (2003), but different from the findings reported by Svensson *et al.* (2011). It has been reported that only three isolates carried known types of SCC*mec* – type III, whereas many strains contained multiple copies of *ccr* gene complexes and one class of *mec* gene complex. It is thought that the presence of different types of *ccr* complex in SCC*mec* elements might be due to rearrangements of types of SCC*mec* in bacterial cells (Hanssen *et al.*, 2006). In our studies, only three strains could not be assigned to known SCC*mec* types because *ccr* gene in these strains could not be determined. These strains contained *mec* complex B.

In conclusion, our results demonstrate the conservation of SCC*mec* element of *S. epidermidis* clinical isolates. These strains constitute a reservoir of SCC*mec* type IV. Although we found that the majority of *S. epidermidis* strains showed resistance to several antibiotics, no isolate showed resistance to daptomycin and tigecycline and only few isolates were resistant to rifampin which are the most efficient antibiotics against *S. epidermidis* biofilm-associated infections.

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SHORT COMMUNICATION

Qualitative and Quantitative Characteristics of Selected Bacterial Groups in Children with Inflammatory Bowel Diseases

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Abstract

The aim of the study was evaluation of qualitative and quantitative changes in bacterial ecosystem in 109 children with inflammatory bowel diseases. Stools obtained from patients were analysed for selected bacteria and concentration of faecal inflammatory markers (calprotectin, lactoferrin, M2-PK). The number of selected microorganisms depends on the level of clinical activity of disease and is correlated with faecal concentration of inflammatory markers. Differences in microflora disturbance, observed in patients with Crohn's disease and ulcerative colitis, may suggest different causes of development of both pathologies.

Key words: Crohn's disease, faecal bacteria, IBD, inflammatory markers, M2-PK

Crohn's disease (CD) and ulcerative colitis (Colitis ulcerosa, CU), referred to as inflammatory bowel diseases (IBD) are conditions of which the aetiology has not been yet fully understood. Approximately 10–15% of patients with IBD do not meet criteria of CU or CD. Those cases are qualified as inflammatory bowel disease unclassified (IBDU) (Austin *et al.*, 2007).

The role of microbiological factors in the pathogenesis of IBD is advocated by numerous evidence. A renowned analysis by Sellon *et al.* (1998) indicated that introduction of physiological, non-pathogenic microflora to the environment of germ-free mice caused the development of colitis. In another experiment, development of inflammatory condition occurred following transfer of ileostomy content into the healthy part of the intestine (D'Haens *et al.*, 1998). Also, efficacy of antibiotics in therapy of IBD, and changes in composition of bacterial ecosystem in IBD patients and animal models may constitute evidence.

A search for a single pathogen leads to contradictory conclusions. Many microorganisms are being analysed, *e.g. Escherichia coli, Yersinia, Listeria*, but none of them have been isolated from all patients with IBD (Lakatos *et al.*, 2006; Wank *et al.*, 2014; Maukonen *et al.*, 2015). Some researchers associate causes of the disease with *Bacteroides fragilis*. Fragilisin – an endotoxin of high proteolytic activity produced by the strain – participat-

ing in the destruction of tight junctions, and in consequence, to loss of selectivity of the intestinal barrier and destruction of intestinal membrane. Kamińska et al. (2004) demonstrated that in children with moderate to acute IBD the discussed group of bacteria constituted a significant majority, and in the study by Andoh and Fujiyama (2006) those bacteria were even the only microorganism detected in all examined patients with CD. Also a role of *Clostridium difficile* and *Candida* spp. was widely discussed in relation to IBD (Gerard et al., 2015; Monaghan et al., 2015). Zwolińska-Wcisło et al. (2004) demonstrated the much higher level of Candida albicans occurrence in children with CU (78%) compared to healthy children (7%). The introduction of anti-mycotic agents caused a significant improvement in a patient's clinical condition. Noteworthy are data indicating the lower number of Faecalibacterium prausnitzii in patients with IBD (Gałęcka et al., 2013). F. prausnitzii is extremely important for colonic homeostasis. Intestinal system of IBD patients is strongly quantitatively and qualitatively altered compared to healthy individuals, therefore maybe causes of IBD should be sought not in excessive proliferation of potentially pathogenic bacteria, but in reduction of count of beneficial microorganisms. It is commonly known that intestinal microbiota is a key factor in human health. The aim of our study was to determine if changes in the profile of microorganisms

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Table I Number of patients at individual levels of clinical activity of disease

| Diag- | The level of clinical activity of disease | | | Total | |
|-------|---|------|----------|--------|-----|
| nosis | No activity | mild | moderate | severe | |
| UC | 0 | 19 | 9 | 8 | 37 |
| CD | 18 | 9 | 12 | 8 | 47 |
| IBDU | - | - | - | - | 25 |
| Total | 18 | 28 | 21 | 16 | 109 |

UC - ulcerative colitis

CD – Crohn's disease

IBDU - inflammatory bowel disease unclassified

could be an useful marker preceding progression of disease, and correlate with inflammatory markers.

Hundred and nine children (64 boys and 45 girls) with IBD, hospitalised between 2009-2011, at the Department of Paediatric Gastroenterology and Metabolic Diseases of Poznan University of Medical Sciences participated in the study. All patients were between 3-16 years of age. Diagnosis was made on medical history, physical examination, laboratory tests, endoscopic and radiographic examinations as well as histopathological examinations. Activity of the disease was evaluated by the Paediatric Activity Index (PCDAI) for patients with CD and by the Truelove-Witts index (Ryzko and Woynarowski, 1995) for children with CU. Table I contains characteristic of each group. Patients in all study groups did not differ significantly in antibiotics and probiotics administration. Stool samples for microbiological culture and evaluation of inflammatory markers were collected from all children. Faeces were inoculated on a set of selective-differentiating and proliferative media. Count of the following bacteria was analysed: E. coli (including mycotic and lactosenegative strains), Proteus spp., Pseudomonas spp., other proteolytic bacteria (Klebsiella spp., Citrobacter spp., Enterobacter spp., Serratia spp.), Enterococcus spp., Bifidobacterium spp., Bacteroides spp., Lactobacillus spp. (including hydrogen peroxide strains), Clostridium spp. and total bacterial count. Stools collected from patients were also analysed for yeasts and moulds. Concentration of analysed markers was evaluated using immunoenzymatic tests (ELISA): M2-PK (ScheBo), calprotectin (Immundiagnostik) and lactoferrin (Techlab).

Stool samples were collected from each patient following obtaining an informed consent on participation in the analysis (patient or patient's guardian in case of children under the age of 16 years). The study obtained approval from the Ethical Committee at the Poznan University of Medical Sciences. All determinations were performed at the Institute of Microecology in Poznan.

Normality of distribution was verified using the Shapiro-Wilk test. Determination of the significance

of differences between particular groups of patients was performed using a non-parametrical Kruskal-Wallis test. Spearman R correlation test was used for analysis of correlation between the level of clinical activity of the disease and microbial count. The same test was also used for analysis of correlation between bacterial count and various variables, including: patient's age, time elapsed since diagnosis and levels of inflammatory condition markers.

No statistically significant correlations were found between age of participating children and the count of analysed microorganisms. Similarly, time elapsed since diagnosis seem to have no effect on count of analysed microbiota.

A count of selected bacteria compared to established physiological norm was estimated to prepare characteristics of intestinal microflora of IBD patients. A median count of individual microorganisms in the test group of children was used. The percentage results of selected bacteria in particular diseases are presented in Table II.

Protective microflora. Bacteroides spp. (normal range: $\geq 10^9$ CFU/g of faeces). In all analysed pathologies count of those bacteria fell within the lower part of the normal range (median values: $CU - 1 \times 10^9$, CD -2×10^9 , IBDU -3×10^9); *Bifidobacterium* spp. (normal range: $\geq 10^9$ CFU/g of faeces). Only in children with IBDU the count of those bacteria fell within the normal range; and the value in patients with CU and CD was significantly reduced (median values: $CU - 7 \times 10^8$, CD -8×10^8 , IBDU -1×10^9); Lactobacillus spp. (normal range: $\geq 10^5$ CFU/g of faeces). – In all analysed pathologies count of discussed bacteria fell within the lower part of the normal range (median values: $CU - 2 \times 10^6$, CD – 4×10^6 , IBDU – 9×10^5). In all analysed pathologies count of those bacteria fell within the normal range; $H_2O_2 - Lactobacillus$ (normal range: $\geq 10^5$ CFU/g of faeces). In all analysed pathologies the counts of the discussed bacteria fell within the lower part of the normal range (median values: $CU - 8 \times 10^5$, $CD - 6 \times 10^5$, IBDU – 1×10^{5}).

Immunostimulatory microflora. *E. coli* (normal range: $\geq 10^{6}$ CFU/g of faeces) – count of *E. coli* bacteria did not exceed the upper limit of normal range in any analysed pathologies (median values: CU – 4×10^{7} , CD – 7×10^{7} , IBDU – 2×10^{7}); *Enterococcus* spp. (normal range: $\geq 10^{6}$ CFU/g of faeces). In all analysed CD – 7×10^{7} , CI – 2×10^{6}).

Other proteolytic bacteria. *Clostridium* spp. (normal range: $\leq 10^5$ CFU/g of faeces). Count of *Clostridium* spp. fits within the normal range in all subgroups of included children (median values: CU – 0, CD – 2×10⁴, IBDU – 0); Mycotic, lactose-negative bacteria of *E. coli* genus (normal range: <10⁴ CFU/g of faeces) – the bacterial count fell within the normal range in all pathologies (median values: CU, CD, IBDU – 0); *Proteus* spp.

Short communication

| | | Perce | ntage | |
|--|-----------|-----------|-------|-------|
| Call count | Diagnosis | | | |
| Cell coulit | Total | Diagnosis | | |
| | | <u> </u> | CD | IBDU |
| Bacteroides spp.: < 10 ⁹ | 33.0 | 37.1 | 37.2 | 18.2 |
| Bacteroides spp.: 10 ⁹ – 10 ¹¹ | 67.0 | 62.9 | 62.8 | 81.8 |
| Bacteroides spp.: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| <i>Bifidobacterium</i> sp.: < 10 ⁹ | 64.0 | 65.7 | 67.4 | 54.5 |
| <i>Bifidobacterium</i> sp.: $10^9 - 10^{11}$ | 36.0 | 34.3 | 32.6 | 45.5 |
| Bifidobacterium sp.: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| <i>Lactobacillus</i> spp.: < 10 ⁵ | 15.0 | 11.4 | 9.3 | 31.8 |
| Lactobacillus spp.: $\geq 10^5$ | 85.0 | 88.6 | 90.7 | 68.2 |
| Lactobacillus spp.: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| H2O2-Lactobacillus: < 10 ⁵ | 38.0 | 31.4 | 39.5 | 45.5 |
| $H2O2$ -Lactobacillus: $\geq 10^5$ | 62.0 | 68.6 | 60.5 | 54.5 |
| H2O2-Lactobacillus: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| <i>E. coli:</i> < 10 ⁶ | 23.0 | 20.0 | 27.9 | 18.2 |
| <i>E. coli</i> : $\geq 10^6$ | 77.0 | 80.0 | 72.1 | 81.8 |
| <i>E. coli:</i> Total | 100.0 | 35.0 | 43.0 | 22.0 |
| Enterococcus spp.: < 10 ⁶ | 26.0 | 22.9 | 23.3 | 36.4 |
| <i>Enterococcus</i> spp.: $\geq 10^6$ | 74.0 | 77.1 | 76.7 | 63.6 |
| Enterococcus spp.: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| <i>Clostridium</i> spp.: $≤10^4$ | 54.0 | 57.1 | 48.8 | 59.1 |
| <i>Clostridium</i> spp.: > 10 ⁴ | 46.0 | 42.9 | 51.2 | 40.9 |
| Clostridium spp.: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| E. coli lactose neg.: < 10 ⁴ | 73.0 | 71.4 | 74.4 | 72.7 |
| <i>E. coli lactose neg.</i> : $\geq 10^4$ | 27.0 | 28.6 | 25.6 | 27.3 |
| E. coli lactose neg.: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| Proteus spp.: <10 ⁴ | 88.0 | 80.0 | 88.4 | 100.0 |
| <i>Proteus</i> spp.: $\geq 10^4$ | 12.0 | 20.0 | 11.6 | 0 |
| Proteus spp.: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| <i>Pseudomonas</i> spp.: < 10 ⁴ | 100.0 | 100.0 | 100.0 | 100.0 |
| <i>Pseudomonas</i> spp.: $\geq 10^4$ | 0 | 0 | 0 | 0 |
| Pseudomonas spp.: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| Other proteolytic bacteria: < 10 ⁴ | 59.0 | 62.9 | 53.5 | 63.6 |
| Other proteolytic bacteria: $\geq 10^4$ | 41.0 | 37.1 | 46.5 | 36.4 |
| Other proteolytic bacteria: Total | 100.0 | 35.0 | 43.0 | 22.0 |
| Total bacterial count: < 10 ¹¹ | 89.0 | 94.3 | 88.4 | 81.8 |
| Total bacterial count: $\geq 10^{11}$ | 11.0 | 5.7 | 11.6 | 18.2 |
| Total bacterial count: Total | 100.0 | 35.0 | 43.0 | 22.0 |

 Table II

 Percentage distribution of the number of selected microorganisms in different diseases

CU – ulcerative colitis

CD – Crohn's disease

IBDU - inflammatory bowel disease unclassified

(normal range: $< 2 \times 10^4$ CFU/g of faeces) – bacterial count fell within the normal range for all pathologies (median values: CU, CD, IBDU – 0); *Pseudomonas* spp. (normal range: $< 10^4$ CFU/g of faeces) – in all analysed pathologies counts of those bacteria fell within the physiological normal range (median values: CU, CD, IBDU – 0); Other, proteolytic bacteria – *Klebsiella*, *Citrobacter*, *Enterobacter*, *Serratia* (normal range: $<10^4$ CFU/g of faeces). In all analysed patients counts of proteolytic bacteria fell within the normal range (median values: CU, CD, IBDU – 0); Total bacterial count (normal range: 10^{11} – 10^{12} CFU/g of faeces) was reduced

in all discussed groups of patients (CU – 4×10^{10} , CD – 3×10^{10} , IBDU – 5×10^{10}).

Fungi. *Candida* spp. (normal range: <10³ CFU/g of faeces) – total count of yeasts was increased in all analysed subgroups of patients. The increase was most prominent in children with CD (median values: CU – 7×10^3 , CD – 1×10^4 , IBDU – 1×10^3). Physiological range of yeasts count was observed in only 32% of children with CU, 21% of children with CD and 50% of children with IBDU.

Another stage of the study was estimation if there is a correlation between count of analysed microorganisms and the level of clinical activity of the disease. Because of the insufficient data relating to the use of nonsteroidal anti-inflammatory drugs in the analyzed group of patients we did not asses their use at levels of calprotectin in the stools which is the limit of presented analysis. A negative correlation was demonstrated between the level of clinical activity of the disease and count of *Bifidobacterium* (p = 0.01; R = -0.28), *Bacte*roides (p = 0.006; R = -0.31) and total bacterial count (p=0.002; R=-0.35). We have observed a higher number of mentioned bacteria in the lighter stage of the disease. The level of clinical activity of the disease was positively correlated with count of yeasts (p = 0.01; R = 0.28). Correlations between individual bacteria in particular types of IBD were estimated. In patients with CU no correlation between the clinical activity of the disease and count of analysed microorganisms was found. On the other hand, in children with CD at various stages of activity of the disease there were statistically significant differences regarding count of Clostrid*ium* spp. (p=0.01), total bacterial count (p=0.04) and count of yeasts (p = 0.02).

The analysed inflammatory markers were found to correlate with the level of activity of disease. Obtained results were compared to count of analysed faecal bacteria. A statistically significant, negative correlation was found between the number of Bifidobacterium and M2-PK concentration (p = 0.05), so we have observed higher number of Bifidobacterium and lower M2-PK concentration. Negative correlation was also found and between the number of Bacteroides and concentration of all analysed markers (M2-PK p=0.02; calprotectin p=0.03; lactoferrin p=0.01). A negative correlation was also observed between the total bacterial count and concentration of M2-PK and lactoferrin (M2-PK p=0.02; lactoferrin p=0.007), so we have observed higher number of total number of bacteria and lower concentration of M2-PK and lactoferrin. A positive correlation was observed between the number of Candida and concentration of M2-PK and lactoferrin (M2-PK p = 0.001; lactoferrin p = 0.003).

Considering that the role of microbiological factor in IBD aetiology seems almost certain (among other factors, such as: genetic predisposition and immunological condition) authors decided to evaluate presence and count of selected bacteria in a group of paediatric patients. Increasing incidence rate of the disease in that group of patients, diagnostic and therapeutic difficulties constitute one of the most important challenges of contemporary medicine. Early diagnosis of the disease and determination of its actual aetiology are necessary for introduction of efficient therapy and ensuring optimal conditions of growth and development for affected child. Existence of correlation between bacterial factor and analysed faecal inflammatory markers was also studied. This analysis involved count of individual microorganisms in patients with particular type of IBD and stage of clinical activity of the disease. That approach was caused by postulated differences in causing factors of CD and CU.

Contrary to speculations, the composition of microflora in the group of analysed paediatric patients with IBD was not significantly different from accepted quantitative normal range (Gibson and Roberfroid, 1995). The authors demonstrated a significant increase in the number of yeasts in the analysed group. Similar observations were made by Zwolińska-Wcisło (2004) studying a group of children with CU. In the cited study, it was shown that the incidence rate of significant mycotic colonisation was significantly higher in the study group compared to the control (78% vs. 7%). That observation is very important, because the authors evaluated that a real probability of occurrence of mycotic infections was growing proportionally to time of the disease. Therefore, systematic monitoring of yeasts count is necessary, as well as rational use of antibiotics in that group of patients (Rosińska et al., 2007). The authors demonstrated a decrease in the count of Bifidobacterium in the analysed patients. An appropriate gut concentration of Bifidobacterium is obligatory for maintenance of homeostasis of the organism. Reduction in count of Bifidobacterium may cause an inflammatory condition. As stated in the literature, a reduction in count of Bifidobacterium is accompanied by excessive proliferation of anaerobic Clostridium. Those observations seem to be confirmed in this analysis. Clostridium count in children with CD was increased compared to other patients. Despite the fact that the count did not exceed the admissible physiological normal range in none of analysed pathologies, in children with CD the count was close to the upper limit of the normal range. Significantly lower counts of Clostridium were observed in children with CU and IBDU. Despite the lack of statistical significance, the observed differences in count of discussed microorganisms suggest the participation of separate microorganisms in the aetiology of both diseases. Another abnormality demonstrated in the group of participating children was the reduction

of total bacterial count. A conclusion on disorders of intestinal ecosystem may be drawn on that basis. As the strongest reduction was observed in relation to *Bifidobacterium*, it seems that reduction of total count may be attributed mostly to the protective group of bacteria. Maybe, elimination of observed dysbiosis and restoration of desirable bacterial conditions in the intestine is a prerequisite of successful therapy of IBD.

Another stage of the study was an evaluation of correlation between count of individual microorganisms and the level of clinical activity of the disease. No correlations of that kind were found in children with CU. However, in children with CD a significant correlation was detected between activity of the disease and total bacterial count, count of yeasts and Clostridium genus. This is another argument for the stronger influence of microbiota on development and/or maintenance of CD, compared to CU. Maybe, complexity of bacterial interactions, affecting development and maintenance of inflammatory condition, is a cause of lack of success of probiotic therapy in that pathology. Observed proliferation of proteolytic bacteria of Clostridium genus and of yeasts is an unfavourable condition, facilitating further development of pathogenic intestinal flora. Excessive development of proteolytic bacteria causes damage to intestinal epithelium (production of toxic metabolites) and increases pH value of intestinal environment, intensifying the disease. On the other hand, excessive count of yeasts, being usually a result of dysbacteriosis, leads to increased pool of secreted mycotoxins. Further analyses are necessary to evaluate a real correlation of microorganisms discussed here with exacerbation of patient's condition. Elimination of dysbiosis may contribute to improved health condition of a patient.

Analysis of correlation between bacterial count and the level of inflammatory markers demonstrated existence of numerous associations. Increased level of all inflammatory markers was accompanied by reduced count of Bacteroides, constituting the most numerous part of intestinal microflora. M2-PK and lactoferrin concentration was reversely proportional to count of protective bacteria, as well as to count of Bifidobacterium - the bacterial count decreased with increasing activity of the disease. Another word - the more intensified inflammatory process, the less protective flora is present, or otherwise: the lower the count of lactobacilli, the more intensified inflammatory process is. Count of yeasts and of proteolytic bacteria increased with increasing marker concentration. It remains to be established if increased quantity of produced proteolytic enzymes (participating in destruction of tissues) and of mycotoxins may be a direct cause of inflammation. A correlation between clinical activity of the disease and changes in intestinal ecosystem should be studied intensively in the analysed group of patients. A correlation between activity of the disease, inflammatory marker levels, and intestinal bacterial count may contribute to the development of a new, effective scheme of diagnostics and evaluation of a patient's condition.

The final aetiological factor of IBD remains unknown. It is worth to emphasize that the cause of IBD can be either a pathogenic infection, or a reduction of tolerance of bacterial symbiotic antigens to initiate the pathogenic sequence. However, in analysed patients, both significant qualitative and quantitative changes of intestinal microflora were observed, being a valuable diagnostic and therapeutic indication. Observed microbiological changes may be associated both with a type of IBD and with its clinical activity. Basically, a trend for reduction of beneficial bacteria count is observed with simultaneous increase in count of potentially pathogenic microorganisms. Currently, determination if observed abnormalities are a cause or a result of the disease is a crucial problem. Evaluation of type of changes of intestinal ecosystem is important for development of effective therapy in IBD. An appropriate modification of microflora could play a role in therapy of exacerbation periods of the disease and maintenance of remission.

Conflict of interest

All of the authors have no conflict of interest to declare.

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SHORT COMMUNICATION

Cytotoxic and Bacteriostatic Activity of Nanostructured TiO, Coatings

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Abstract

Nanostructures are structures, mainly synthetic (nanosurfaces, cylindrical nanotubes, and nanospheres), which range between 1-100 nm in at least one dimension and can be engineered to a wide range of physical properties. This paper aims to explore the bacteriostatic and cytotoxic characteristics of nano-TiO₂ coated specimens of glass, stainless steel and ceramic with different thickness and roughness. The results show that stainless steel and glass specimens with a nano-TiO₂ coating thickness of 200 nm have a bacteriostatic effect of 97% and 100%, respectively after 30 minutes of UV exposure. Glass specimens with a nano-TiO₂ coating thickness of 750, 200 and 50 nm have a bacteriostatic effect of 86%, 93% and 100% after 60 minutes. Nano-TiO₂ coatings show a great bacteriostatic but not a cytotoxic effect, thus representing a valuable alternative for biomedical applications.

Key words: bacteriostatic effect, cytotoxic activity, TiO, coatings

Bulk titanium oxide (TiO₂) does not exist in nature but is mainly derived from the industrial process of ilmenite (Tao et al., 2013). It is an n-type wide bandgap semiconductor with 3 crystalline phases, anatase, rutile and brookite, with rutile as the most stable form (Liao et al., 2012; Wang et al., 2014). Rutile and Anatase TiO₂ are widely used as photocatalysts although a mixed form of these two phases have displayed an enhanced photocatalytic ability (Kho et al., 2010; Li et al., 2008). Due to its high refractive index small amounts of TiO₂ can be used to achieve an opaque coating with a great resistance to discolor under UV light (Mikkelsen et al., 2011). In the last decade great interest arose from the field of synthesis and applications of nanostructured TiO, materials (Kulkarni et al., 2015). In this regard examples of applications are, for instance, dyesensitized solar cells, sensors, rechargeable batteries, electrocatalysis, self-cleaning and antibacterial surfaces, photocatalytic cancer treatment and dental implants surface modification to prevent infections (Petrini et al., 2006; Seo et al., 2007; Roy et al., 2011; Cai et al., 2013; Lee et al., 2014; Behzadnia et al., 2014). An interesting application of TiO₂ is related to its use in coating different surfaces such as glass and tiles, providing them with deodorant, bactericidal (Pleskova et al., 2011; Yu et al., 2013) and self-cleaning (Xi et al., 2012; Khataee *et al.*, 2013) activity once exposed to the UV light (Cai *et al.*, 2013).

Based on such features it is also reasonable to hypothesize a possible use of this nanostructured material for biomedical applications such as operating rooms floor coatings, laboratory bench surface coating, as well as surgical instruments coating.

The aim of this study was to assess the bacteriostatic and cytotoxic activity of TiO_2 coatings on different bars made of glass, ceramic and stainless steel by means of a photocatalytic reaction following UV exposure. TiO₂ photocatalysis involves generation of hole (hþ) and electron (e) pairs and a subsequent reaction of surfacebound hydroxyl ions (OH⁻) and/or H₂O on TiO₂ particles with the holes to generate hydroxyl radicals (OH⁻) (Zuo *et al.*, 2015).

 TiO_2 coatings were performed by means of the electric arc physical vapour deposition (EA-PVD) technique (Kepenek *et al.*, 2004) modified with the Electro Magnetic Controlled Arc Stirring (EMCAS). The first technique is characterized by a fast deposition rate, a good control of the process parameter in respect of conventional evaporation sources and the capability to coat very thin films of nanometric thickness (Giolli *et al.*, 2007). As to the second technique, an electromagnetic device controls the electric arc movement

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| Code | Sample | Dimensions (mm) | Thickness (nm) | Roughness (µm) |
|------|--------------------|--------------------|-------------------|-------------------|
| AL | polished glass | 6×50 | 200 | 0.08 |
| AH | acid treated glass | 6×50 | 200 | 0.3 |
| AS | sandblasted glass | 6×50 | 200 | 4.6 |
| BL | polished glass | 6×50 | 100 | 0.08 |
| BH | acid treated glass | 6×50 | 100 | 0.3 |
| BS | sandblasted glass | 6×50 | 100 | 4.6 |
| CL | polished glass | 6×50 | 50 | 0.08 |
| CH | acid treated glass | 6×50 | 50 | 0.3 |
| CS | sandblasted glass | 6×50 | 50 | 4.6 |
| DL | polished glass | 6×50 | 750 | 0.08 |
| DH | acid treated glass | 6×50 | 750 | 0.3 |
| DS | sandblasted glass | 6×50 | 750 | 4.6 |
| EL | polished glass | 6×50 | 750 | 0.08 |
| EH | acid treated glass | 6×50 | 750 | 0.3 |
| ES | sandblasted glass | 6×50 | 750 | 4.6 |
| FL | polished glass | 6×50 | 750 | 0.08 |
| FH | acid treated glass | 6×50 | 750 | 0.3 |
| FS | sandblasted glass | 6×50 | 750 | 4.6 |
| ST | undeposited glass | 6×50 | - | - |
| ST | stainless steel | 6×50 | 200 | 0.3 |
| ST | ceramic tile | 25×25 | 100 | 4.6 |

Table I Physical features of sample used for the study

(scanning speed and position), the dimension of electric arc spot, and the time of permanence of spot in a desired position on the target so that it is possible to control the plasma density, the plasma temperature, the target wear and the evaporation zone of the target (Giolli *et al.*, 2007). It is possible to distinguish two separate and different electric circuits of the deposition apparatus: 1) high voltage (up to 1000 V) and low current (< 5 A) for obtaining a plasma able to clean the substrates, and 2) relatively low voltages (5–30 V) and high currents (50–150 A) for coating the substrates; moreover, it is possible to apply to the substrates to be coated a negative bias.

All samples characteristics, provided by VACUUM SURTEC S.r.l., Parma, Italy, have been summarized in Table I.

Before any biological assay the surface of glass, ceramic and stainless steel specimens was previously observed and characterized by means of an environmental scanning electron microscope (ESEM, Quanta 200, Fei Company, Holland) and Energy Dispersive X-ray Analysis (EDS) for the presence of contaminants. Subsequently, samples were also analyzed by means of surface-conduction electron-emitter display (SED) and back-scattered electrons (BSE) analysis, at a low vacuum (0.98 Torr), 25–30 KV spectrum with different spots and at a distance of 10 mm (data not shown).

Before each analysis each sample was sterilized in an autoclave at 120°C for 20 minutes.

In-vitro assays. Specimen cytotoxicity was assessed according to the standard EN/ISO 10993/5 using Neutral Red (Sigma Aldrich St. Louis Missouri; USA) and 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) (Life Technologies, Monza, Italy), whereas bacteriostatic activity was tested against *Staphylococcus aureus* by means of a Wood lamp (G.C.M., Milan, Italy) application at a 365 nm peak and at a distance of 10 cm which was previously recognized as safe in terms of bacterial death.

Each specimen was first rinsed with ethanol, then with acetone in a ultrasonic bath for 10 minutes and eventually accurately washed with deionized water for other 10 minutes. According to EN 30993-12 the ratio between sample area (>1 mm) and the extraction liquid [(DULBECCO'S MEM, GIBCOTM, UK) with Penicillin/ Streptomycin (GIBCOTM, UK) 100 IU/100 µg/ml, 2 mM L-glutamine, 1 mM Sodium Pyruvate and 10% fetal calf serum (Euroclone, Milan, Italy)] was: A/V = 3 cm²/ml. Each sample was then incubated at 37°C with 5% CO₂.

As to Neutral Red and MTT assay we used murine fibroblasts L929 at a concentration of 5×10^4 and 10×10^3 and uniformly distributed in 6 and 96 MW in 3 and 0.2 ml of MEM, respectively.

Copper was used as a positive control for both assays whereas a cell culture without specimen was used as a negative control.

Microscopic evaluations were performed by means of an optical microscope (Nikon Eclipse E600 microscope, Japan) whereas spectrophotometric analyses of wells were performed by a HP 8452 diode array spectrophotometer (St. Paul. GMI Inc. USA) at 540 nm.

As to cytotoxic evaluations, 100 mm plastic petri dishes (Life Technologies, Monza, Italy) were used to place both specimen and 3 ml (4 ml only for ceramic specimen) of a 5 mM saline solution which was achieved from 3 serial dilutions of a 0.5 M solution with *Staphylococcus aureus* ATCC 6538. Serial dilutions were used to allow the operator to easily count the remaining colonies following UV light exposure. Then, before starting the UV light exposure (time 0), after 30 minutes of exposure (time 30) and after 60 minutes of exposure (time 60) 10 µl of solution was withdrawn from the petri dish and placed onto a blood Agar (TSA with 5% Sheep Blood)/MacConkey plate (Life Technologies, Monza, Italy).

Statistical analysis. Data were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as the means \pm standard error of the mean and were first checked for normality using the D'Agostino-Pearson normality test. Differences between samples were analyzed using Friedman Test with Dunn's multiple comparisons test. p < 0.05 was considered significant.

Neutral Red assay. Neutral Red assay highlighted a great viability and numerosity of cells in contact with AL, AS and AH as well as clearly colored as for the negative control (data not shown). Conversely, cell numerosity resulted quite reduced for BH, BL and BS specimens, less stained and rod shaped meaning a suffering condition which was the same of that observed for the negative control (data not shown).

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MTT assay. MTT assay on TiO_2 -coated polished and sandblasted glass clearly indicated that cell proliferation was quite the same as for negative control, thus meaning the lack of any cytotoxic activity (Fig. 1).

Microbiological assay. Before each experiment stainless steel, glass and ceramic specimens were first placed in respective petri dishes along with a 5 mM saline solution with *S. aureus* ATCC 6538, as previously described in the materials and methods section. Then before starting the UV light exposure (time 0), at a 10 cm distance, after 30 minutes of exposure (time 30) and after 60 minutes of exposure (time 60) 10 μ l of solution were withdrawn from the petri dish and placed in triplicate into blood Agar/MacConkey plates. Then, all plates of all specimens were placed at 37°C overnight and a mean of all colonies for each specimen was determined.

Firstly, a comparative analysis of ceramic, stainless steel and glass (AL) was performed to better evaluate differences, in terms of bacteriostatic activity, between different specimens (Fig. 2A).

Figure 2A clearly shows a marked bacteriostatic effect of stainless steel and glass specimens (97% and 100%, respectively), with respect to the positive control, already after 30 minutes of UV light exposure, thus highlighting the possible enhanced bacteriostatic activity of TiO_2 -coating. On the contrary, the ceramic specimen seemed to share the same trend of positive control, thus evidencing the absence of a possible bacteriostatic activity induced by TiO_2 -coating. As to negative control, it showed the normal trend of *S. aureus* ATCC 6538 which had not been exposed to UV light, thus confirming the bacteriostatic activity of all specimens.

Based on these result the bacteriostatic activity on glass specimens was evaluated with different TiO_2 -coating thickness in order to observe any differences in terms of bacterial growth inhibition (Fig. 2B).

A marked bacteriostatic effect of glass specimens AL, CL, and FS, was observed with respect to the positive control, as well as an overall decrease of *S. aureus* ATCC 6538 colonies of 100%, 93%, and 86% respectively after 60 minutes of UV light exposure, thus highlighting the possible enhanced bacteriostatic activity of TiO_2 -coating. DS specimen showed a good bacteriostatic effect with a decrease of *S. aureus* ATCC 6538 colonies of 66% after 60 minutes of UV light exposure.

As for BH and EH specimens, a poor bacteriostatic activity was observed since there was only a 49% and



Fig. 1. Graphical representation of cytotoxic activity of TiO₂-coated glass with different treatments.
(A) polished, (B) sandblasted, (C) acid treated; (*p<0.05).

56% decrease of S. aureus ATCC 6538 colonies after 60 minutes of UV light exposure.

This study aimed to evaluate the response of stainless steel, glass and ceramic specimens with TiO₂ layers





(A) Before starting the UV light exposure (time 0), after 30 minutes of exposure (time 30) and after 60 minutes of exposure (time 60). The positive control was *S. aureus* ATCC 6538 exposed to UV light, while the negative control was the same strain but not exposed to UV light; (B) Before starting the UV light exposure (time 0), after 30 minutes of exposure (time 30) and after 60 minutes of exposure (time 60) on glass specimens with different TiO_2 -coating thickness. The positive control was the *S. aureus* ATCC 6538 exposed to UV light, while the negative control was the same strain but nuexposed to UV light.

of different thickness coated by means of the electric arc physical vapour deposition technique. Neutral Red assay pointed out that BL, BH and BS specimens showed a marked reduction of bacterial proliferation with respect to positive control while AL, AH and AS specimens only showed a slight reduction. Conversely, the MTT assay evidenced the almost lack of cytotoxicity of the specimens. This is in agreement with previous literature reports which showed the biocompatibility of TiO₂ so that it is commonly used for different biomedical applications (He *et al.*, 2013; Kulkarni *et al.*, 2015).

We hypothesized that the lack of a cytotoxic effect was either due to a partial penetration of UV light which was unable to trigger the photocatalytic effect or due some surface structure alterations. As to bacteriostatic effects, these resulted particularly enhanced with AL, EH, CL and FS glass specimens.

In conclusion, this study revealed that both the bacteriostatic and cytotoxic effects depended on the

kind of material and on its TiO_2 thickness. Thus, further experiments are required to overcome the lack of information about nanostructured surfaces for future biomedical applications.

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SHORT COMMUNICATION

Enteroviruses Associated with Aseptic Meningitis in Poland, 2011–2014

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Abstract

A 4-year study (2011–2014) of patients with meningitis was performed. Out of the 686 cerebrospinal fluid samples, 465 (67.8%) were positive for eneteroviruses using RT-PCR and out of 334 clinical samples, 216 (64.7%) were positive for enteroviruses using cell culture methods. The highest detection rate was observed in the summer and autumn. In total, 185 enteroviruses were identified by using neutralization test. Echovirus 6 and 30 were the most common (41.7% and 37.5% respectively). The highest frequency of neurological infections (32.7%) occurred in children aged 5–9 years, mostly males (63.9%).

Key words: aseptic meningitis, cerebrospinal fluid, diagnostic PCR, enteroviruses

Human enteroviruses (HEVs) are members of the *Picornaviridae* family, a large and diverse group of small RNA viruses characterized by a single-positive-strand genomic RNA. They are classified in four species: enteroviruses A, B, C and D. More than 100 serotypes are described. They affect millions of people worldwide each year, and are often found in the respiratory secretions and stool of an infected person. Infection can result in a wide variety of symptoms ranging from mild respiratory illness (common cold), through hand, foot, and mouth disease, acute haemorrhagic conjunctivitis, and myocarditis, to severe neonatal sepsis-like disease and acute flaccid paralysis, but the most common neurological manifestation is aseptic meningitis.

In temperate climates enteroviral meningitis is more than 5 times more common in summer than in winter and spread is predominantly through the faecaloral route. More than 90% of infected people remain asymptomatic. However, since most HEV infected individuals are asymptomatic, it is difficult to prevent further spread of the virus. The majority of symptomatic patients develop only mild febrile illness, less than 5% develop meningitis. Enteroviral meningitis may affect all age groups and usually is self-limiting. However, some patients show complications such as seizure, coma, and movement disorders. The predominant serotypes identified in enteroviral meningitis outbreaks are echovirus (E) 6, E9, E11, E13, E19 and E30 (Hayashi *et al.*, 2012). Enteroviral meningitis is confirmed by either virus isolation followed by identification of virus by neutralization assay using type-specific antisera or polymerase chain reaction (PCR) using cerebrospinal fluid (CSF). Molecular methods are faster and more sensitive than viral cell culture. The 5'UTR (5' untranslated region) is the most conserved region among EVs and is therefore targeted in many diagnostic tests (Richter *et al.*, 2006). Early diagnosis is optimal for patient management because it helps to avoid unnecessary antibiotic treatment.

This paper examines the epidemiology of HEVs in Poland over a four-year period with special attention to serotypes associated with meningitis.

A total of 686 CSF samples that were sent to the National Polio Laboratory in Warsaw for analysis between January 2011 and December 2014, were included in this study. Most of the samples were obtained from patients with viral meningitis of suspected enteroviral etiology, hospitalised in neurological and infectious disease departments of hospitals mainly in two voivodships podlaskie and mazowieckie (>90% of all samples). The clinical samples were tested with diagnostic pan-enterovirus RT-PCR (EV PCR). Viral RNA was extracted from 140 μ l of sample using spin columns (Qiagen) following the manufacturer's instructions. RT-PCR was carried out using Pan-enterovirus primers for enterovirus detection based on the WHO manual (WHO, 2004). This set of primers produces a product

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Fig. 1. Detection frequency of enterovirus in cerebrospinal fluid samples, 2011–2014

of 114 bp and has been designed to detect and amplify a genome segment present at the 5'-UTR of the enterovirus genomes. RT-PCR amplification was performed: one cycle of reverse transcription at 45°C for 20 min; one cycle of denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 70°C for 30 s followed by one cycle of elongation at 70°C for 7 min. Reaction mixtures were then held at 4°C. Amplification products were analysed in 2% agarose gels, GelRed-stained and examined under a UV DNA trans-illuminator. When the volume of CSF collected was sufficient positive samples were inoculated onto cell cultures.

In clinical samples that were tested with diagnostic pan-enterovirus RT-PCR, enteroviral RNA was detected in 465 (67.8%) of the 686 CSF specimens. EVs were detected throughout the four-year study period: 19 of 48 (39.6%) in 2011, 71 of 117 (60.7%) in 2012, 53 of 121 (43.8%) in 2013 and 322 of 400 (80.5%) in 2014. Figure 1 shows the seasonal distribution of EV infections cases, confirmed by PCR. Although some cases were notified throughout the year, they mostly occurred during summer and autumn months (III and IV quarter of year).

EV-positive patients' ages range from newborns to 73-years-old. As shown in Fig. 2, the highest rate among positive patients was observed in children 5–9 years old. Infected children were predominately males (63.9%) with a male-to-female ratio of 1.77:1, with 297 male and 168 female cases.

For virus isolation in cells, total of 334 clinical samples (cerebrospinal fluids - 127, stools - 202, throat swabs - 5), obtained from patients with viral meningitis, were analysed for enterovirus (EV) isolation in cell cultures during a 4-year period (2011-2014). Viruses have been isolated from cerebrospinal fluid (CSF), throat swabs and stool specimens by conventional cell culture methods using WHO recommendations. Viral isolation was performed on RD-cells (human rhabdomyosarcoma). In order to exclude the involvement of polioviruses L20B-cells (transgenic mouse cell with the human poliovirus receptor) were also used for virus cultivation. RD and L20B cells were cultivated in minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS). A volume of 200 µl of sample was inoculated into tubes with RD and L20B cells. The tubes were incubated at 36°C and were examined daily. After 7 days, the tubes were frozen and thawed and repassaged, and another 7-day examination was performed. Each specimen underwent two passages in RD and L20B cells. Samples demonstrating viral cytopathic effect (CPE) were identified by neutralization assay using specific antisera (National Institute for Public Health and the Environment, the Netherlands).

A total of 334 clinical samples were analysed for enterovirus by isolation in cell cultures. The study found that 216 (64.7%) samples were positive (78 – cerebrospinal fluid, 134 – stool, 4 – throat swabs). Out of the 334 samples analysed by cell culture isolation, 177 came from patients with positive EV PCR results,



Fig. 2. Age distribution of patients with a cerebrospinal fluid sample positive for enterovirus, 2011-2014

51 came from patients with negative EV PCR results and 106 samples were not analyzed earlier for enteroviruses. Among the samples from patients with positive EV PCR results, 135 (76.3%) were positive in cell culture isolation, but also among the 51 samples from patients with negative EV PCR results, 14 (27.4%) were positive in cell culture isolation. Out of the 106 samples not analyzed earlier for enteroviruses, 67 (63.2%) were positive in cell culture isolation.

Table I shows the number and types of enteroviruses detected during 2011–2014. During these 4 years,

Table I Enterovirus serotypes detected in viral culture by year the specimen was received at NPL – Warsaw; January 1, 2011 to December 31, 2014; Poland

| Enterovirus | 2011 | 2012 | 2013 | 2014 | Total |
|-----------------------------|------|------|------|------|-------|
| CVA9 | - | - | 2 | - | 2 |
| CVB | - | - | 3 | 1 | 4 |
| E6 | 7 | 55 | 22 | 6 | 90 |
| E7 | - | - | - | 1 | 1 |
| E11 | 2 | 1 | 2 | 2 | 7 |
| E30 | - | 3 | 33 | 45 | 81 |
| Untyped enteroviruses | - | 2 | 2 | - | 4 |
| Not done | - | - | - | 27 | 27 |
| Total enteroviruses/year | 9 | 61 | 64 | 82 | 216 |
| Total specimens tested/year | 33 | 72 | 113 | 116 | 334 |

the positive EV isolation ratio was fluctuated between 27.3% in 2011 and 84.7% in 2012. Two hundred and sixteen enteroviruses were isolated. Serotyping was performed on 189 isolates, and serotypes of E6, E7, E11, E30, CVA9 and CVB were revealed from 185 isolates, while the other 4 isolates could not be typed by serological method. The most common were echovirus 6 (E6) (90, 41.7%) and echovirus 30 (E30) (81, 37.5%). Together these two genotypes represented 79.2% of all EVs isolated. These predominant strains circulated in each year of the study with the exception of E30 in 2011. In 2011, echovirus 6 was predominant and E11 was also represented. In 2012, E6 constituted a high percentage (90.2%) of enteroviruses detected, and E11 and E30 were also represented. In 2013, E30 and E6 were predominant and Coxsackievirus A9 (CVA9), CVB and E11 were also represented. In 2014, E30 constituted the highest percentage (54.9%) of enteroviruses detected, and CVB, E6, E7 and E11 were also represented.

The present study describes the epidemiological and laboratory characteristics of enteroviral aseptic meningitis cases in two regions in Poland (mazowieckie and podlaskie), between January 2011 and December 2014. The majority of previous studies have shown that HEVs are responsible for high percentages of all aseptic meningitis cases, ranging from 43 to 83% (Gharbi *et al.*, 2006; Kumar *et al.*, 2013; Papadakis *et al.*, 2013). Others studies, however, demonstrated that enteroviral

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meningitis is less prevalent and ranged between 16% and 27% (Tao *et al.*, 2014). The incidence of the enteroviruses varies globally according to different populations and methodologies. The present study displayed that EVs were responsible for 67.8% (from 39.6% in 2011 to 80.5% in 2014) of cases of CNS infections suspected of having viral etiology.

In temperate climates, EV infections predominate in the summer and fall seasons. In the current study, PCR results for EV meningitis demonstrate a clear seasonality. Positive results were reported mainly in III and IV quarter of year. However, infections were also detected at other times of year, indicating year-round sporadic infections.

Among the 465 enterovirus positive patients, 385 (82.8%) were 5–19 years of age. Surveillance data from several countries have shown that approximately 29% to 44% of CNS-associated EV infections occur in young children under the age of one year (Harvala *et al.*, 2014). For example, in the United State, the peak age for children with aseptic meningitis is reported to be <1 year old. The age distribution of meningitis cases varies, possibly due to different causative agents. Others studies described a large proportion of teenagers and young adults infected during previous E30 outbreaks (Khetsuriani *et al.*, 2006; Savolainen-Kopra *et al.*, 2011; Takamatsu *et al.*, 2013).

Aseptic meningitis is generally commoner in males with a male-to-female ratio of 1.2–2.3:1, although the exact reason for this in unknown. In the current study the male-to-female ratio was 1.77:1.

Isolation of the enterovirus in cell culture is the traditional method used to identify the causative agent of viral meningitis, and it also makes serotyping of the isolated enterovirus possible, but it usually has low sensitivity, ranging from 60 to 75%. In our study, among the samples from patients with positive EV PCR results, 23.7% were negative in cell culture isolation, but also among the samples from negative patients, 27.4% were positive in cell culture isolation. The presence of enteroviruses does not necessarily generate positive PCR results. Negative results are probably a consequence of the presence of compounds that inhibit RT or PCR (Kopecka et al., 1993). Obtaining false negative results in cell culture can be due to the presence of noninfectious virus particles or slow growing enteroviruses, lack of sensitivity of cell line, low titre of virus in the specimens and toxic factors. Not all enteroviruses cause cytopathic effects in cell lines and thereby PCR assay detect a wider variety of viruses than cell culture method. A number of studies have demonstrated that RT-PCR is more sensitive than cell culture for enterovirus detection. Sensitivity and specificity of enterovirus RT-PCR are estimated at >95%. It should also be considered that in years with high activity of types

growing well in cell culture, the sensitivity of cell culture can be higher than in years with lower enterovirus activity (Roth *et al.*, 2007). Nevertheless, a combination of cell culture methods and detection by RT-PCR is more sensitive for detection of enteroviruses than either method alone.

The three predominant genotypes identified were E30, E6, and E11, all of which are members of the species Human Enterovirus B (HEV-B). Members of HEV-B have been widely described as the most common cause of aseptic meningitis cases and outbreaks worldwide (Gharbi et al., 2006; Mirand et al., 2008; dos Santos et al., 2011). Some serotypes of enteroviruses are more commonly associated with aseptic meningitis than others. E6 and E30 were among the most frequently reported serotypes in the United States in 1970 to 2005 (Khetsuriani et al., 2006), and in Europe in 2000s (Antona et al., 2007; Blomqvist et al., 2008; Kapusinszky et al., 2010; Milia et al., 2013). E30 is the most commonly associated with aseptic meningitis. Increases in echovirus 30 activity are characterized by global spread and large-scale aseptic meningitis outbreaks (Martinez et al., 2012; Hyeon et al., 2013; Xiao et al., 2014; Nougairede et al., 2014).

This study focused on EV epidemiology in Poland over a 4-year period. The results described in this study provide valuable information on the circulation of different EV types in the context of limited EV surveillance in Poland. Enterovirus surveillance is important not only for monitoring the changing epidemiology these infections but also for the rapid identification of spread of emerging EV types.

Acknowledgments

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SHORT COMMUNICATION

Monitoring of Virulence Genes, Drug-Resistance in *Campylobacter coli* Isolated from Golden Retrievers

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Abstract

The investigation was performed on 75 of Golden Retriever puppies. Faecal samples were collected on the 42 day of the puppies life (control). Probiotic preparation was administered on 43 day of the puppies life and 10 days after the application of the probiotic, faecal samples were collected again (on 53 day of puppies life). All isolates of *Campylobacter coli* isolated prior to the administration of the probiotic were found to contain the *cad*F gene responsible for adhesion, as well as, the *fla*A gene influencing motility of the examined bacteria. Significant differences (P < 0.05) were recorded only in the case of enrofloxacin.

Key words: Campylobacter coli, dogs microflora, drug-resistance, virulence genes

Campylobacter coli is the most seldom reported Campylobacter spp. in the majority of dog populations sampled (Acke et al., 2009). However, some studies have found Campylobacter jejuni to be the most commonly isolated species in dogs, particularly outside of Europe (Tsai et al., 2007). Other species of Campylobacter such as C. coli and Campylobacter lari have also been isolated from dogs on occasion, but these species are usually of very low prevalence (Rossi et al., 2008). At the moment, it is believed that the following genes are responsible for the pathogenicity of Campylobacter spp.: flaA gene conditioning motility, cadF - affecting adhesion, *cdt*B - responsible for toxin production and iam - determining invasiveness (Krutkiewicz, 2008). The European Food Safety Administration (EFSA) and the European Centre for Disease Control (ECDC) published the second joint report concerning antibiotic resistance of pathogenic bacteria infecting people, animals and food articles. Campylobacteriosis is the most frequently recorded animal-born infection in humans. High resistance of Campylobacter spp. strains to some antimicrobiological substances, including ciprofloxacin constitutes a growing problem in EU countries (EFSA, 2014). The aim of this study was to show varied shares of virulence genetic markers among strains isolated from dogs with diarrhea. The aim of the second stage of experiments was to presents impact of two commonly applied methods on changes in drug – resistance of the obtained isolates.

The investigation was performed on 75 of Golden Retriever puppies. *Campylobacter* sp. isolates were obtained from the rectum using swab kits with transport substrate (Euro Tubo Collection Swab Rubi, Spain). Faecal samples were collected on the 42 days of puppies life (control), (moment of weaning). Probiotic preparation was administered on 43 days of puppies life and 10 days after the application of the probiotic, faecal samples were collected again (on 53 day of puppies life to examine possible changes in *C. coli* drug-resistance).

The applied preparation BioProtect 200 mg (Vet-Expert) contained 5×10^6 CFU of *Lactobacillus acidophilus, Enterococcus faecium, Bifidobacterium longum, Lactobacillus rhamnosus*, and manno-oligosaccharides, fructooligosaccharides. The probiotic was administered 2 capsules daily for 10 days.

Campylobacter isolates were cultured at 42 ± 1 °C in Campy Selective Agar Base Preston (Neogen) for 48 h in an atmosphere composed of 6% oxygen, 10% carbon dioxide and 84% nitrogen. *Campylobacter* spp. identification was performed using PCR for the detection of *C. jejuni* and *C. coli*. The following positive strains: *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were

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also included. All strains were preserved in 20% glycerol at -70°C. Extraction of DNA (Andrzejewska et al., 2011) was performed using CHELEX-100 chelating resin (Bio-Rad). Bacterial colonies were suspended in 100 µl Tris buffer and 45 µl 20% CHELEX and boiled for 10 min. Samples were then immediately placed on ice for 1 min and centrifuged at 13.000 g for 10 min at room temperature. The supernatant (2 µl) was used in PCR. The purity and concentration of DNA were estimated using spectrophotometry at 260 and 280 nm. The presence of the *cadF*, *flaA*, *cdtB* and *iam* genes was determined with the primers given by Nachamkin et al. (1993), Konkel et al. (1999), Carvalho et al. (2001) and Bang et al. (2001). All PCR amplifications (Andrzejewska et al., 2011) were performed in a mixture (25 µl) containing: 2.5 μ l of the PCR buffer (10 × concentrated), 2.5 µl of MgCl₂ (25 mM), 0.5 µl of dNTPs (10 mM), 1 µl of each primer (100 μ M), 0.5 μ l (1 U) of the Tag thermostable DNA polymerase (Promega Corporation), $2 \mu l$ of the bacterial template DNA and $15 \mu l$ nuclease free water. The PCR products were analyzed by electrophoresis in 1.5% agarose gel. The size of the PCR amplicons was compared to the 100 bp DNA marker (Promega Corporation).

In order to assess the resistance of the isolates, the disc method was employed using the following antibiotics (Oxoid): ciprofloxacin (5 µg), enrofloxacin (5 µg), erythromycin (15 µg) and tetracycline (30 µg) Culturing was conducted on nutrient broth (NB Merck) which, after 18 hours of incubation at the temperature of 37° C, was diluted at 1:10.000 in sterile physiological liquid. The suspension (500 µl each) was screened onto plates with Mueller-Hinton (Oxoid) substrate and discs with antibiotics were placed on the agar surface. Following 18-hour incubation at 37° C, inhibition zone diameters were determined. The control of antibiotic activity was carried out with the assistance of the *C. coli* ATCC 33559 reference strain.

E test strips were used in accordance with the manufacturer's instructions. They were removed from -20° C storage and brought to room temperature prior to use. Mueller – Hinton agar plates supplemented with defibrinated 5% sheep blood (Oxoid) were inoculated by swabbing evenly in three directions with a 0.5 McFarland standard of the test organism. Four E test strips were applied to the surface of the plate in an equidistance radial manner, with the lowest concentration toward the centre. Plates were incubated under the same condition as for disc diffusion. MICs were read directly from the test strip at the point where the zone of inhibition intersected the MIC scale on the strip. National Committee for Clinical Laboratory Standards were used for interpretation of the results (Murat *et al.*, 2005).

Results of investigations regarding numbers of microorganisms were subjected to statistical analysis using the *glm* procedure of the SAS (2012) program and the significance of differences was verified by Duncan's test.

All isolates of C. coli isolated prior to the administration of the probiotic were found to contain the cadF gene responsible for adhesion, as well as, the flaA gene influencing motility of the examined bacteria. The *ctd*B gene, involved in preconditioning the development of CDT toxin, was identified in 48% of the isolates, whereas gene iam, affecting invasiveness - in 49.3%. The examination of the isolates obtained after the administration of the probiotic failed to reveal any significant influence of the probiotic on the frequency of occurrence of the above - mentioned genes (Table I). Table II presents the results of the comparison of two methods of determination of resistance of isolates (obtained prior to probiotic administration) to selected antibiotics. Two tests (disc diffusion test and E Test strips) which were used to determine quantities of isolates sensitive (S) and resistant (R) were compared to the applied antibiotic. In case of ciprofloxacin, erythromycin, and tetracycline, the results of the two tests did not differ significantly in terms of statistic. Significant differences (P < 0.05) were recorded only in the case of enrofloxacin. C. coli isolates obtained prior to the administration of the probiotic revealed the highest resistance in relation to ciprofloxacin and enrofloxacin. Numerical data towards susceptible and resistant isolates turned out to be similar to the results obtained in the control group.

As mentioned above, *Campylobacter* spp. are among the most frequently reported bacterial cause of human gastroenteritis worldwide (CDC, 2008). The invasive ability of a *Campylobacter* is strongly affected by its motility, provided by the flagellum. This has been demonstrated by studies that inactivated the *flaA* gene (encodes for the filament of the flagella) or generated mutant bacteria, and found that this affected mobility and thus invasiveness (Konkel *et al.*, 1999). In the carried studies, this gene was identified in 100% of the

 Table I

 Numbers and percentages of virulence genes in C. coli (PCR)

| Teslete amount | Number of positive isolates | | | | |
|---|-----------------------------|-----------|--------------|------------|--|
| Isolate group | cadF | flaA | <i>cdt</i> B | iam | |
| Dogs (control) C. coli ($n = 75$) | 75 (100%) | 75 (100%) | 36 (48%) | 37 (49.3%) | |
| Dogs (diet with probiotic) C. $coli$ (n = 75) | 75 (100%) | 75 (100%) | 38 (50.7%) | 37 (49.3%) | |

Short communication

| Antimicrobial agents | E Test | Disc diffusion | |
|----------------------|---|----------------|--|
| Ciprofloxacin (5 µg) | 38S (*MIC $\leq 1 \ \mu g \ ml^{-1}$) | 38S (≥21 mm) | |
| | $37R (MIC \ge 4 \ \mu g \ ml^{-1})$ | 37R (≤15 mm) | |
| Enrofloxacin (5 µg) | 43S (MIC $\leq 1 \ \mu g \ ml^{-1}$)a | 60S (≥23 mm)b | |
| | 32R (MIC \leq 2 µg ml ⁻¹)a | 15R (≤16 mm)b | |
| Erythromycin (15 µg) | 72S (MIC \leq 0.5 µg ml ⁻¹) | 73S (≥23 mm) | |
| | $3R (MIC \ge 8 \mu g ml^{-1})$ | 2R (≤13 mm) | |
| Tetracycline (30 µg) | 74S (MIC \geq 4 $\mu g~ml^{1})$ | 74S (≥19 mm) | |
| | $1R (MIC \ge 16 \mu g ml^{-1})$ | 1R (≤14 mm) | |

Table II Results of susceptibility testing of 75 isolates *C. coli* by disc diffusion and E test methods for four antibiotics (control)

* Minimum inhibitory concentration (MIC) specified by the National Committee for Clinical Laboratory Standards (2002); S – susceptible, R – resistant; a, b – means in rows designated with the same letters do not differ significantly at the level of P < 0.05

Table III Results of susceptibility testing of 75 isolates *C. coli* by disc diffusion and E test methods for four antibiotics (diet with probiotic)

| Antimicrobial agents | E Test | Disc diffusion | |
|----------------------|---|----------------|--|
| Ciprofloxacin (5 µg) | 37S (*MIC $\leq 1 \ \mu g \ ml^{-1}$) | 37S (≥21 mm) | |
| | $38R (MIC \ge 4 \ \mu g \ ml^{-1})$ | 38R (≤15 mm) | |
| Enrofloxacin (5 µg) | 45S (MIC $\leq 1 \ \mu g \ ml^{-1}$)a | 61S (≥23 mm)b | |
| | $30R (MIC \le 2 \ \mu g \ ml^{-1})a$ | 14R (≤16 mm)b | |
| Erythromycin (15 µg) | 74S (MIC \leq 0.5 µg ml ⁻¹) | 73S (≥23 mm) | |
| | $1R (MIC \ge 8 \mu g ml^{-1})$ | 2R (≤13 mm) | |
| Tetracycline (30 µg) | 73S (MIC≥4 µg ml ⁻¹) | 73S (≥19 mm) | |
| | $2R (MIC \ge 16 \mu g ml^{-1})$ | 2R (≤14 mm) | |

isolates from both experimental groups. According to the investigations by Andrzejewska et al. (2011) and Selwet and Galbas (2012a; 2012b) on C. coli occurrence in people, dogs, cats, and piglets, the presence of the *flaA* gene in all the examined isolates was also recorded (Selwet et al., 2015). The next factor affecting virulence is gene *cad*F, which is responsible for production of adhesines. In the experiments, the *cad*F gene was found also in 100% of isolates from both study groups. Some researchers attribute a high importance of the cadF gene leading to campylobacteriosis in human (Selwet and Galbas, 2012a). Cytokines such as interleukin-8 (IL-8) are secreted by host cells in response to bacterial invasion, acting as early warning signs to the host immune system, and Campylobacter spp. flagellum and cytolethal distending toxin (CDT) are both thought to stimulate the secretion of IL-8 from host cells (Zheng et al., 2008). In the carried studies, the cdtB gene was identified in 48-50.7% of the isolates. The iam gene responsible for invasiveness, was found to occur at a similar level (49.3%). Carvahlo et al. (2001) reported that the iam gene occured less frequently in C. coli. Referring to the EFSA report (2014), antibiotics which are most commonly used in the treatment of people, animals are fluorochinolons (*e.g.* ciprofloxacin) ability to mutation in the gyrase - coding gene, which leads to changes in this protein and reduces affinity to fluorochinolons. According to Krutkiewicz (2008) approximately 55.9% to 59% of Campylobacter sp. strains show resistance to ciprofloxacin. In the experiments, resistance to mentioned antibiotic was observed in both experimental groups at the level of 49.3-50.7%. Tambur et al. (2010) compared the E test strips and disc diffusion methods and observed a distinct increase of the determined antibiotics to which C. coli and C. jejuni were resistant. The two tests, which were used to investigate drug - resistance failed to show any differences in the amount of antibiotics to which C. coli were sensitive. Campylobacter sp. are able of producing many toxins, which can damage red blood cells. Many of these toxins/hemolysins are considered virulence factors because of their ability to increase the availability of iron to the pathogen throughout the process of infection via lyses of erythrocytes and subsequent release of heme from hemoglobin (Istivan et al., 2008; Selwet and Galbas, 2012a). Summing up, the prevalence of C. coli

in dogs are low, suggesting that this population of dogs is unlikely to be a common source of *C. coli* infection for humans. The examined animals were symptomless carriers of these rods. However, great abundance of *cad*F and *fla*A genes as well as smaller of *cdt*B and *iam* genes in strains isolated from those puppies can pose a threat associated with increase of their pathogenicity. In our studies *Campylobacter* strains exhibited high resistance to ciprofloxacin, enrofloxacin and low

resistance to erythromycin and tetracycline. Resistance to ciproflaxin was also determined in strains derived from animals (in particular, from chickens, pigs and cattle) as well as from food articles (EFSA, 2015).

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